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Phylogeography of a deep-sea demersal fish, *Bothrocara hollandi*, in the Japan Sea

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ABSTRACT: The population structure of *Bothrocara hollandi*, a dominant deep-sea demersal fish in the Japan Sea, was analyzed on the basis of the nucleotide sequence of part of the mitochondrial control region. The 296 individuals analyzed were collected at 17 sites at depths between 375 and 1677 m, and could be divided into a distinct monophyletic group of haplotypes and a paraphyletic group that contained the remaining haplotypes. Individuals in the first group were collected only from shallower sites at depths less than 1100 m, and they were found predominantly off the western part of Honshu Island (the Japanese mainland). Individuals in the second group were collected from all sites at which fish were sampled. The genetic diversity of the first group was greater than that of the second group. As a consequence, the genetic diversity of populations showed a tendency to decrease with increasing depth. The divergence of the 2 groups was estimated to have occurred during the early part of the last glacial period. The geographical pattern of distribution of haplotypes might be attributable to the accumulation of unique genetic characteristics in different refuges during the last glacial period and to secondary contacts as a result of migration from such refuges after the last glacial period. No genetic differentiation between the 2 morphotypes of this species was detected.

KEY WORDS: Phylogeography · Deep-sea demersal fish · *Bothrocara hollandi* · Japan Sea · Mitochondrial DNA

INTRODUCTION

The Japan Sea is a semi-enclosed marginal sea that is separated from neighboring waters by relatively narrow and shallow straits. It has been proposed that the Japan Sea might have been more isolated from such waters during glacial periods than during interglacial periods (Ohshima 1990, Tada 1994, Tada et al. 1999). In addition, the input of a significant amount of freshwater into the surface layer of the Japan Sea appears to have caused severe anoxic conditions during the last maximum glacial period (20 000 to 15 000 yr ago), as indicated by the results of taxonomic and geochemical analyses of microfossil assemblages within core samples of sediment (Tanimura 1981, Oba et al. 1991, Tada 1994, Crusius et al. 1999, Tada et al. 1999, Gorbarenko & Southon 2000). A major environmental change of this type should have resulted in the fragmentation and reduction of populations of many marine organisms. As a result, we would expect that significant genetic differences between conspecific populations would have accumulated independently both in the Japan Sea and in neighboring areas, and that marine fauna endemic to the Japan Sea would have been established.

Molecular biological techniques, such as the direct sequencing of products of the polymerase chain reaction (PCR) allow the analysis of the geographical structure of intraspecific genetic variations; the general

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term for such analysis is phylogeography (Avise 1994, 2000). The marine fauna of the Japan Sea should provide good material for phylogeographical studies. Indeed, Kojima et al. (1997) analyzed the population structure of a Japanese turban shell, *Turbo* (*Batillus*) *cornutus*, around the Japanese Islands, and demonstrated significant intraspecific genetic differences between specimens on the Pacific coast and those on the coast of the Japan Sea off Honshu Island (the Japanese mainland). Significant genetic differences were also demonstrated between specimens of another gastropod species, *Omphalius pfeifferi*, from both coasts of Honshu Island (Kojima et al. unpubl. data). Furthermore, allozyme electrophoresis revealed significant genetic differences between populations in the Japan Sea and the northwestern Pacific of an arrow worm, *Parasagitta elegans* (Thuesen et al. 1993), a sea urchin, *Strongylocentrotus nudus*, and a sea star, *Asterine pectinifera* (Matsuoka et al. 1995).

The genetic diversity of populations of deep-sea organisms has attracted the attention of deep-sea biologists for many years. During the 1970s, much research involving allozyme electrophoresis was performed in attempts to reveal the bathymetrical patterns of genetic diversity among populations of marine fauna (Doyle 1972, Goochi & Schopf 1972, Ayala & Valentine 1974, Ayala et al. 1975, Valentine & Ayala 1975). The possible causes of the high genetic diversity that was discovered were discussed for populations of various deep-sea species (Goochi & Schopf 1972, Ayala et al. 1975, Murphy et al. 1976, Soulé 1976). The analytical power of allozyme electrophoresis is somewhat too low for the quantitative resolution of genetic variations over a geographic range, and the results obtained may be biased by natural selection, which might operate in different ways at different depths. For an accurate understanding of the genetic characteristics of deep-sea fauna, more detailed information about the genetic structure of various organisms that inhabit the deep-sea is indispensable. While analytical methods based on DNA sequences can be expected to contribute to such studies (Chase et al. 1998, Etter et al. 1999), phylogeographical analyses of deep-sea fauna using DNA sequences have been rather limited to date except in the case of fauna in deep-sea reducing environments, such as hydrothermal vents and seep areas (Creasey & Rogers 1999, Gage & Young 1999).

Bothrocara hollandi is the dominant deep-sea demersal fish in the Japan Sea, and it is believed to be one of the species that are endemic to the Japan Sea and the Sea of Okhotsk. Although this species was also reported for the Yellow Sea (Nakabo 1993), this report might have been the result of misidentification and there is no clear evidence that it inhabits this sea area (Kinoshita pers. comm.). *B. hollandi* has been collected from the deepest site examined in the Japan Sea, and is distributed from 200 to 1980 m, covering the broadest range of depths of any demersal fish in the Japan Sea. This species has 2 morphotypes that are distributed above and below a depth of 800 m. A large white type inhabits shallower areas and a small black type inhabits deeper areas (Okiyama unpubl. data). In the present study, we analyzed the genetic structure of populations of *B. hollandi* with reference to changes in genetic diversity with depth in the Japan Sea. The analysis was based on the nucleotide sequences of the first half of the control region, which has the highest evolutionary rate within mitochondrial DNA (Lee et al. 1995). Therefore, the analysis of this region is expected to be useful for detecting genetic differences among intraspecific populations formed through relatively recent events such as severe reduction of habitable areas during the last glacial period.

MATERIALS AND METHODS

A total of 296 individual specimens of *Bothrocara hollandi* were collected at 17 sampling stations in the Japan Sea during Cruises KT-96-17, KT97-15 and KT-98-17 of RV 'Tansei-Maru' of the Ocean Research Institute, University of Tokyo (Fig. 1, Table 1). A single specimen of a congeneric species, *B. tanakae*, which was used as an outgroup for phylogenetic analysis, was collected in the north Western Pacific off Northeastern Japan (39° 24.39′ N, 142° 51.55′ E; depth

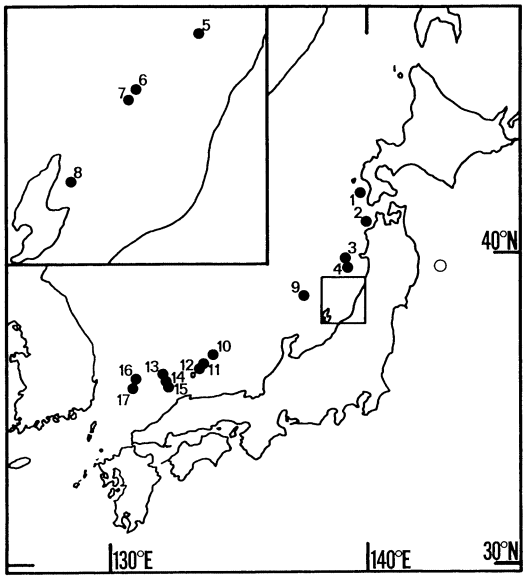

Fig. 1. Locations of stations at which *Bothrocara hollandi* was collected (●); details in Table 1. (○) Station at which congener *B. tanakae* was collected. Inset: enlarged view of area within square in main map

Table 1. List of sampling stations. Station numbers correspond to those in Fig. 1

Stn	Position	Depth (m)	Cruise	N
1	41° 38.62′ N, 139° 44.89′ E	1438	KT96-17	15
2	40° 58.95′ N, 139° 53.99′ E	816	KT97-15	17
3	39° 53.43′ N, 139° 04.72′ E	1474	KT96-17	24
4	39° 41.05′ N, 139° 09.28′ E	1065	KT96-17	24
5	38° 57.01′ N, 139° 25.05′ E	542	KT97-15	14
6	38° 40.20′ N, 139° 01.01′ E	594	KT97-15	20
7	38° 37.00′ N, 138° 58.18′ E	600	KT96-17	15
8	38° 12.24′ N, 138° 35.69′ E	637	KT97-15	20
9	38° 42.51′ N, 137° 50.84′ E	1677	KT97-15	15
10	36° 54.03′ N, 133° 51.32′ E	1544	KT96-17	10
11	36° 43.37′ N, 133° 37.56′ E	1164	KT96-17	23
12	36° 38.63′ N, 133° 27.41′ E	509	KT96-17	14
13	36° 19.60′ N, 132° 05.09′ E	1561	KT96-17	20
14	35° 53.31′ N, 132° 14.44′ E	519	KT98-17	20
15	35° 50.97′ N, 132° 15.22′ E	401	KT98-17	20
16	35° 55.00′ N, 131° 11.11′ E	1076	KT98-17	15
17	35° 47.72′ N, 131° 09.07′ E	375	KT98-17	10

1654 m) during Cruise KT-96-16 of RV 'Tansei-Maru' (open circle in Fig. 1). All samples were collected by a beam trawl with a 3 m span, and were kept in a freezer (–20°C) prior to analysis.

A small piece (about 0.1 cm^3) of muscle tissue from each fish was minced in 500 µl of a solution of 100 mM EDTA, 50 mM Tris (pH 8.0), 1% sodium dodecyl sulfate (SDS), and 100 µg ml^{-1} proteinase K and incubated at 50°C overnight. Each homogenate was extracted with buffer-equilibrated phenol, and DNA was precipitated with 2.5 volumes of cold ethanol, rinsed with 70% ethanol, dried and resuspended in 50 µl of double-distilled water (DDW).

Using 1 µl of each sample of DNA, we amplified the control region of the mitochondrial DNA by the polymerase chain reaction (PCR) using the primers Pro-L, 5′-CTACCTCCAACTCCCAAAGC-3′, and 1612SAR-H, 5′-ATAGTGGGGTATCTAATCCCAGTT-3′ (Palumbi et al. 1991), and rTaq DNA polymerase (Toyobo, Inc., Tokyo, Japan). The parameters for amplification were 94°C for 1 min and then 30 cycles of 92°C for 40 s, 55°C for 60 s and 72°C for 90 s. GeneReleaser™ (BioVenture, Inc., Murfreeboro, Tennessee, USA) was used to sequester products of cell lysis that might have inhibited the polymerase.

The nucleotide sequence of the first half of the control region was determined by the dideoxynucleotide chain-termination method with a Sequenase™ PCR product-sequencing kit (United States Biochemical Corp., Cleveland, Ohio, USA) with primers Pro-L, CR-1 (5′-CCACYGGTTAWGATATACG-3′) and/or CR-2 (5′-CRAATACTTGTCCCTCACCCTC-3′). The primers CR-1 and CR-2 were synthesized on the basis of determined sequences, and their positions are shown in Fig. 2.

We constructed a phylogenetic tree by the maximum parsimony method using the heuristic search approach of the computer program 'Parsimony', which was kindly provided by Dr K. Tamura of Tokyo Metropolitan University. The genetic diversity of each population was estimated by 2 indices, namely, gene diversity, i.e. the probability that 2 randomly chosen haplotypes are different (Nei 1987), and nucleotide diversity, which is expressed by the probability that 2 randomly chosen homologous nucleotides are different (Tajima 1983, Nei 1987). Genetic distance between haplotypes was calculated by the method of Tajima & Nei (1984). The significance of population structure and genetic differentiation between morphotypes were tested by the analysis of molecular variance (AMOVA) using a permutational approach (Excoffier et al. 1992). We performed the analysis with the computer program package 'Arlequin' (Schnider et al. 1996).

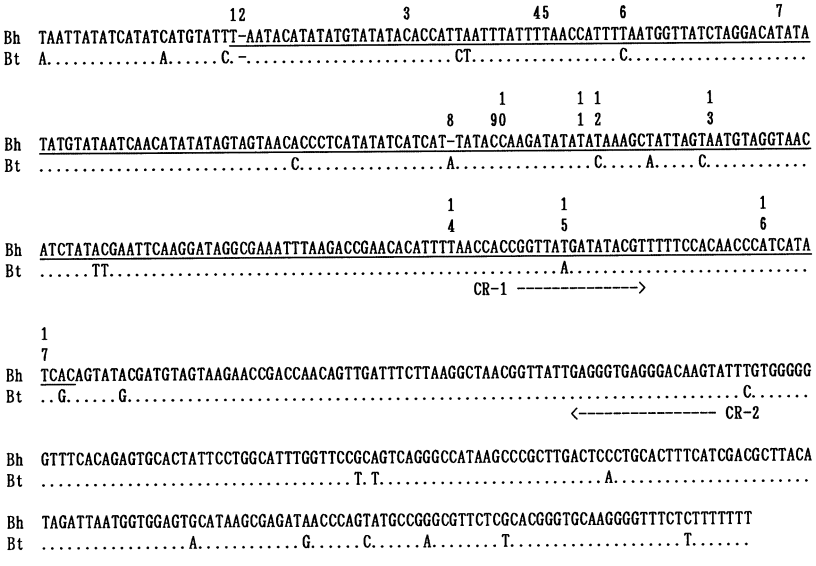

Fig. 2. *Bothrocara hollandi* (Bh) *B. tanakae* (Bt). Nucleotide sequence of part of the mitochondrial control region of the dominant haplotype (No. 1 in Table 2). Dots indicate nucleotides that are identical in both sequences. Sequence of the underlined region was determined for all specimens. –: site at which a nucleotide deletion was detected; numbers: polymorphic sites within sequences from specimens of *B. hollandi*. Positions of primers CR-1 and CR-2 are indicated. The nucleotide sequences will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases under Accession Nos. AB056505 (Bh) and AB056506 (Bt)

	Site																
										1	1	1	1	1	1	1	1
Type	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7
1	T	–	C	T	T	T	T	–	C	C	T	T	A	T	T	A	T
2	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
3	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.
4	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.
5	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C
7	A	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.
8	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C
9	.	A	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.
10	.	.	.	.	.	.	.	A	T	.	.	.	.	.	.	.	.
11	.	.	.	.	.	.	.	A	.	T	.	.	.	.	.	.	.
12	.	.	.	.	.	.	.	A	.	.	.	C	.	.	.	.	.
13	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C
14	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C
15	.	A	T	.	.	.	.	.	.	.	.	.	.	.	A	.	.
16	.	A	.	.	.	.	.	A	.	.	.	.	.	.	A	.	.
17	.	.	T	.	.	.	.	A	.	.	.	C	.	.	.	.	.
18	.	.	T	.	.	.	.	A	.	.	.	.	.	.	A	.	.
19	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	G	C
20	.	.	.	.	.	C	.	A	.	.	.	C	.	.	.	.	.
21	.	.	.	.	.	.	.	A	.	.	.	C	.	A	.	.	.
22	.	.	.	.	.	.	.	A	.	.	.	C	.	.	.	.	C
23	.	A	T	.	.	.	.	A	.	.	.	.	.	.	A	.	.
24	.	A	.	.	.	C	.	A	.	.	.	.	.	.	A	.	.
25	.	A	.	.	.	.	.	A	.	.	C	.	.	.	A	.	.
26	.	A	.	.	.	.	.	A	.	.	.	.	.	.	A	.	C
27	.	A	T	.	.	C	.	A	.	.	.	.	.	.	A	.	.
28	.	A	T	.	.	.	.	A	.	.	.	.	.	.	A	.	C
29	.	A	.	.	.	C	.	A	.	.	.	.	T	.	A	.	.
30	.	A	.	.	.	.	C	A	.	.	C	.	.	.	A	.	.
31	.	A	T	.	.	C	.	A	.	.	.	.	.	.	A	.	C
32	.	A	T	.	.	C	.	A	.	.	.	.	.	.	A	G	C

Fig. 3. *Bothrocara hollandi*. Nucleotides at polymorphic sites in part of the mitochondrial control region. Numbers of sites refer to positions shown in Fig. 2. Dots indicate nucleotides that are identical to those in the top line

RESULTS

The nucleotide sequence of the first half of mitochondrial control region (about 530 base pairs) of the dominant haplotype of *Bothrocara hollandi* is shown in Fig. 2. Only 1 polymorphic site was detected within the posterior part (that lacking underlining in Fig. 2) when the sequences of 18 individuals were compared. Thus, the anterior part, which was about 240 base pairs in length (underlined in Fig. 2), was sequenced for all remaining specimens. When we examined this region from all 296 samples, we found that 17 sites were polymorphic (Fig. 2). There were 2 nucleotide deletions/insertions and 15 nucleotide substitutions. The 296 individuals had a total of 32 haplotypes, as shown in Fig. 3. Two individuals exhibited heteroplasmy. One was collected at Stn 11 and had Mitochondrial Types 1 and 2; the other was collected at Stn 17 and had Mitochondrial Types 23 and 28 (Table 2).

Fig. 4 shows the phylogenetic relationships among the resultant haplotypes. Fourteen haplotypes (Nos. 9, 15, 16, 18 and 23 though 32) formed the most derivative cluster. All the maximum parsimonious trees supported the monophyly of this cluster. This group and the group that included all the remaining haplotypes are hereinafter referred to as Group A and Group B, respectively.

Individuals in Group B had been collected from all sampling stations while those in Group A had been collected from stations at depths <1100 m (Table 2, Fig. 5). In addition, individuals in Group A were distributed predominantly off the western part of Honshu Island and their easternmost occurrence was at a depth of 600 m at Stn 7, off Awa-shima Island, Niigata Prefecture (Table 2, Fig. 5). At Stn 6, approximately 8 km northeast of Stn 7 (Fig. 1), at a depth of 594 m, no individuals belonging to Group A were collected (Fig. 5). The genetic diversity of all individuals in Group B was lower than that of individuals in Group A (Table 3).

Genetic differentiation between the 2 morphotypes of *Bothrocara hollandi* was tested for 3

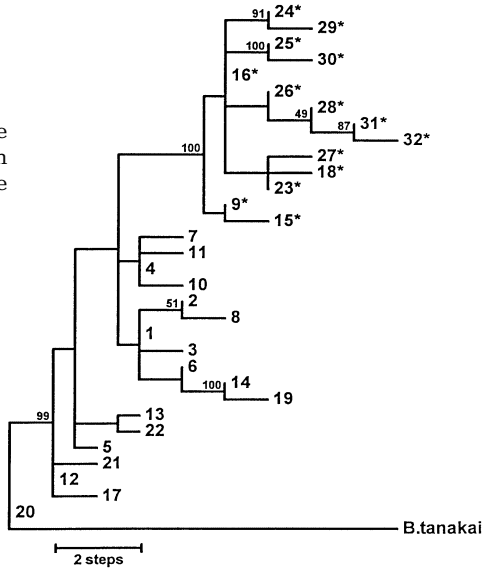

Fig. 4. *Bothrocara hollandi*. 50% majority rule consensus phylogram of 42 steps, showing results of the maximum parsimony analysis of haplotypes of *B. hollandi*, with *B. tanakae* as the outgroup (CI [Consistency Index] 0.385; RI [Retention Index] 0.771). Consensus values of more than 50% are shown above branches. Asterisks indicate hap-lotypes in Group A

Table 2. *Bothrocara hollandi*. Haplotype (H) composition of each population. Sampling stations arranged in order of increasing depth. *Haplotypes of Group A

H	Stn																	Total
	9	13	10	3	1	11	16	4	2	8	7	6	5	14	12	15	17	
1	9	15	7	13	8	16[a]	9	14	10	4	6	9	6	5	1	0	2	134[a]
2	0	0	0	0	0	1[a]	0	0	0	0	0	0	0	0	0	0	0	1[a]
3	2	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	4
4	2	1	1	4	1	4	1	5	3	2	2	4	1	3	1	0	1	36
5	0	1	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	4
6	0	1	0	0	2	0	0	1	0	0	0	0	0	0	0	0	0	4
7	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
8	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1
9*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
10	0	1	0	1	0	0	1	0	1	0	0	1	0	0	0	0	0	5
11	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
12	1	0	0	3	2	0	1	3	2	7	3	5	4	5	2	6	0	44
13	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
14	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	2
15*	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	2
16*	0	0	0	0	0	0	1	0	0	1	0	0	0	2	1	3	1	9
17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
18*	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	2
19	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
20	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
21	1	1	1	2	1	0	0	1	0	0	0	1	0	0	0	0	0	8
22	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1
23*	0	0	0	0	0	0	1	0	0	2	1	0	0	1	3	4	5[a]	17[a]
24*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
25*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	2
26*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
27*	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	2
28*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	1[a]	4[a]
29*	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	2
30*	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1
31*	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1	3
32*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1

[a]Includes 1 heteroplasmic individual (see 'Results' for details)

transects along which samples from each station were composed of a single morphotype (Table 4). For all 3 transects, no genetic differentiation between the 2 morphotypes was detected (AMOVA, $p > 0.05$). Genetic structures were also compared among the 3 sea areas (Table 5): off Hokkaido Island (Stns 1 and 2), off northern Honshu Island (Stns 3 to 9), and off western Honshu Island (Stns 10 to 17); and among 4 depth ranges (Table 6): <500 m (Stns 15 and 17), 500 to 1000 m (Stns 2, 5, 6, 7, 8, 12 and 14), 1000 to 1500 m (Stns 1, 3, 4, 11 and 16) and >1500 m (Stns 9, 10 and 13). Although no genetic differentiation was detected among sea areas (AMOVA, $p > 0.05$), a clear genetic structure was evident along the depth gradient (AMOVA, $p < 0.01$).

Fig. 6 shows the relationships between the genetic diversity of the population and the sampling depth for *Bothrocara hollandi*. Genetic diversity decreased significantly with increasing depth for all individuals sampled ($p < 0.001$) as well as for those in Group B alone ($p < 0.05$).

DISCUSSION

Our phylogeographical study of the dominant deep-sea demersal fish *Bothrocara hollandi* in the Japan Sea revealed a clear genetic structure. On the basis of the nucleotide sequences of part of the mitochondrial control region of 296 individuals collected from 17

Table 3. *Bothrocara hollandi*. Genetic diversity of the 2 groups

Diversity index	Group A	Group B
Gene diversity	0.85 ± 0.04	0.66 ± 0.03
Nucleotide diversity	0.00552 ± 0.00385	0.00265 ± 0.00227

Table 4. *Bothrocara hollandi.* Result of analysis of molecular variance (AMOVA) for genetic differentiation between morphotypes collected from stations on 3 transects. p: probability of having a more extreme variance component and ϕ-statistic than observed values by chance alone (1000 permutations)

Transect	Sampling stations with black type	white type	Variance components	Variance	% of var.	ϕ-stat.	p
I	3, 4	5	Among morphotypes	0.026	7.29	0.073	0.316
			Among populations within morphotypes	−0.008	−2.22	0.051	0.541
			Within populations	0.339	94.94	−0.024	0.227
II	10, 11	12	Among morphotypes	0.408	46.30	0.463	0.316
			Among populations within morphotypes	−0.023	−2.59	0.437	0.669
			Within populations	0.496	56.29	−0.048	<0.001
III	13	14, 15	Among morphotypes	0.145	17.08	0.171	0.326
			Among populations within morphotypes	0.049	5.74	0.228	0.117
			Within populations	0.657	77.17	0.069	<0.001

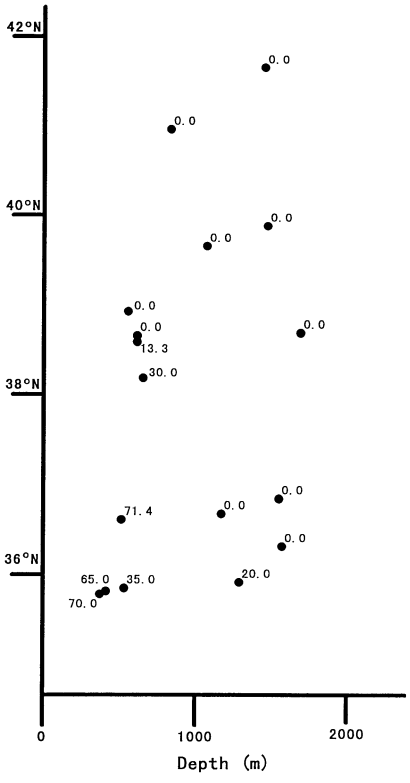

Fig. 5. *Bothrocara hollandi.* Distribution of the members of 2 groups of haplotypes (Group A and Group B). Geographical position (latitude) at each sampling station is plotted against depth, with the proportion (%) of Group A individuals in all the samples obtained at each station indicated beside each data point

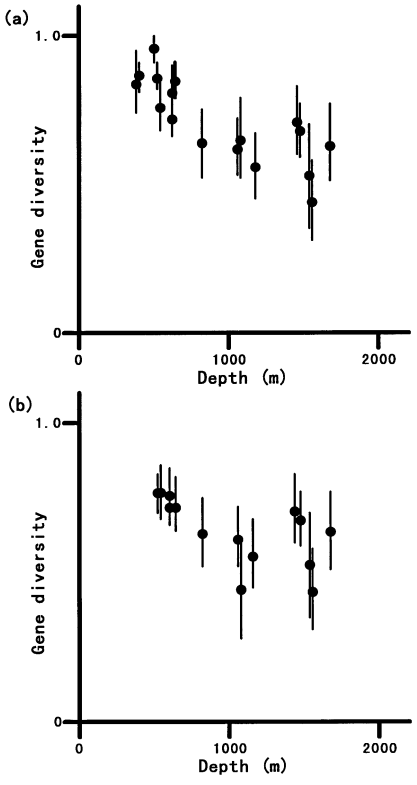

Fig. 6. *Bothrocara hollandi.* Relationships (means ± 1 SD) between depth and gene diversity at each sampling station based on individuals in both A and B groups (a) and on individuals in Group B only (b). Only statiFons for which data were available for >10 individuals were used to generate these plots

sites between depths of 375 and 1677 m, we were able to separate the collected specimens into 2 genetically distinct groups, namely a clearly monophyletic group (Group A) and a paraphyletic group that included the remaining haplotypes (Group B). The distribution of individuals in Group A was limited to the coastal area with depths shallower than 1100 m in the western part of the Japan Sea, while members of Group B were collected at all sampling sites (Fig. 5). In addition, the genetic diversity of individuals in Group B was lower than that of individuals in Group A (Table 3).

During the last maximum glacial period, the Japan Sea may have been an unsuitable habitat for marine

organisms because of severe anoxic conditions (Tanimura 1981, Oba et al. 1991, Tada 1994, Crusius et al. 1999, Tada et al. 1999, Gorbarenko & Southon 2000). *Bothrocara hollandi* might have survived this period by taking refuge at suitable sites in the Japan Sea and/or adjacent sea areas. The accumulation of unique genetic characteristics at these sites and subsequent secondary contact upon migration to the present habitats might have led to the present geographical pattern of distribution of haplotypes. The paraphyly of haplotypes of Group B, with exclusion of the haplotypes of Group A (Fig. 4), suggests that members of Group A might be derived from a relatively small population of immigrants from the common ancestral population of both groups.

Table 5. *Bothrocara hollandi.* Result of analysis of molecular variance (AMOVA) for genetic structure among sea areas. p: probability of having a more extreme variance component and ϕ-statistic than observed values by chance alone (1000 permutations)

Variance components	Variance	% of var.	ϕ-stat.	p
Among sea areas	0.030	4.97	0.050	0.080
Among populations within sea areas	0.075	12.30	0.173	<0.001
Within populations	0.505	82.73	0.129	<0.001

Table 6. *Bothrocara hollandi.* Result of analysis of molecular variance (AMOVA) for genetic structure among depth ranges. p: probability of having a more extreme variance component and ϕ-statistic than observed values by chance alone (1000 permutations)

Variance components	Variance	% of var.	ϕ-stat.	p
Among depth ranges	0.072	11.66	0.117	0.008
Among populations within depth ranges	0.041	6.69	0.184	<0.001
Within populations	0.505	81.64	0.076	<0.001

Using the method proposed by Jukes & Cantor (1969), we calculated the genetic difference between the dominant haplotype in Group A (No. 23) and that in Group B (No. 1) to be 0.0081. Accepting the rate of evolution of the mitochondrial control region of fishes as 0.12 per million years (Brown et al. 1993, Bremer et al. 1995, Chenoweth et al. 1998), we estimate that the divergence between these 2 groups occurred about 34 000 yr ago, at a time corresponding to the early part of the last glacial period.

Specimens of *Bothrocara hollandi* in Group A were found predominantly in a shallower and more westerly part of the range of distribution of this fish than those in Group B. The highest relative frequency of Group A (71.4%) was observed at Station 17, which was situated in the westernmost part of the Japan Sea (Fig.1). The present distributional trend of Group A might be explained by the hypothesis that its ancestral population lived, isolated from Group B, in refuges located in a relatively shallow region in the westernmost part of the Japan Sea during the last glacial period, and that Group A is now expanding its distribution. Gorbarenko & Southon (2000) showed that the sea area on the western shelf of the Tsushima Strait did not become anoxic during the last glacial period, probably because of constant inflow of fresh seawater through the Tsushima Strait. This fact supports our above hypothesis. It is plausible to speculate that, while the severe environment of other areas of the Japan Sea during the last glacial period might have reduced the genetic diversity of the population in Group B, the high genetic diversity of Group A might have been maintained under the relatively moderate environmental conditions of its refuges.

We detected no genetic differentiation between the 2 morphotypes of *Bothrocara hollandi* (Table 4), and some dominant haplotypes were shared by both morphotypes. Phylogenetic analysis based on the nucleotide sequence of a mitochondrial gene for 16S ribosomal RNA also failed to reveal any genetic difference between the morphotypes (Yamaguchi pers. comm.). The morphological differences between the 2 morphotypes could be the results of local factors that govern the growth and coloration of the fish, such as food availability and predation.

The genetic structure of the population of *Bothrocara hollandi* appears to be determined by depth rather than geographical distance (Tables 5 & 6). We also found a significant decrease in the genetic diversity of populations of *B. hollandi* with increasing depth (Fig. 6a). This decrease was not so clear in Group B; the individuals of Group A, more genetically diverse than Group B, decreased in occurrence with increasing depth. This indicates that the bathymetric trend in the genetic diversity of the population mainly resulted from the individuals in Group A.

Although genetic differentiation between Groups A and B is relatively small and they are not reciprocally monophyletic (Fig. 4), the possibility that they have already reproductively isolated from each other still remains. If they represent sibling species, the discordance between grouping based on mitochondrial haplotypes and that based on morphotypes could also be attributable to hybridization between the 2 sibling spe-

cies. Analyses using neutral genetic markers coded in the nuclear DNA will greatly help in revealing the exact taxonomic relationship between the 2 groups.

The effects of changes in the marine environment can be expected to persist in the genetic structure of populations of marine organisms over a long period of time. Thus, it is possible to obtain information about the history of variations in the marine environment from the phylogeography of marine species (Avise 1992, Magoulas et al. 1996, Kojima et al. 2000). In order to evaluate more accurately the relative importance of paleoceanographic effects on the formation of the genetic structure of populations, comparative studies of various aspects of the life history, such as modes of development and feeding strategies, of many marine species are necessary. Marine organisms in the Japan Sea are ideal subjects for such phylogeographic studies because of the dramatic environmental changes that have occurred in the Japan Sea on the millennial timescale over the past 200 000 yr (Tada et al. 1999). We have started phylogeographic studies of some marine species, such as intertidal gastropods, starfishes, barnacles and deep-sea brittle stars, in both the Japan Sea and adjacent sea areas. Analysis of a variety of marine species, based on samples collected from waters not only around the Japanese Islands but also off Russia, China and Korea, should provide more detailed information that will help us to understand the genetic diversification of marine organisms associated with environmental variations in the Japan Sea.

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