

**A note on the possibility of identifying individual J stock animals from a mixed assemblage based on mitochondrial DNA analysis**

Mutsuo Goto<sup>1</sup>, Zang Geun Kim<sup>2</sup>, Hideaki Abe<sup>1</sup> and Luis A. Pastene<sup>1</sup>

*1 The Institute of Cetacean Research  
4-18 Toyomi-cho, Chuo-ku,  
Tokyo 104-0055  
Japan*

*2 National Fisheries Research and Development Institute  
408-1 Shirang-ri, Kijang-up, Kijang County,  
Pusan City, 619-900,  
Korea*

**ABSTRACT**

A total of 863 mtDNA control region sequences of the western North Pacific minke whales were examined to determine whether or not individual assignment in the J stock is possible. Samples were available from past coastal commercial whaling in Japan and Korea (sub-areas 6, 7 and 11), from by-catches in the Sea of Japan (sub-area 6) and from JARPN surveys in sub-areas 7, 8, 9 and 11. Phylogenetic reconstruction of unique sequences was based on the neighbor-joining method. The tree showed an informative cluster containing predominantly haplotypes occurring in the Sea of Japan or sharing J stock predominant haplotypes '3' and '5' of a previous RFLP analysis. Under the criteria that all the haplotypes in this cluster are unique to the J stock, we estimated the number of J stock individuals sampled by JARPN surveys in sub-areas 7, 8, 9 and 11. Very low frequencies were found in sub-areas 7, 8 and 9 but a larger frequency was found in sub-area 11. The alternative assumption is that the haplotypes contained in the cluster are predominant in the J stock but they also occur in the O stock perhaps in lower frequencies. If this second assumption is correct, the estimate that considered the haplotypes in the cluster as exclusive of the J stock, could be overestimated. Although at this stage we can not be sure whether or not the haplotypes contained in the cluster are absolute markers for the J stock, several biological studies that used our criteria to separate J stock individuals found significant differences between this group and other assumed to be from the O stock,

**INTRODUCTION**

Several studies on stock identity have been conducted on the western North Pacific minke whales since 1956 to the decade of the 80', and they supported the view of two different stocks occurring on either side of Japan (see review by Pastene *et al.* this

meeting). These stocks have been recognized by the IWC as the J and O stocks (IWC 1994). DNA-based studies conducted under the Japanese Whale Research Program under Special Permit in the North Pacific (JARPN) have confirmed the striking differences between these two stocks.

Both allozymes studies conducted in the decade of the 80' as well DNA-based studies conducted in the decade of the 90' were based on a single set of samples representative of the J stock. Such set was taken from former coastal whaling operation in Korean from an operation carried out in September-October 1982 in sub-area 6. Recent genetic studies based on these samples have been possible by means of the use of the polymerase chain reaction (PCR) technique, which permit the DNA analysis from old or from very small amount of tissue samples.

However, no samples from the J stock have been available for other kind of biological studies such as morphological, conception date, ecological markers, pollutant burden, which are conducted under the JARPN. It is well known that J and O stocks animals mix to each other in the southern part of the Okhotsk Sea (sub-area 11) (Wada, 1991; Best and Kato, 1992; Pastene *et al.* 1998). Using maximum likelihood methods, the proportion of these stocks in the mixed assemblage of sub-area 11, have been calculated by sex on a monthly basis (Pastene *et al.*, 1998). On the other hand, JARPN surveys have been conducted in this sub-area in August 1996 and July 1999 and 30 and 50 individual minke whales were sampled, respectively.

If the genetic assignment of J stock individuals is possible in sub-area 11, then these individuals can be used as representative of the J stock for other kind of biological studies conducted under the JARPN.

The aim of this study is to use a phylogenetic approach of mtDNA control region sequences, to explore the possibility of individual assignment in the J stock. The analysis is based on the total sequences obtained from both past commercial whaling operations in Japan and Korean and JARPN surveys. In addition we used a new set of samples obtained from by-catches in the Sea of Japan.

## **MATERIAL AND METHODS**

### **Samples**

Tissue samples used in this study were from past coastal whaling operation in Korea (sub-area 6, n=28), by-catches in the Korean side of sub-area 6 (n=8), by-catches in the Japanese side of sub-area 6 (n=10), past coastal whaling operation in Japan in sub-area 7 (n=146) and 11 (n=173), JARPN surveys in sub-areas 7 (n=139), 8 (n=91), 9 (n=188) and 11 (n=80). A total of 863 samples were used. By-catches samples were available from a co-operative sampling effort between Korea and Japan.

### **DNA extraction and sequencing analysis**

Laboratory procedures are as described in Goto and Pastene (this meeting). Apart from the by-catch samples, all the sequences of the mtDNA control region have been already reported elsewhere (Goto and Pastene, 1999; this meeting; Goto *et al.*, this meeting).

### **Phylogenetic approach**

The evolutionary distance between two nucleotide sequences was calculated according to Kimura's two parameters method (Kimura, 1980). Phylogenetic reconstruction of unique sequences (haplotypes) was made using the neighbor-joining (NJ) method (Saitou and Nei, 1987) as implemented in the PHYLIP package (Felsenstein, 1993). To evaluate the confidence intervals, we used the bootstrap method (Felsenstein, 1985).

As the sampling location of each individual is known, we investigated the relationship between a given cluster in the tree and geographical locality.

## **RESULTS AND DISCUSSION**

### **Variability of mtDNA control region sequences**

The total number of sequences examined was 863. A region of consensus of 487bp of the mtDNA control region was used. A total of 34 informative sites defined a total of 83 unique sequences (haplotypes) (Fig. 1). Apart one transversion and two deletions, all substitutions were transitions.

### **Phylogenetic tree**

Fig. 2 is a neighbor-joining-based phylogenetic tree showing the phylogenetic relationships among western North Pacific minke whales unique sequences. A sequence from the North Atlantic minke whale is used as an outgroup (Arnason *et al.* 1993).

Of the eleven haplotypes occurring in the Sea of Japan (sub-area 6), nine are located within the cluster specified in the box, but it should be noted that such cluster is not supported by a high bootstrap value. A mtDNA control region RFLP analysis (Goto and Pastene, 1997) had discriminated eight haplotypes and of them, haplotypes '3' and '5' were the predominant in the sample from the J stock in sub-area 6. The distribution of these two haplotypes is also shown in the tree. RFLP haplotypes '3' and '5' are also predominantly located within the box in Fig. 2.

Within the box in Fig. 2, we have defined two sub-groups on the basis of the position of informative sites. The first group is characterized either by a base C in position 290, 311 and 406 or by a base C in position 311 and A in position 463. The second group is characterized by a base G in position 298. These site positions could characterize J stock individuals. It should be noted that only haplotype 72 in the box is out of these characterizations.

One assumption is that individuals having some of the haplotypes marked in the box (all apart sequencing haplotypes '40', '72', '34' and '68') belong to the J stock, which is

predominantly distributed in the Sea of Japan. As some individuals having these haplotypes are distributed in other sub-areas, we can assume that some J stock individuals could migrate into the Okhotsk Sea or into the Pacific side of Japan. An alternative assumption could be that some of these haplotypes are normally present in both J and O stocks at different frequencies.

Based on the first assumption we calculated the number of J stock animals in the sub-areas surveyed by the JARPN (Table 1). We made two estimations, one considering all the haplotypes in the box in Fig. 2 (Estimation A) and the other excluding haplotypes '40', '72', '34' and '68', which neither are not represented in the Sea of Japan nor they are not characterized by RFLP haplotypes '3' and '5' (Estimation B).

Both estimations A and B give a low proportion of J stock individuals in sub-areas 7, 8 and 9 but a higher proportion in sub-area 11. Estimations for sub-area 11 correspond to the total sample obtained in July and August.

As mentioned earlier, we can also assume that the sequencing haplotypes in the box in Fig. 1 are predominant in the J stock but they also occur, with a lower frequency, in the O stock. This is an alternative explanation to the occurrence of these haplotypes in the Pacific side of Japan. From Fig. 2 we can observe that some sequencing haplotypes occurring in the Sea of Japan (haplotypes '62' and '11') are out of the cluster specified in the box. A possible explanation is that some O stock individuals could migrate into the Sea of Japan.

At this stage we can not establish whether the sequencing haplotypes in the box of Fig. 2 are absolute markers for the J stocks or alternatively they also occur in the O stock in lower frequencies. Several studies conducted under the JARPN have used all or part of the individuals listed in Table 1 (Estimation B) as representative sample of the J stock. These studies involved morphometry (Hakamada and Fujise, this meeting), fat index (Tamura *et al.*, this meeting), biological parameters (Zenitani *et al.*, this meeting), stable isotope analysis (Mitani *et al.*, this meeting) and ecological markers (Kuramochi *et al.*, this meeting). Most of these studies found, in at least one of the parameters examined, significant differences between the J stock sample (as defined by our mtDNA analysis) and the rest of the individuals assumed to be from the O stock.

Abe *et al.* (this meeting) used the mtDNA criteria for separating J and O stock individuals in sub-area 11. Their dendrogram based on microsatellite information was constructed first on the basis of the total JARPN individuals in sub-area 11. This sub-area was grouped close to sub-areas 7, 8 and 9 in the Pacific side of Japan and divergent from sub-area 6 in the Sea of Japan. The next dendrogram considered sub-area 11 separated into two groups: sub-area 11 (J) (J-stock) and sub-area 11 (O) (O stock), based on our mtDNA criteria (Table 1, Estimation B). This time, the dendrogram showed sub-area 11 (J) stock clustered with sub-area 6, even the length of the branch was relatively large, while sub-area 11 (O) clustered with sub-areas 7, 8 and 9. These results suggest that sub-area 11 (J) could contain also individuals from the O stock.

Despite these interesting results and as established earlier, at this stage we can not be sure whether the haplotypes in the box in Fig. 2 belong exclusively to the J stock. Further multidisciplinary research is necessary to clarify whether or not individual assignment to a given stock is possible. In this regard we used a new set of samples from by-catches from the Sea of Japan but the number of samples were still small. They were collected in a co-operative effort between Korean and Japan and this effort will continue in the future.

## REFERENCES

- Abe, H, Goto, M. and Pastene, L.A. This meeting. Population structure in the western North Pacific minke whale inferred from microsatellite analysis. Paper SC/F2K/J10.
- Arnason, U., Gullberg, A. and Widegren, B. 1993. Cetacean mitochondrial DNA control region: sequences of all extant baleen whales and two sperm whale species. *Mol. Biol. Evol.* 10:960-70.
- Best, P.B. and Kato, H. 1992. Report of the sub-committee on North Pacific minke whales, Appendix 5. Possible evidence from foetal length distributions of the possible mixing of different components of the Yellow Sea-East China Sea-Sea of Japan-Okhotsk Sea minke whale population(s). *Rep. Int. Whal. Commn* 42: 166.
- Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using bootstrap. *Evolution* 44: 1097-1108.
- Felsenstein, J. 1993. PHYLIP 3.5c (Manual and program available from Joe Felsenstein, University of Washington, WA, Seattle)
- Goto, M. and Pastene, L.A. 1997. Population structure of western North Pacific minke whale based on an RFLP analysis of mitochondrial DNA control region. *Rep. Int. Whal. Commn* 47: 531-537.
- Goto, M and Pastene, L.A. 1999. Genetic population structure in the western North Pacific minke whale examined by mtDNA control region sequencing analysis. Paper SC/51/RMP8 presented to the IWC Scientific Committee, May 1999 (unpublished). 12pp.
- Goto, M. and Pastene, L. A. This meeting. Population structure in the western North Pacific minke whale based on RFLP and sequencing analyses of mtDNA control region, using data from 1994-1999 JARPN surveys. Paper SC/F2K/J11.
- Goto, M., Abe, H. and Pastene, L.A. This meeting. Estimation of the mixing proportion of the J and O stocks in sub-area 11 using uni- and bi-parental genetic markers. Paper SC/F2K/J27.

- Hakamada, T. and Fujise, Y. This meeting. Preliminary examination on spatial difference of external measurement of minke whales in the western part of the North Pacific, using data collected during 1994-1999 JARPN surveys. Paper SC/F2K/J15.
- International Whaling Commission. 1994. Report of the Working Group on North Pacific minke whale management trials. *Rep. int. Whal. Commn* 44: 120-144.
- Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16: 111-120.
- Kuramochi, T., Araki, J., Uchida, A., Moriyama, N., Takeda, Y., Hayashi, N., Wakao, H., Machida, M. and Nagasawa, K. This meeting. Summary of Parasite and Epizoit Investigations during JARPN Surveys 1994-1999, with Reference to Stock Structure Analysis for the Western North Pacific Minke Whales. Paper SC/F2K/J19.
- Mitani, Y., Bando, T., Takai, N. and Sakamoto, W. This meeting. Diet records and stock structure of minke whales *Balaenoptera acutorostrata* around Japan examined by  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  analyses. Paper SC/F2K/J20.
- Pastene, L.A., Goto, M. and Kishino, H. 1998. An estimate of mixing proportion of 'J' and 'O' stocks minke whale in sub-area 11 based on mitochondrial DNA haplotype data. *Rep. Int. Whal. Commn* 48: 471-474.
- Pastene, L.A., Goto, M., Fujise, Y. This meeting. Review of the studies on stock identity in the minke whale *Balaenoptera acutorostrata* from the North Pacific. Paper SC/F2K/J1.
- Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic tree. *Mol. Biol. Evol.* 4: 406-425.
- Tamura, T., Ohsumi, S. and Fujise, Y. This meeting. Some examinations on the fatness of the western North Pacific minke whales. Paper SC/F2K/J25.
- Wada, S. 1991. Genetic heterogeneity in the Okhotsk Sea-west Pacific stock of minke whales. Paper SC/43/Mi32 presented to the IWC Scientific Committee, May 1991 (unpublished) 17pp.
- Zenitani, R., Kato, H. and Fujise, Y. This meeting. Analysis on some biological parameters in the western North Pacific minke whales, from views point of the stock identification. Paper SC/F2K/J13.

Table 1: Number of J stock animals in the sub-areas surveyed by the JARPN according two estimations (see text). In parenthesis are the percentage of occurrence.

| Sub-area | Total sample size | Estimation A | Estimation B |
|----------|-------------------|--------------|--------------|
| 7        | 139               | 9 (0.065)    | 7 (0.050)    |
| 8        | 91                | 10 (0.110)   | 5 (0.055)    |
| 9        | 188               | 6 (0.032)    | 3 (0.016)    |
| 11       | 80                | 25 (0.313)   | 24 (0.300)   |

|       | 10          | 20          | 30          |       |
|-------|-------------|-------------|-------------|-------|
|       | 1           | 1111222222  | 2222223333  | 3444  |
|       | 11288892    | 2377000125  | 7788991346  | 7066  |
|       | 6907002905  | 6234489803  | 2489081213  | 3638  |
| Cons. | ATTACTTCCA  | CCGTGCCATG  | TCAGTGTAGG  | CCAA  |
| Hap27 | -.G.....    | ...A.....   | ...CA.....  | ...   |
| Hap28 | -.G..C...   | ...A...A... | ...CA.....  | ..G.  |
| Hap74 | -.G..C.T.   | ...A...A... | ...CA.....  | ..G.  |
| Hap36 | -.GT.C...   | ...A...A... | ...CA.....  | ..G.  |
| Hap47 | -.GT.C.T.   | ...A...A... | ...CA.....  | ..G.  |
| Hap30 | -.G.....    | ...A.....   | ...CA.....  | ..G.  |
| Hap53 | -.G...T.    | ...A.....   | ...A.....   | ...   |
| Hap09 | -.G...T.    | ...A.....   | ...A.....   | ..G.  |
| Hap78 | -.G...TT.   | ...A.....   | ...A.....   | ..G.  |
| Hap16 | -.G.....    | ...A.....   | ...A.....   | ..G.  |
| Hap50 | -.G.....    | ...A.....   | ..G..A..... | ..G.  |
| Hap35 | -.G.....    | ...A.....   | ...A.....   | ..GG  |
| Hap41 | -.G.....    | ...CA.....  | ...A.....   | T.GG  |
| Hap62 | -.G...T.    | ...A.....   | ...CA.....  | ..G.  |
| Hap40 | -.G...T.    | ...A.....   | ...CAC...   | ..G.  |
| Hap72 | -.G.....    | ...A.....   | ...AC.A.    | ..G.  |
| Hap34 | -.G.C.....  | ...A.....   | ...CAC...   | ..G.  |
| Hap69 | -.G.C.....  | ...A.....   | ...AC.....  | ...   |
| Hap46 | -.G.....    | ...A.....   | ...CAC...   | ..G.  |
| Hap03 | -.G.....    | ...A.....   | ...CAC...   | ...   |
| Hap05 | -.G...T.    | ...A.....   | ...CAC...   | ...   |
| Hap22 | -.G.....    | ...A.....   | ...C.C...   | ..G.  |
| Hap68 | -.G...T.    | ...A.....   | ...C.C...   | ..G.  |
| Hap63 | -.G.....    | ...A.....   | ...C.C...   | ...   |
| Hap70 | -.G...T.    | ...A..G..   | ...C.C...   | ...   |
| Hap64 | ..G.....    | ...A.....   | ...C.....   | ..G.  |
| Hap80 | ..G.....    | ...A.....   | ...C.....   | ...   |
| Hap67 | ..G...T.    | ...T.....   | ...C.....   | ...   |
| Hap04 | .....       | .....       | .....       | ..G.  |
| Hap02 | .....       | .....       | ...T...     | ...   |
| Hap82 | .....       | ...T...C    | .....       | ...   |
| Hap01 | .....       | .....       | ...A...C    | ...   |
| Hap42 | .....       | .....       | ...A...C    | ...   |
| Hap48 | .....       | ...A.....   | ...A.....   | ...   |
| Hap10 | -.G.....    | ..A.A.....  | C...CA....  | ..TG. |
| Hap06 | -.G...T.    | ...A.....   | C...CAC...  | ..TG. |
| Hap21 | -.G...T.    | ...A.....   | C...CA....  | ..TG. |
| Hap56 | -.G...T.    | ...A.....   | C...CA....  | ..TG. |
| Hap39 | -.G.....    | ...A.....   | ...CA....   | ..TG. |
| Hap14 | -.G.....    | ...A.....   | ...CA.G...  | ..TG. |
| Hap51 | -.G.....    | ...A.....   | ...CA.G...  | ..G.  |
| Hap44 | -.G.....    | ...A.....   | ...CA.G...  | ..TGG |
| Hap45 | -.G...T.    | ...A.....   | ...CA.G...  | ..GG  |
| Hap32 | -.G...T.    | ...A.....   | ...CA....   | ..T.. |
| Hap54 | -.G.....    | ...A.....   | ...CA....   | ..T.. |
| Hap65 | -.G.....    | ...A.....   | ...CA....   | ..T.. |
| Hap11 | -.G...T.    | ...A.....   | ...CA....   | ..TG. |
| Hap37 | -C.G...T.   | ...A.....   | ...CA....   | ..TG. |
| Hap15 | -.G...T.    | ...A.T...   | ...CA....   | ..TG. |
| Hap08 | -.G...G     | ...A.T...   | ...CA....   | ..TG. |
| Hap18 | -.G...TG    | ...A.T...   | ...CA....   | ..TG. |
| Hap52 | -.G...T.    | ...A.....   | ...AC....   | ..TG. |
| Hap66 | -.G...T.    | ...A.....   | ...A....    | ..TG. |
| Hap79 | -.G.....    | ...A.....   | ...AC....   | ..TG. |
| Hap60 | -.G.....    | ...A.....   | ..G..A....  | ..TG. |
| Hap81 | -.G.....    | ...A.....   | ...A....    | ..TG. |
| Hap07 | -.G...T.    | ...A.....   | ...CAC...   | ..TG. |
| Hap77 | -.G...TT.   | ...A.....   | ...CAC...   | ..TG. |
| Hap55 | -.G...T.    | ..T..A....  | ...CAC...   | ..TG. |
| Hap61 | -.G...T.    | ...A.....   | ...CAC..C   | ..TG. |
| Hap29 | -.G.....    | ...A.....   | ...CAC...   | ..TG. |
| Hap38 | -.G..C...   | ...A.....   | ...CAC...   | ..TG. |
| Hap73 | -.G.....    | ...A.....   | ...CAC...   | ..TG. |
| Hap83 | ..G.C....   | ...A...C.   | ...CAC...   | ..TG. |
| Hap26 | ..CG.C....  | ...A.T.C.   | ...CAC...   | ..TG. |
| Hap25 | ..CG.C....  | ...A...C.   | ...CAC...   | ..TG. |
| Hap57 | ..CCG.C.... | ...A...C.   | ...CAC...   | ..TG. |
| Hap33 | ..CG.C....  | ...AT.T..   | C...CAC...  | ..TG. |
| Hap19 | ..CG.C....  | ...AT..C.   | ...CAC...   | ..TG. |
| Hap23 | ..CG.C....  | ...AT..C.   | C...CAC...  | ..TG. |
| Hap31 | ..CG.C....  | ...T..C.    | ...CAC...   | ..TG. |
| Hap71 | ..G.C....   | ...T..C.    | ...CAC...   | ..TG. |
| Hap58 | -.G.C....   | ...A.....   | C...A....   | ..TG. |
| Hap59 | -.G.C....   | ...A.....   | C...AC....  | ..TG. |
| Hap43 | -.CG.C..T.  | ...A.....   | C...CAC...  | ..TG. |
| Hap13 | -.G.C..T.   | ...A.....   | C...CAC...  | ..TG. |
| Hap76 | -.G.C..T.   | ...A.....   | C...CAC...  | ..TG. |
| Hap17 | -.G.C....   | ...A.....   | C...CAC...  | ..TG. |
| Hap20 | -.G.C....   | T...A....   | C...CAC...  | ..TG. |
| Hap12 | -.G.C....   | ...A.....   | C...CACG..  | ..TG. |
| Hap24 | -.G.C....   | ...A.T...   | C...CACG..  | ..TG. |
| Hap49 | -.G.C..T.   | ...A.T...   | C...CAC...  | ..TG. |
| Hap75 | -.G.C....   | ...A.T...C  | ...CAC...   | ..TG. |
|       | 1222222222  | 2222222322  | 2222222222  | 2222  |

Fig. 1. Variable sites defining 83 North Pacific minke whale unique sequences (haplotypes) in the mtDNA control region. The column on the left is haplotype ID. The numbers above list the nucleotide position of the polymorphic sites from the 5 end of the mtDNA control region. Cons. means the consensus sequence which is same sequence with haplotype 1 (Hap1).



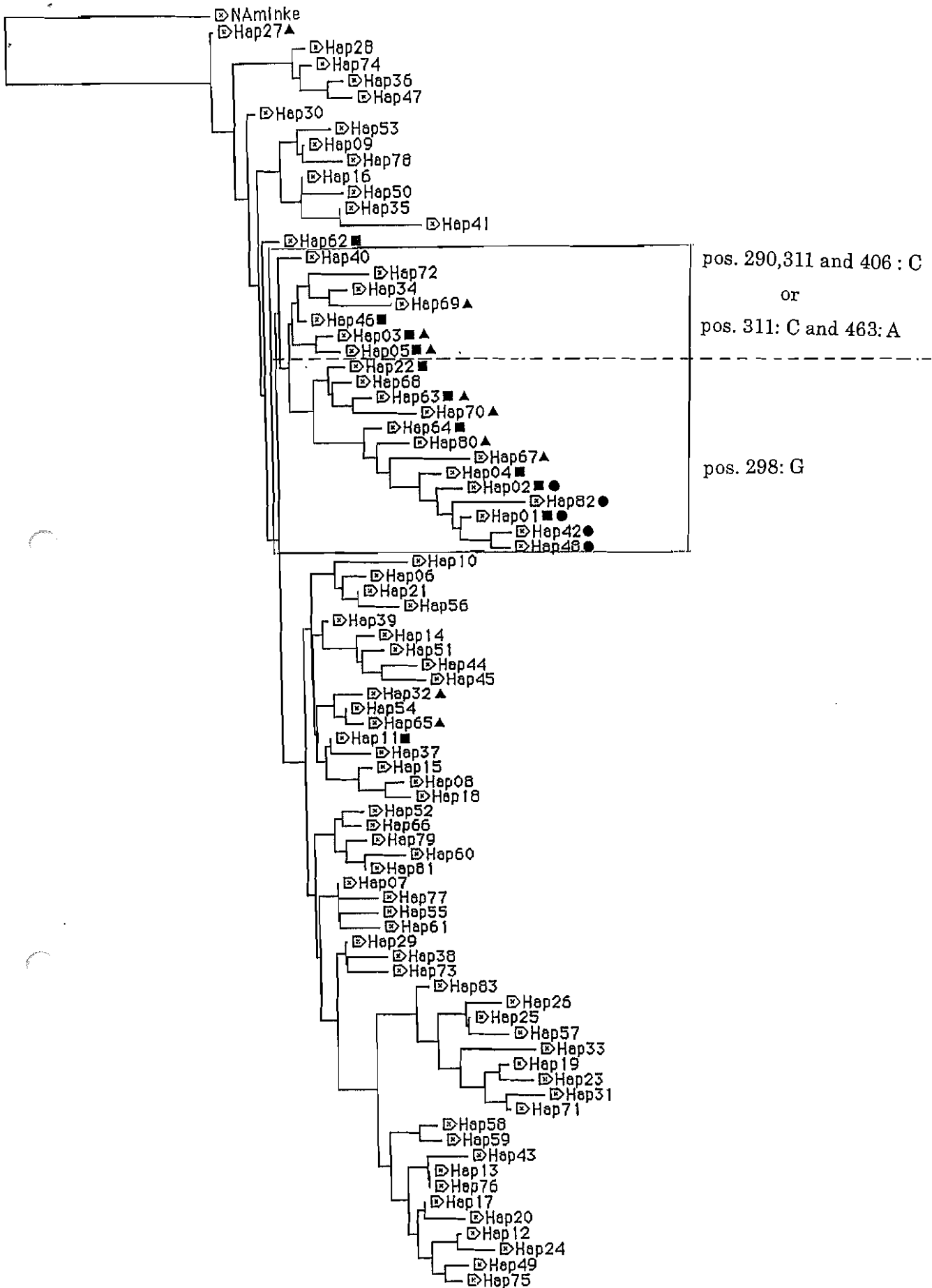


Fig. 2. Phylogenetic relationship of the 83 unique mtDNA control region sequences constructed by the neighbour-joining method. The sequence of North Atlantic minke whale (NAminke) is added as outgroup. ■ : Haplotypes occurring in samples from Sea of Japan, ▲ : RFLP haplotype 3 and ● : RFLP haplotype 5.