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Typing of Northeast Atlantic Minke Whales, *Balaenoptera acutorostrata*, by Arbitrary Amplification of Polymorphic DNA

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ABSTRACT

This work describes the conditions for application of arbitrary amplification of polymorphic DNA for stock determination in the minke whale. We screened 192 10-mer primers, 19% of which were useful to detect polymorphisms. Seven of these primers were used to analyze 31 minke whales caught along the Norwegian coast, Bear Island and Spitsbergen. Although due to the small number of animals and primers analyzed no final conclusions must be drawn, our results do not support the existence of distinct breeding stocks in these areas. The assertion is based on: the low percentage of primers which revealed polymorphisms; the high number of alleles with high frequencies (6 out of 14 had a frequency higher than 0.77 and 3 of those had a frequency higher than 0.90); the fact that the average fraction of bands matched was sometimes greater across than within areas and the lack of important systematic variation revealed by principal component analysis. The extreme bias which exists in the sex distribution of minke whales across the mentioned areas did not affect the above preliminary conclusions.

INTRODUCTION

The arbitrarily primed polymerase chain reaction, AP-PCR [24] or random amplification of polymorphic DNA, RAPD [28], are two variants of the same technique. The technique generates reproducible fingerprints from any organism (fowl parasites [19]; bacteria [13,14,15,27], insects [2,4], fish [6], plants [4,25], mink [29]) without a need for previous knowledge of specific DNA sequences, since fragments of DNA from any source can be amplified with arbitrary primers. Some of these fragments are polymorphic, *i.e.*, present in some individuals but absent from others. These polymorphisms, which are usually frequent, are useful as genetic markers and are inherited in a Mendelian fashion [24,26,28]. It has been proposed that the RAPD technique may be useful to generate individual fingerprints in the minke whale, *Balaenoptera acutorostrata* [11].

However, most of the polymorphisms generated by this technique are scored as dominant markers, and therefore heterozygotes cannot be distinguished accurately [24,28]. In addition, there are several technical factors that must be taken into consideration when standardizing this technique: the pattern of amplified fragments obtained is not only dependant on the primer-template combination, but also on the concentrations of DNA template, primer and magnesium

included in the reaction mixture [7]; the denaturing, annealing and extension times [31]; type of DNA polymerase and thermocycler used [16] and on the method of DNA extraction [13].

Here, we describe the application of this technique to the study of genetic variation among minke whales from the Northeast Atlantic. Genetic variability among minke whale stocks has been studied by different techniques including, enzyme electrophoresis [5]; simple-sequence length polymorphisms [20], restriction fragment length polymorphisms and subsequent probing [21]; and sequencing of the mitochondrial D-loop [3]. Different techniques have rendered contradictory results in some cases. Thus, while enzyme electrophoresis indicated that minke whales from West Greenland and Iceland represented two different breeding populations [5], restriction fragment analysis of mitochondrial and ribosomal DNA from minke whales from the same areas did not reveal significant differences [18]. However, as the authors of both papers indicated, the sex ratio of the samples may have influenced their results. The relationship between minke whales from Norway, West Greenland and Iceland was also studied by Danielsdottir *et al.* [5], who concluded that Norway represented a third breeding stock. This conclusion, however, is based on 12 animals of unknown sex and should be interpreted with care, as the authors indicate.

Due, not only to the scarcity of data on the Norwegian minke whales, but also on the contradictory results mentioned above, it is necessary to carry out an extensive study of minke whales from the Northeastern Atlantic to settle this controversy and provide the management authorities with sounded data on the boundaries between or among breeding populations. Of all the possible techniques, except perhaps isoenzyme analysis, arbitrary amplification of polymorphic DNA offers the highest output, speed to render results, and lowest cost.

MATERIALS AND METHODS

Animal samples

The minke whales used in this work were captured in four different areas in Norwegian and adjacent waters (Fig. 1) during the Norwegian scientific whaling expedition in 1994. Data on the samples are given on table 1; for additional data see [8,10]. Samples of muscle were excised and frozen at -20°C.

Extraction of genomic DNA

Genomic DNA was prepared according to Miller *et al.* [17] with some minor modifications. Briefly, about 100 mg of sample were excised, minced and washed with 500µl of sterile ddH_2O. Followed by a brief centrifugation, 5min at 14,000 rpm; the pellet was resuspended in 300µl of 10mM tris, 2mM EDTA, 400mM NaCl, pH 8.0. Another 300µl of the same buffer containing 1.6% SDS and 400µg/ml proteinase K were added to the tubes. After an incubation of 4 to 6 h at 55°C, 280µl of a 6M NaCl (saturated) solution was added to the tubes which were then vortexed for 3min, followed by a centrifugation of 20min at 14,000 rpm. Eight hundred µl of the supernatant were added to 960µl of isopropanol, the tubes were inverted several times and kept at -80°C for 1-2h. Following a centrifugation of 20min at 14,00rpm the supernatants were eliminated and the pellets washed with 500µl of 70% ethanol, which was subsequently removed. The washed pellets were allowed to dry for about 15min at 55°C, and then resuspended in 200µl ddH_2O. To fully redissolve the DNA, the tubes were incubated at 55°C for 12-16h. Then, they

were centrifuged for 10min at 14,000 and the clear supernatants transferred to new tubes for further analysis.

Arbitrary amplification of DNA

To establish the range of DNA template concentration which gave reliable results, *i.e.* bands dependant on the primer-template combination and not concentration-dependant bands *i.e.*, bands dependent on the amount of template DNA [7,13]; four serial dilutions of each sample (1:25; 1:125; 1:625 and 1:3,125) were analyzed in combination with different primers. Two of the concentrations which gave consistent patterns were chosen for subsequent analyses.

Amplifications, essentially as described by [24,28], were carried out in 30µl volumes containing 50mM Tris-HCl, pH 9.1, 16mM ammonium sulfate, 6.5mM MgCl2 [7], 100µg/ml BSA, 100µM each of dATP, dCTP, dGTP and dTTP, 0.4µM primer, 1.5 units Klen Taq1 DNA polymerase (Ab Peptides Inc. St Louis Missouri) and genomic DNA (different concentrations as shown in the figures). The reaction mixtures were overlaid with 20µl of Chill Out liquid wax (MJ Research Inc., Watertown, Massachusetts) and amplification was performed on a PTC-100 programmable thermal controller (MJ Research, Inc.) programmed as follows: an initial step of 94°C, 2 min, followed by 40 cycles of 94°C 20 sec, 35°C 20 sec, 72°C 2min, one step of 72°C 5min, [31] and a final step of 15°C 5sec.

Fifteen µl of the products obtained were loaded into the wells of 2% agarose gels (3:1 Seakem LE: Nusieve, FMC products), and the fragments were resolved by electrophoresis in 0.5xTBE. The gels were stained in the same electrophoresis buffer containing 0.5µg/ml ethidium bromide, destained and photographed.

Selection of primers

A total of 192 10-mer primers from Operon Technologies (Alameda, California) were screened. The screening process was as follows: each of the 192 primers was used to amplify the DNA from two or four minke whales from different areas and usually also of different sex. The fragments were resolved by gel electrophoresis, as described above. The "patterns" obtained fell into 4 categories (see results): none or very few products; concentration-dependant patterns, blurred patterns, and well resolved patterns. Not all the primers which generated well resolved patterns disclosed polymorphic fragments on the minke whales used for the screening.

For this work, we chose 7 primers which generated well resolved and polymorphic patterns: OPG-18 (5'GGCTCATGTG-3'), OPH-12 (5'ACGCGCATGT-3'), OPH-18 (5'GAATCGGCCA-3'), OPL-01 (5'GGCATGACCT-3'), OPU-11 (5'AGACCCAGAG-3'), OPV-07 (5'GAAGCCAGCC-3') and OPV-20 (5'CAGCATGGTC-3').

Treatment of the data

Only polymorphic fragments that were clearly distinguishable were scored. A matrix of absence (0) and presence (1) for each fragment and each minke whale was constructed and treated in two different ways.

Relatedness among minke whales was calculated as described by [2]. RAPD loci segregate independently and usually more than 95% of the alleles are dominant [28]. The dominant

phenotype is expressed as the presence of an amplified DNA fragment of a specific molecular weight, and the recessive is the absence of that band. As described by [2], we measured the similarity of pairs of minke whales by examining both the shared absence and presence of each polymorphic band. The fraction of matches (M) was estimated using the formula:

$$M = N_{AB}/N_T$$

where N_{AB} is the total number of matches in individuals A and B (both present or both absent), and N_T is the total number of loci scored in the study. An M value of 1 indicates that two individuals have identical patterns and an M value of 0 indicates that two individuals have completely different patterns. Values of M were calculated among all n(n-1)/2 (=465) pairs of n (=31) minke whales.

Ideally, however, one wished to find a "marker" (polymorphic band) that is absent in all animals belonging to one given stock (all of them homozygote recessives for the particular band), and present in animals from other stocks (heterozygotes or homozygote dominants). The second best choice would be a marker with very different frequencies among stocks (very high in one stock and very low in another). Accordingly, to establish whether we had some allele able to reveal such systematic variation among the minke whales; whether there was geographical systematic variation among the minke whales and therefore whether minke whales from different locations would form clusters [12], the initial matrix of absence/presence of polymorphic bands was subjected to Principal Component Analysis with full cross-validation. The programm used was Unscramble [23].

RESULTS

DNA concentration

Figure 1 shows the effect of varying the concentration of DNA on the RAPD profile. Dilutions of 1:25 and 1:125 of the initial DNA gave the same patterns but with further dilutions (notably with 1:3,125), the pattern started to changed. After carrying out the titration of all 31 samples, two of the DNA concentrations which gave consistent results were chosen for further analysis.

Primers

To carry out a primer screening to select primers which can give useful genetic markers, one should use at least two individuals with each primer, with two different concentrations of DNA and the individuals should differ in the characteristic one wants to "mark". Either male/female, to find sex-linked markers; or different possible fathers [29], different families, stocks, etc. In our case, not knowing the relationships between the individuals, they were arranged so that individuals to be screened with the same primer were from different locations (possible different stocks) and usually of different sex. With this arrangement of individual minke whales, 19% of the 192 10-mer primers screened produced very few or no products; 17% caused concentration dependent bands; 9% blurred patters; 36% gave clear patterns, but no polymorphic bands were evident and the rest 19% did produce polymorphic bands (Fig. 2). This means that only the last 19% of the primers would have be chosen after an initial screening by using our samples. This number is much smaller than those reported in the literature: 25% [29], 30% [1], 100% [13,30]. The manufacturers of the 10-mer primers (Operon Technologies) indicate that their customers

report getting useful markers from about 50-98% of their 10-mer sequences. We may have overlooked possible useful primers due to the disposition of samples during the screening, as illustrated in Fig. 2f: primer OPH-18 was shown to produce 1 clear polymorphic fragment with the 4 minke whales shown in the figure, however, it would not have produced any if it had been screened with only the last two minke whales, although this still indicates small differences among minke whales from the four different areas covered by this work. Anyway, to check the possibility of having underestimated the number of possible useful primers, 2 of the primers which gave clear patterns and no polymorphisms were used to analyzed the 31 minke whales. No polymorphims were detected in this case either, thus supporting the previous observation.

Relationships among the minke whales

Seven of the possibly useful primers were chosen to screen the 31 minke whales and 14 polymorphic bands were scored. The distribution of frequencies of each band is shown in Fig. 3: 6 of the polymorphic bands had a frequency higher than 0.77, 6 bands had a frequency between 0.10 adn 0.60 and the remaining 2 bands were present in 1 and of the 2 minke whales respectively. These results contrast with those of Apostol *et al.* [2] in their study of family relationships among mosquito populations. They found that only 13% of their scored polymorphic bands had frequencies below 0.1, while the rest was comprised between 0.1-0.6. This result indicates, again, a close genetic relationship among the minke whales included in the present work.

Although only those bands with a frequency between 0.1-0.6 can be useful to discriminate family groups [2], a combination of primers which originate bands with extremely either high low frequency in our samples could be the most useful to identify minke whales belonging to different breeding stocks. In addition, not knowing the relationships among our sample, to carry out the analysis with only those alleles of frequency comprised between 0.1-0.6 would be meaningless.

The distribution of M values (fraction of matched bands) is shown in Fig. 4: the average was 0.70 ± 0.13 (STD) with a median of 0.71 (Fig. 4), and the total individual average M values are shown in Fig. 5. The average M values within and across areas is shown in Fig. 6. Although the area whose individuals showed the lowest average band match values, within and across areas was Bear Island, none of their individual M values was outside the range covered by the other areas. Moreover, the lowest individual average M values were, in order, due to one animal from Spitsbergen (0.58 ± 0.11) followed by another from Finnmark (0.59 ± 0.11). M values of 1 (full matches) on the other hand, were observed both across and whithin areas: individual 3, from Spitsbergen showed a full match with individuals 24 and 26 from Lofoten-Vesterålen; individual 4 from Spitsbergen, with 28 from Lofoten-Vesterålen; individual 12 from Bear Island, with 22 from Finnmark and finally, the only intra-area match was individuals 20 and 23, both from Finnmark.

Principal component analysis of the matrix of absence/presence (0/1) of the 14 scored alleles in the 31 minke whales revealed similar information. A model with 4 principal components explained only about 60% of the total calibration variance and about 40% of the total validation variance (Fig. 7), indicating a high degree of non-systematic, and unexplained, variability in the data set. The plots of the scores of each minke whale on each principal

component (Fig. 8) did not display clusters of minke whales according to area of capture, which should have been expected had there been either distinct breeding stocks in any of the areas included in this work. There was no clustering either according to the sex of the minke whales, indicating that none of the polymorphic bands were linked to the sex of the animals. The animals which had a full match, M value =1, appear, as expected, superimposed in the scores plots.

DISCUSSION

Amplification of polymorphic DNA using arbitrary primers is a useful a technique to study genetic relationships among minke whales, as has been proven for all other species studied. It does not require extremely pure DNA, as previously shown in other publications [13,14] and confirmed in this paper. The concentration of DNA, however must be within a range in order to obtain consistent results and at least two different concentrations should be analyzed [13,24]. Otherwise, primers which give what has been called "concentration-dependant" bands may be wrongly considered to give individual-dependant patterns. The patterns obtained are usually consistent within laboratories, but for this technique to be used to make interlaboratory comparisons, the variables mentioned in the introduction which influence the bands obtained [7,13,16,31] should be standardized. Such standardization would permit to create a database where one introduced the patterns (or fingerprints) obtained with primers of relevance. Unknown samples could simply be introduced individually in the database and calculations made to see in which stock the unknown fits. If not, each laboratory should carry out the analysis of all necessary samples to perform identifications, classifications etc.

The two analyses of relationships among minke whales were consistent and none of them indicated the presence of distinct breeding stocks within these areas. PCA offers some additional advantages to detect animals from different stocks: *i*) it allows to evaluate the "weight" that each variable (the scored bands in this case) has on the principal components (Fig. 9) thus helping in the selection of primers which contain the most relevant information to expose differences among stocks, *ii*) it exposes clusters of individuals, revealing simultaneously which primers helped most in the clustering, indicating not only whether we have animals from one or more stocks in the data set, but also the primers that uncovered that particular information and *iii*) it allows the detection of "outliers", or individuals that do not fit in the general trend of the initial data set, if the data set contained only one of a few individuals from a different stock than the bulk. Moreover, the relationship outlier/principal component would also indicate whether the outliers may come from the same or from different stocks.

We must emphasise that this is a preliminary work and that it has to be extended to *i*) more primers, *ii*) more minke whales from the same locations included in this work and *iii*) more minke whales from other locations and from known different breeding stocks. However, all the present results point towards the same direction: small genetic differences among the minke whales captured in the areas of Spitsbergen, Bear Island, Finnmark and Lofoten-Vesterålen. This assertion is based on: the low percentage of primers which revealed polymorphisms (192 primers is an important number, and yet few revealed polymorphisms); the high number of alleles with high frequencies (6 out of 14 had a frequency higher than 0.77 and 3 of those had a frequency higher than 0.90); the fact that the average M values were sometimes smaller within than across areas; as well as the higher frequency of full matches across than within areas (3 *versus* 2) and

the lack of important systematic variation revealed by PCA. Moreover, when full matches were observed across areas, they did not necessary implicated neighbouring areas: 2 animals from Spitsbergen showed full matches with 3 individuals from Lofoten-Vesterålen, *i.e.*, the most separated locations.

Consequently, the present results support Bakke and El-Gewely's work [3]. They also found a very close genetic relationship among minke whales captured along the Norwegian coast. It has been noted however, a severe bias in the sex distribution of minke whales across the four areas included in this work (see also 8,9,22): the percentage of females is Spitsbergen is always much higher than that of males. The number of males increases towards the south, around Bear Island they constitute about 50% of the captured animals; their contribution to the total continues increasing in Finnmark and they clearly predominate in Lofoten-Vesterålen. Thus, although there were no detectable genotypic differences among minke whales which could be attributed to the location of capture, the distribution of males and females shows clearly a pattern. A sexually biased sample of minke whales, together with a small number of animals may have had an influence in previous works (see the discussion in Danielsdottir *et al.* [5] and [18]) in overstating differences among minke whales from different locations. RAPD analysis together with PCA, offers the additional advantage of revealing when polymorphic bands are linked to sex. In that case, the researcher can chose those primers for purposes other than interstock relationship studies.

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Table 1. Data on the minke whales used in this work. M, male; F, female. Month, latitude and longitude refer to the time and location of capture.

Area of capture	Minke whale no.	Sex	Length (cm)	Month	Latitude	Longitude
Spitsbergen	1	M	654	July	77° 35'	10° 15'
	2	F	555	"	78° 28'	09° 28'
	3	F	690	"	79° 18'	07° 34'
	4	F	700	"	79° 25'	08° 18'
	5	F	699	"	79° 31'	08° 34'
	6	F	686	"	77° 33'	10° 40'
	7	F	632	"	77° 36'	10° 14'
Bear Island	8	F	765	"	74° 23'	24° 01'
	9	M	620	"	74° 04'	15° 59'
	10	F	648	"	74° 25'	24° 10'
	11	M	755	"	74° 11'	15° 23'
	12	F	670	"	74° 05'	16° 00'
	13	M	810	"	74° 11'	15° 23'
	14	F	805	"	74° 04'	15° 59'
	15	M	800	September	73° 46'	18° 03'
Finnmark	16	F	620	July	71° 25'	30° 17'
	17	M	625	"	71° 33'	30° 02'
	18	F	765	"	70° 50'	30° 30'
	19	M	730	"	69° 58'	30° 47'
	20	F	523	September	70° 55'	29° 26'
	21	M	785	July	69° 59'	31° 04'
	22	M	789	"	71° 40'	28° 56'
	23	M	815	"	70° 39'	21° 10'
Lofoten-Vesterålen	24	F	680	"	68° 08'	14° 57'
	25	M	816	"	68° 42'	12° 34'
	26	F	610	September	67° 21'	12° 48'
	27	M	580	July	68° 41'	12° 33'
	28	M	752	"	69° 17'	15° 02'
	29	M	790	"	69° 21'	15° 48'
	30	M	825	"	69° 21'	15° 45'
	31	M	815	"	67° 11'	12° 30'

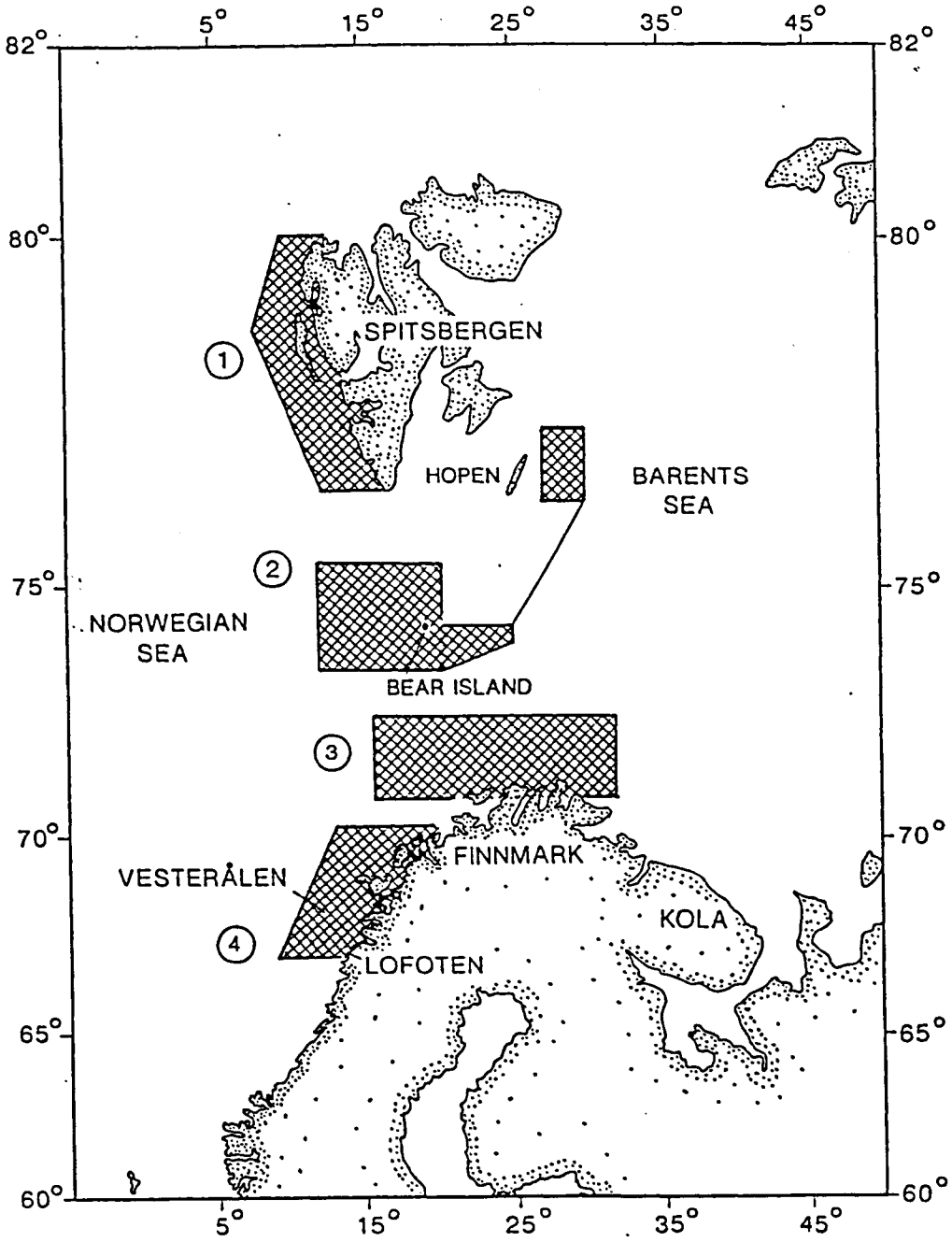

Figure 1.- Chart showing the four areas where the minke whales used in this work were captured. Areas: 1, Spitsbergen; 2, Bear Island; 3, Finnmark and 4, Lofoten-Vesterålen.

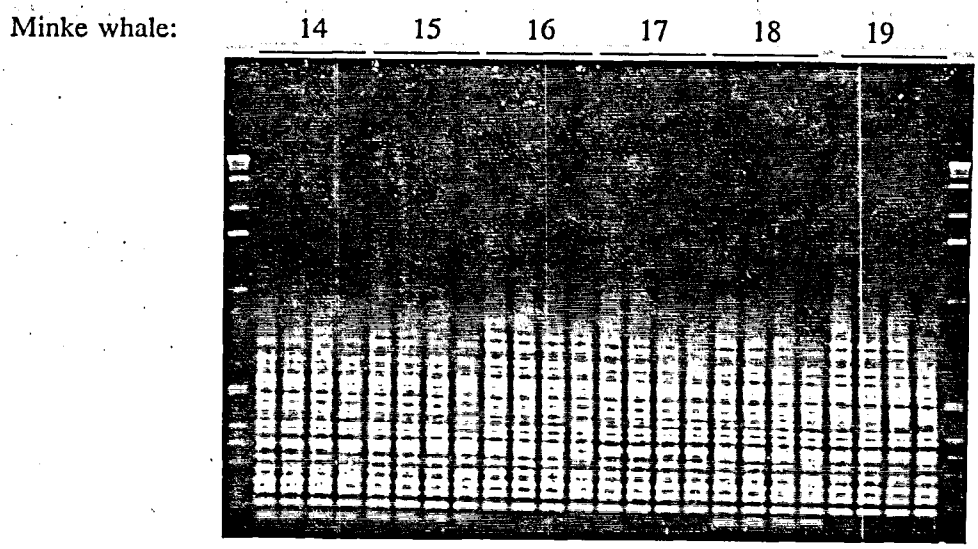

Figure 2.- Effect of the concentration of template DNA on the RAPD profile with primer OPG-18. The figure shows the results of the analysis of 4 different dilutions (from left to right: 1:25, 1:125, 1: 625 and 1:3,125) from the original DNA extract of 6 different minke whales. The arrowhead indicates one polymorphic band. Note that the pattern is altered at the highest dilution.

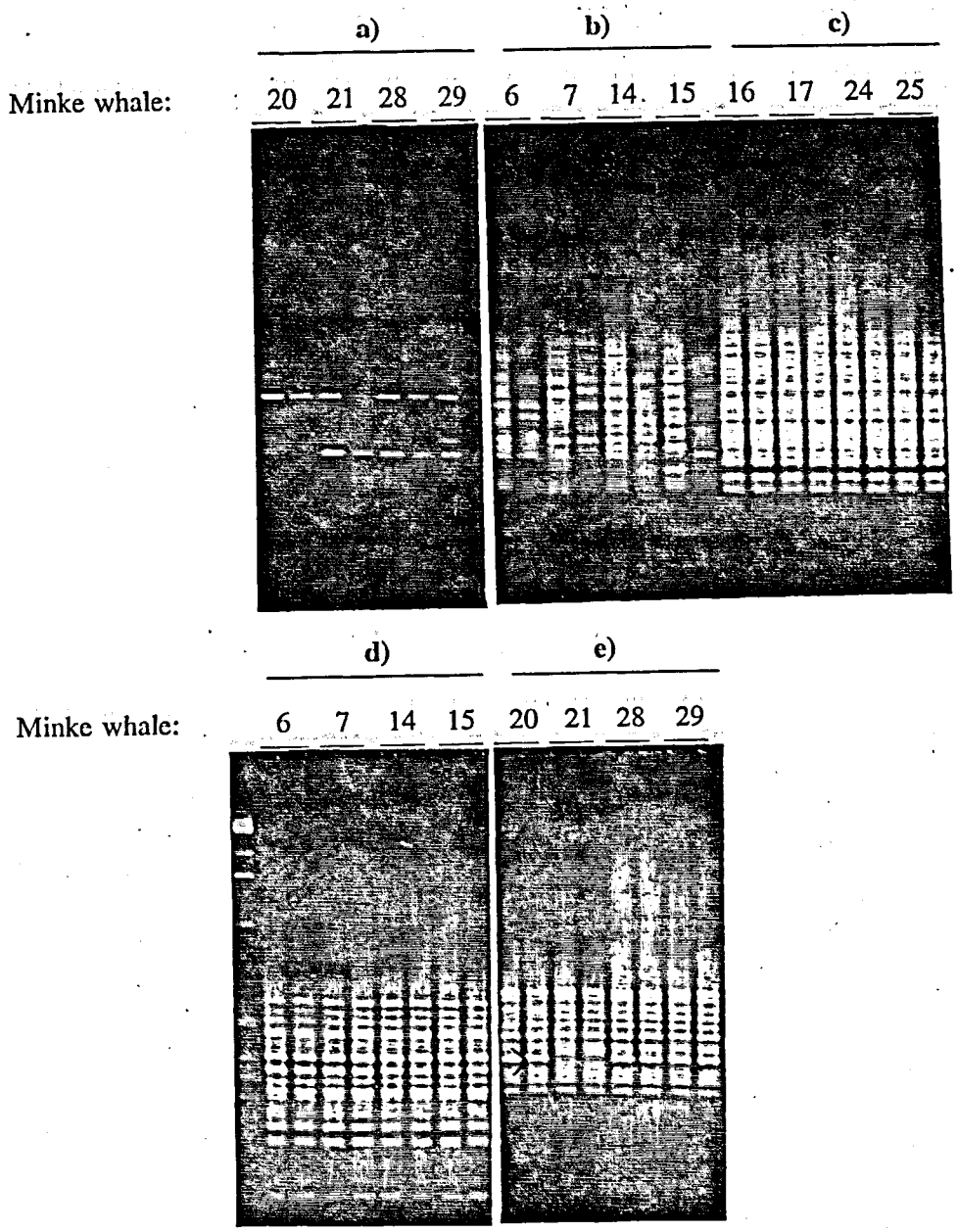

Figure 3.- Primer screening showing the 4 types of RAPD profiles obtained. a) a few bands (primer OPT-17); b) concentration-dependant bands (primer OPS-18); c) blurred patterns (primer OPS-19); d) clear bands but no polymorphisms (primer OPH-15) and e) clear bands and evidence of polymorphic bands (primer OPH-18). Each primer in the figure was used to analyze 2 concentrations of DNA from each of 4 minke whales. Two of the animals always belonged to different areas, and when possible, also to different sex. The arrowhead indicates one clear polymorphic band.

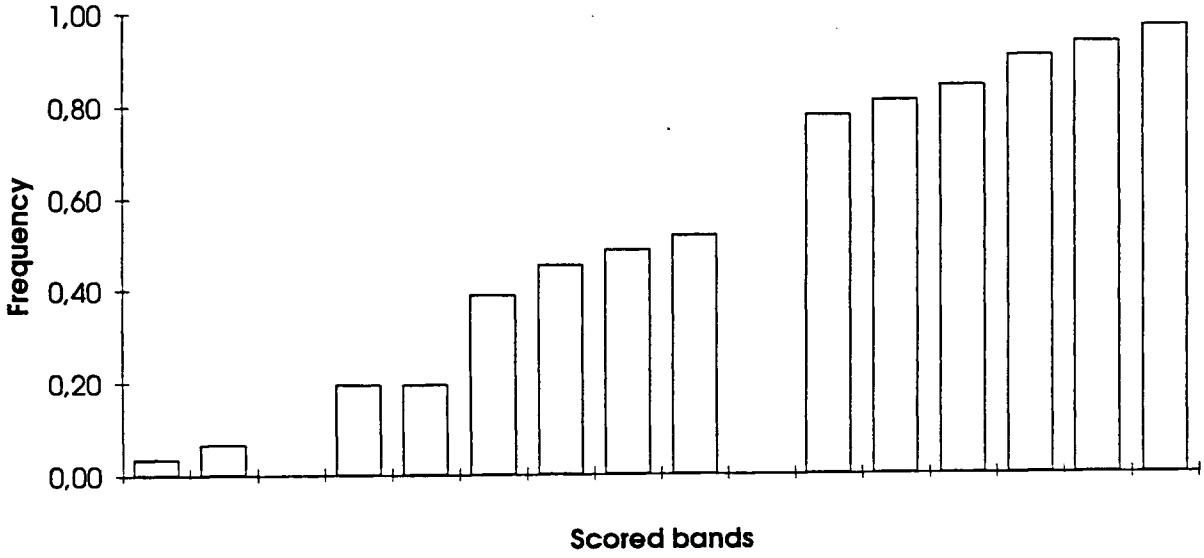

Figure 4.- Distribution of frequencies of the 14 polymorphic bands scored in the present study.

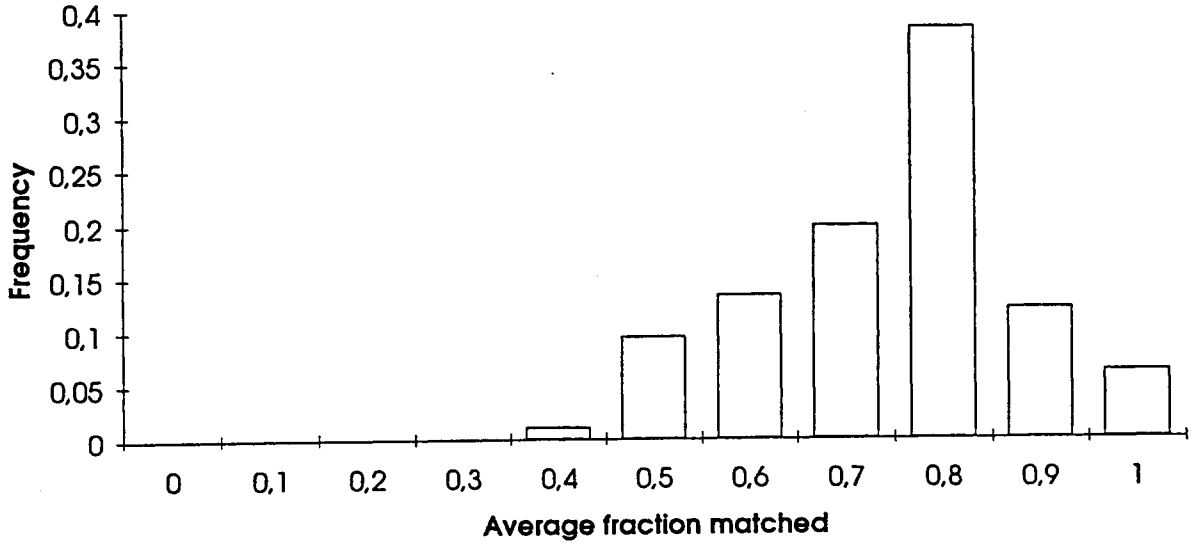

Figure 5.- Distribution of frequencies of M values (fraction of bands matched) among the 31 minke whales.

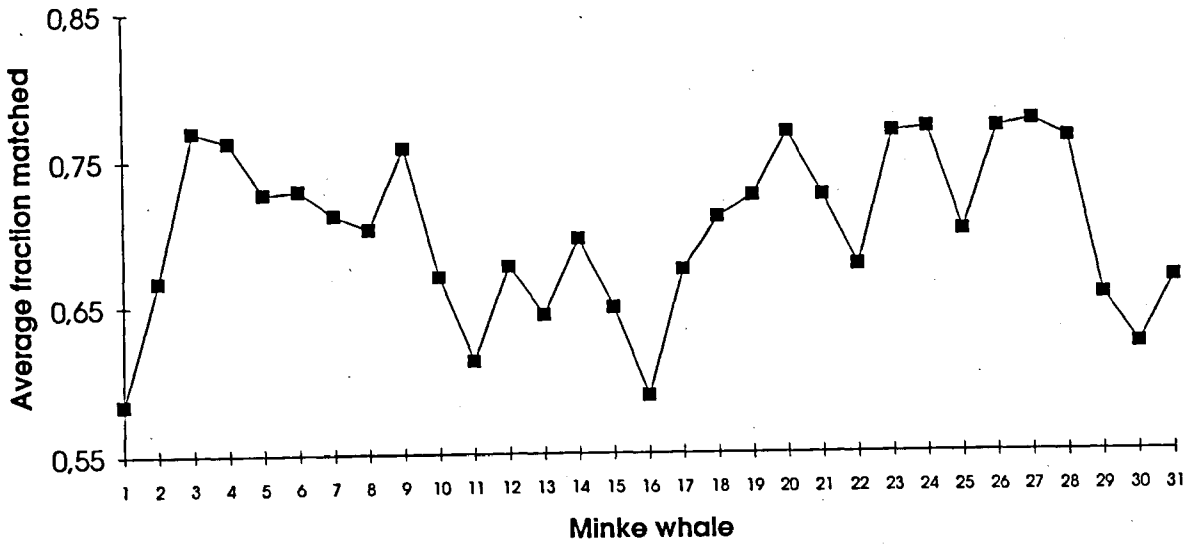

Figure 6.- Average fraction matched for each individual for the 14 scored polymorphic bands.

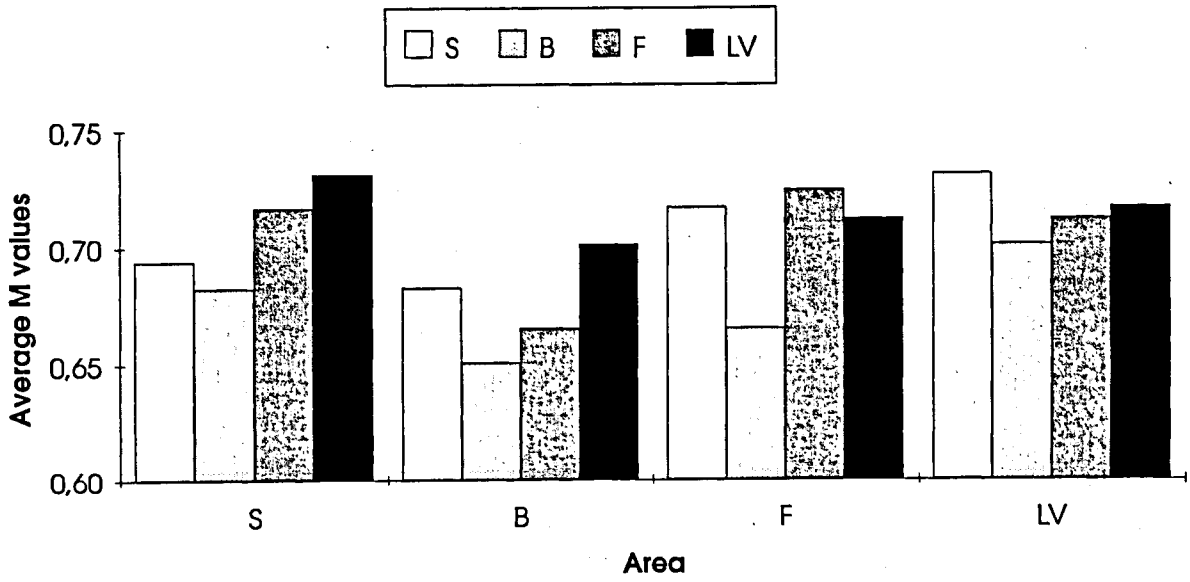

Figure 7.- Average fraction matched, of the 14 polymorphic bands, within and across areas. S, Spitsbergen; B, Bear Island; F, Finnmark; LV- Lofoten-Vesterålen.

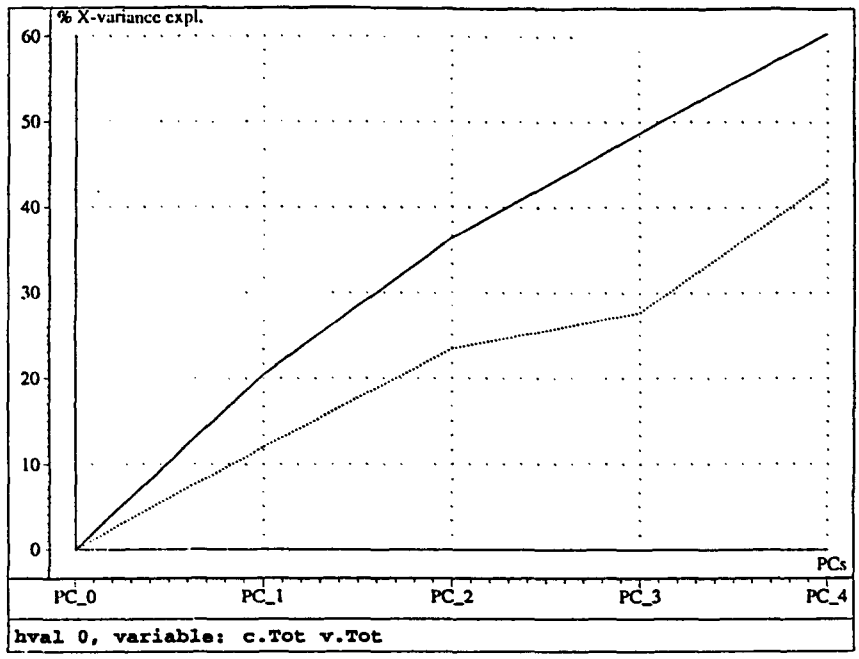

Figure 8.- Plot of the percent variance explained by a principal component analysis model with 4 principal components. Solid line, calibration variance; dotted line, validation variance.

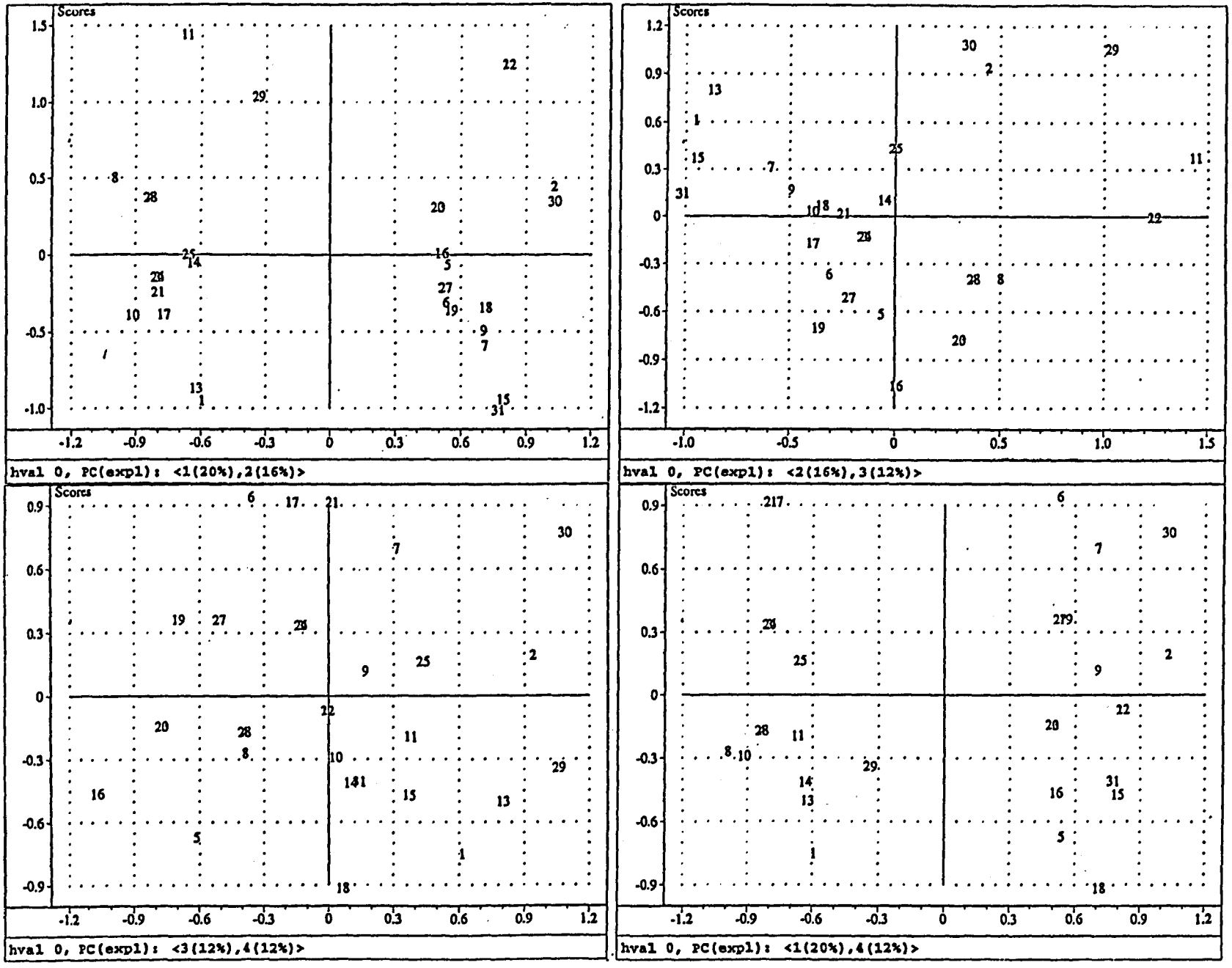

Figure 9.- Scores plot of the PCA model with 4 principal components. Upper left, PC1 (OX) versus PC2(OY); upper right, PC2 (OX) versus PC3 (OY); lower left, PC3 (OX) versus PC4 (OY) and lower right, PC1 (OX) versus PC4 (OY). The numbers represent the individual minke whales. Individuals 3, 24 and 26; 4 and 28; 12 and 22; and 20 and 23 appear superimposed.

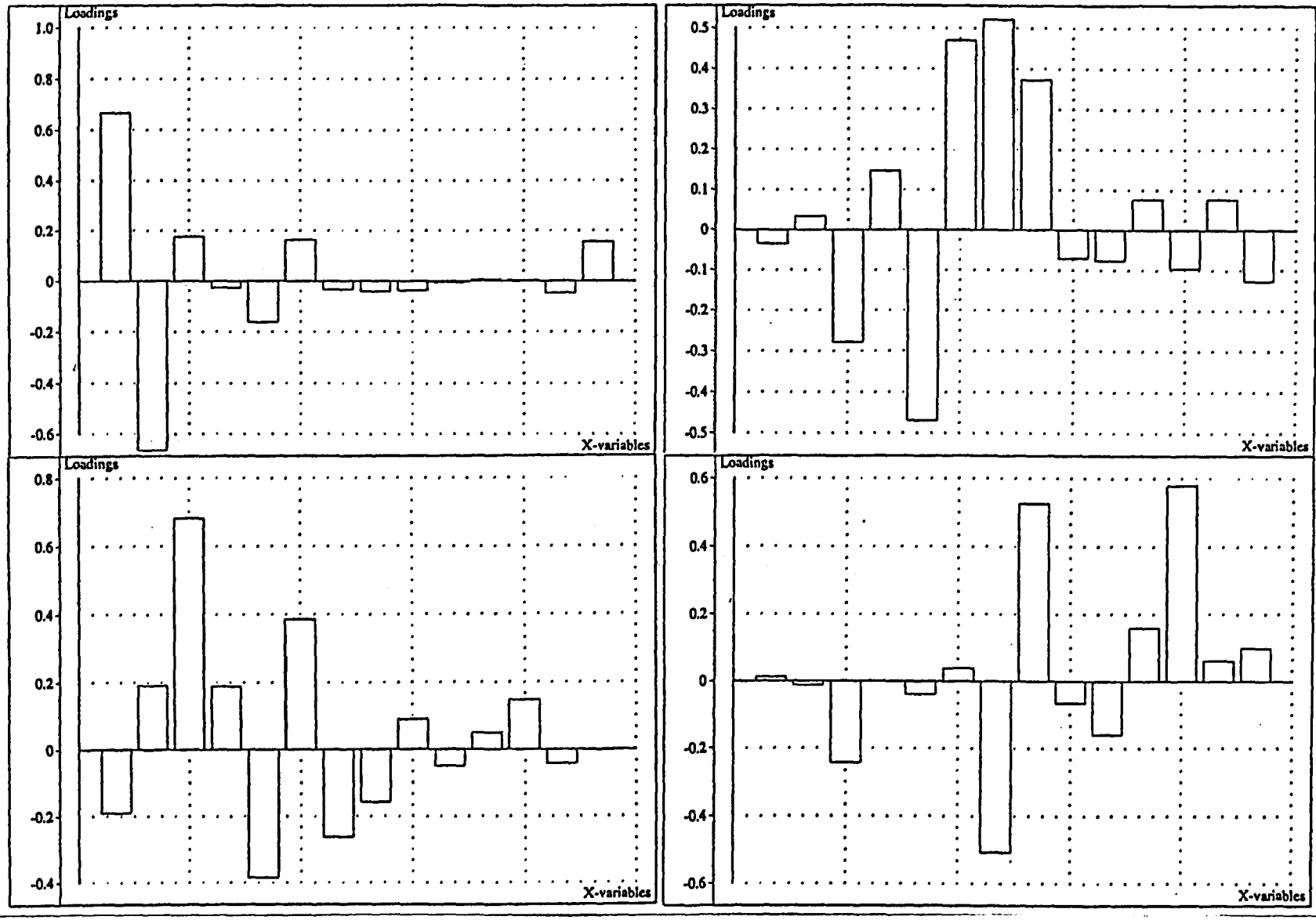

Figure 10.- Loadings plot of the PCA model with 4 principal components. Upper left, PC1; upper right, PC2; lower left, PC3 and lower right, PC4. Each bar represents one scored band.