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POLYMORPHISM OF SERUM TRANSFERRIN IN ATLANTIC SALMON

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ABSTRACT

Sera of Atlantic salmon have been analysed with regard to transferrin types by starch/agar electrophoresis. Three main patterns made up of two molecular types have been described.

Distributions of observed transferrin patterns from progenies of three different matings were in agreement with expected Mendelian distributions in offspring of known parentage, implying that the bands have their origin in two codominant alleles. In the majority of the samples of wild salmon the genetic basis of transferrin variation could be demonstrated by nonsignificant differences between observed and expected distributions when the Hardy-Weinberg formula was applied.

Significant differences in frequency of the Tf^A allele in samples from different rivers and within the same river were demonstrated. It is assumed that interchange of stocks has had influence on the values of the different gene frequencies found.

INTRODUCTION

Serum proteins which bind ferric ions are called transferrins. The presence of these proteins in salmon (*Salmo salar* L.) has been shown by electrophoresis and autoradiography (Møller and Naevdal, 1967) and polymorphism has been reported (Møller, 1970; Wilkins, 1969, 1970; see also de Ligny, 1969).

Transferrins have been useful for the identification of fish populations, particularly for those of cod (see Jamieson and Jonsson, 1970) and the work here reported on salmon was done with this in mind. Earlier investigators have indicated genetic diversity in the species (Nyman, 1966, 1967a, 1967b, 1970; see also de Ligny, 1969).

This report describes transferrin polymorphism in salmon, discusses the genetic origin of the observed differences, and gives values of gene frequency in samples from different localities in eastern Canada and United States.

MATERIALS AND METHODS

Specimens of sera were obtained from approximately 400 juvenile salmon and 1600 grilse and large salmon (Table 1). Most of the pre-smolt parr came from three lots of different hatchery-reared progenies, each from two parents only. Two progenies were kept together after one was marked by clipping the adipose fin; the third was kept separately. All the other specimens were from wild animals trapped in research counting fences or caught by nets for hatchery purposes. Individuals from 16 different localities from Sand Hill River, Labrador, in the north to Narraguagus River, Maine, in the south were represented in the material (Table 1 and Fig. 1).

The fish were anesthetized with tertiary amyl alcohol or MS222-Sandoz before bleeding. From grilse and large salmon the blood was obtained by cardiac puncture; for smaller fish it was drawn by cutting the tails. To prevent haemolysis, which commonly occurs during clotting, all samples were heparinized. After sampling, the large fish were released; the whole blood was kept cool during storage and transport to the laboratory where specimens were centrifuged before the serum was pipetted off and deep-frozen for subsequent analyses.

The electrophoretic technique used was a modification of the starch/agar gel electrophoresis on microscope slides (75 x 25 mm) (Sick, 1965; Møller, 1966). The buffer giving best separation of the selected proteins was found to be a Tris buffer (Aronsson and Grönwall, 1957) made up of:

60.6 g Tris(hydroxymethyl)methylamine
12.8 g Boric acid, and
6.0 g Ethylenediaminetetra-acetic acid in
1 litre distilled water.

The pH of the buffer made up from these components with these quantities varied between 8.0 and 8.2. Control of pH at 8.1, the preferred level, was achieved by mixing two stock solutions, one with 40 g of boric acid instead of 12.8 g and the other with no boric acid.

The microscope slides were covered by 3 ml of gel, and after application of the sera specimens the gels were covered with a sheet of thin plastic.

Each electrophoretic run contained 20 specimens, two on each slide, and lasted for 5 1/2 hours with approximately 6 volt/cm gel. After the run, the proteins were fixed in a 5:5:2 mixture of methanol, water, and glacial acetic acid, then stained with amido black 10B. The gel was then destained in the fixative and dried.

Centrifuging, pipetting of specimens, and electrophoretic analysis were all performed in a refrigerated room (-2 to +2°C). However, heat generation still made it necessary to have ice in the compartment underneath the slides.

Where the patterns were difficult to define, the specimens were reanalysed with particular care, this time with only one specimen on the slide.

RESULTS

Pattern description

Fig. 2 shows the three common patterns of serum proteins found in Atlantic salmon by starch/agar electrophoresis. Most of the individuals possess a slow moving (anodic) component (1) in common. Next to this band the individuals may show either one or both of the proteins (2 and 3) studied in this paper. Still faster bands (4 and 5) are present in most animals.

The bands 1, 4, and 5 (Fig. 2) are not identified. Both their appearance and their intensity seem to vary occasionally, and their presence, therefore, could depend upon environmental factors. Blood samples with a high degree of haemolysis could result in electrophoretograms showing haemoglobin patterns also. The haemoglobin pattern was always poor in frozen specimens, but it did not obscure the identification of bands 2 and 3.

Band 2 has already been shown to bind ferrous ions (Møller and Naevdal, 1967). The difference in electrophoretic mobility between bands 2 and 3 is now interpreted as phenotypic expression of codominant alleles. Three phenotypes are distinguished, Tf AA, Tf AC, and Tf CC, involving one or both of the bands Tf A (band 3) and Tf C (band 2).

As well as the three common transferrin patterns shown in Fig. 2, other patterns are shown diagrammatically in Fig. 3 as types 4 to 11. In type 4, Tf C is found together with a band with the same intensity as Tf C but with somewhat faster anodic mobility. The band is believed to be another transferrin and it is therefore called Tf B. However, in routine analyses it became apparent that it was difficult to distinguish Tf BC from Tf BB or for that matter Tf AB from Tf AC. When detected, therefore, the band was ignored and the patterns typed as Tf CC or Tf AC, respectively.

In the patterns 5 to 11 (Fig. 3) one or two faint bands are present together with Tf A and/or Tf C. These bands are probably transferrin subtypes. So far nothing definite can be said about these bands or the genetic base of these patterns. However, the specimens were not rejected; in general types 5 to 9 were denoted Tf AC, while types 10 and 11 were called Tf CC and Tf AA respectively.

The electrophoretic patterns were always clearly distinct when using fresh sera up to about one week old. The intensity of the bands varied however. As a rule the one band patterns were stronger than the two band patterns, and in the Tf AC pattern, Tf C could have a more pronounced appearance than Tf A. Thawed sera usually gave reliable results over several months, but freezing and thawing affected the distinctness of the bands; types 5 to 11 (Fig. 2) were hardly recognizable and the Tf AC pattern was not always well defined.

Genetic considerations

The observed distributions of the three patterns Tf AA, Tf AC, and Tf CC in the three groups of progeny (samples 17, 18, and 19) are quite different. They appear to represent three different crosses between the three genotypes of the two allele system postulated above. However, in sample 18 a single individual had a Tf AA pattern; whereas, all of the others had the Tf AC patterns. The pre-smolts providing this sample were still available as the sample had been frozen for another purpose. A single fish in this sample had a clipped adipose fin, indicating that it came from a different pair of parents than the other fish in the sample. It was assumed that this individual gave the anomalous result.

In Table 2, the observed distributions of the transferrin patterns are compared to expected Mendelian distributions in offspring of known parentage. Pairs of distributions fit, and the two codominant alleles involved are called $\underline{Tf}^A$ and $\underline{Tf}^C$.

Distributions of the transferrin patterns and gene frequency

Table 3 shows the distribution of the transferrin patterns of the samples (obs.), the frequency of the Tf^A allele (q^A), and the expected distributions of the types (exp.) according to the Hardy-Weinberg law of genotypic distributions in large random mating populations. These values have also been calculated in observed distributions of subtotals in order to obtain reliable values for identification purposes. Two of the subtotals (samples 6-8 and 12-14) combine samples collected at the same locality; the last subtotal (samples 15-16) combines specimens from two neighbouring rivers. The gene frequency (q^A) in the samples within each of these units varies only slightly.

The agreement between observed and expected distributions has been tested by means of the Fisher chi-square test (Table 3), and the value of X^2 and the corresponding probability intervals for one degree of freedom are given for those samples which showed large deviations.

There is good agreement between observed and expected distributions in all samples except those from the Miramichi River (samples 7, 8 and 9) and the combined samples (12, 13, and 14) from the St. John River. The significant difference between observed and expected distributions and the excess of homozygotes in these units indicate mixtures of two or more Meldelian populations.

The frequency of the $\underline{Tf}^A$ allele varies greatly. The lowest value, 0.0714, is found in Adies Stream, Nfld., while the highest value, 0.6042, is present in the sample from Narraguagus River, Maine. However, there is no indication that this variation follows a latitudinal cline (see Table 3 and Fig. 1).

The mean gene frequency and the approximate 95 percent confidence limits are shown for each sample in Fig. 4. The cross-hatched bars represent values calculated from the three units of samples, the samples from NW Miramichi River, St. John River, and the two adjacent rivers in Maine. Because of the low number of specimens in samples 15 and 16, the values of q^A in each of these samples are omitted from the figure.

The frequency values of q^A make it possible to divide the samples into three different groups: samples 1 and 2 from Labrador and Newfoundland, samples 3 to 14 from New Brunswick and Nova Scotia, and samples 15-16 from Maine. A large area was unsampled between samples 2 and 3; the discontinuity between these two samples, therefore, is not surprising. This cannot apply to the second case, however, because the distance between the river mouths is only about 120 km and there are only about half a dozen salmon-producing streams in between.

The second group is variable for gene frequencies; differences in frequencies of q^A may be characterized as significant between samples 6-8 and sample 9, and the samples 11 and 12-14. The samples from the Miramichi River area (samples 5-9) are interesting in this respect. Samples 6-8 are all from the Northwest Miramichi River, and sample 9 is from the Southwest Miramichi; both are main branches of the same river system. Even so, the two groups of samples show significant difference

in the values of q^A; sample 5, collected in the estuary, has an intermediate value.

DISCUSSION

The main purpose of this investigation was to describe transferrin polymorphism in Atlantic salmon, demonstrate its genetic basis, and use the system in a preliminary study of salmon populations of the region.

Transferrin polymorphism

The transferrins in Atlantic salmon appear as well-defined bands in starch/agar gel electrophoresis. Comparisons between salmon specimens of Canadian and Norwegian origin did not show any pronounced differences in the main system (Naevdal, pers. comm.). Even in the small numbers of Norwegian salmon specimens investigated, existence of different faint bands similar to the Canadian ones was also demonstrated. However, of the high intensity bands, the Norwegian specimens included only individuals of the Tf CC type.

Tf A has not been shown to have ironbinding capacity. However, its strength, position, and its appearance alone, or together with Tf C, conform to the characteristics of a second transferrin molecule.

Tf B is very rare, and it has not been possible to test this protein autoradiographically. Both the position and the strength, however, are comparable to characteristics of Tf A and Tf C, and the protein is interpreted as a transferrin.

The rare patterns (see types 5-11, Fig. 3) were not always distinct in electrophoretograms from the available frozen specimens. In some cases the bands were hardly visible; in other cases, one of the rare bands was prominent enough to make it difficult to distinguish between it and Tf A. This often happened if Tf A occurred together with a strong Tf C. Therefore, it was not possible to get good figures for further studies of the faint bands.

Types 5-8 (Fig. 3) are all referred to type Tf AC because the faint bands were present in positions belonging to Tf A and Tf C, and because of the difficulty in separating the Tf AC pattern from types 5 and 6. However, these types were rare, and the "observed" distributions (Table 3) do not show an excess of the Tf AC pattern.

The genetic studies strongly support the two-allele hypothesis by which the observed transferrin variation is explained, and the majority of the samples of wild salmon, showing nonsignificant differences between observed and expected distributions of the transferrin patterns (Table 3), demonstrated the genetical basis of the individual variation. The patterns did not change with the age of the fish; there was no real difference in gene frequency between smolts and adults (samples 6, 7, and 8, Table 3). Nor was it possible to see any difference between patterns obtained in hatchery-reared smolts and patterns in wild fish.

Transferrins have been demonstrated in brook trout; genetic studies have shown that three codominant alleles are involved (Hoffman, 1966), and investigations of some physio-chemical properties have been carried out (Hershberger, 1970). The brook trout transferrins were designated A, B, and C, and their appearance in polyacrylamide gels seems to be very similar to those of salmon. However, the form A which has a much faster anodal migration than B and C, was lethal in the homozygous condition. The frequencies of the transferrin alleles have also

been investigated in several hatchery and wild populations of brook trout (Wright and Atherton, 1970). The frequencies were found to vary, and it was apparent that, generally, there was a much higher percentage of heterozygotes in hatchery than in wild brook trout stocks.

Three main sera patterns are found in Atlantic salmon by starch/agar electrophoresis. The patterns are composed of two different bands. One is identified as serum transferrin, and the patterns are shown to represent two codominant alleles, $\underline{Tf}^{A}$ and $\underline{Tf}^{B}$.

Identification of populations

Differences in gene frequency between groups of individuals of the same species, and disparities between observed and expected distributions as a result of an excess of homozygotes, are characteristics commonly used to identify genetically different Mendelian populations.

The variation of the frequency of the $\underline{Tf}^{A}$ allele reflects the genetic variability in Atlantic salmon. The similarity between the observed distributions and the expected distributions of the transferrin patterns, and the similarity in gene frequencies at the same locality (samples 6, 7, and 8; and 12, 13, and 14, Table 3) and between neighbouring localities (samples 3 and 4; and 15 and 16) support the conclusion that the statistically significant differences found represent differences between populations.

Two groups of samples, those from the Miramichi River (samples 7, 8, and 9, Table 3) and the combined samples from St. John River (samples 12, 13, and 14) showed significant differences between the observed and the expected distributions of the transferrin patterns. The material is too limited for any final conclusion, but the excess of homozygotes in these rivers may signify the existence of different populations further upstream.

Genetic variability has been shown before in other salmonids. Brannon (1967, 1969) and Raleigh (1967, 1969) experimented with progeny of sockeye salmon (*Oncorhynchus nerka*) taken from parents spawning above and below lakes. The inlet and outlet fry differed significantly in their rheotactic and phototactic responses, and in both cases the rheotactic response resulted in a greater proportion of each population finding its way to its nursery lake. In that study, the fry were kept in a controlled environment from the time of fertilization until the final tests of behaviour, so that any differences must be assumed to be genetic in origin.

The present results for Atlantic salmon are, thus, in accordance with the view that nearly all species are made up of genetically distinct populations.

Two questions can be raised, however. Over the years there has been a great amount of interchange of stocks within West Atlantic salmon which could have contributed greatly to the present heterogeneity. Most of these introductions have been of progenies from a relatively few parental fish which, by chance, could have had a quite different gene frequency from the parental stock. The influence of such interchange of stocks on the genetic diversity found, cannot be ignored.

However, interchange of stocks can hardly explain all the observed genetic diversity. No recorded interchange of fish has occurred between either Sand Hill River (sample 1) or Humber River (sample 2) in the north, and any other river. The difference in gene frequency between these rivers and the rivers further south is, therefore, probably of natural origin.

Nor can the stocking explain the observed pattern of distribution. If anything, frequent introductions would break down the isolation mechanisms and lead to panmixia (Calaprice, 1969), but this does not seem to have happened. The reason could be the common occurrence of the efficient homing instinct (Carlin, 1969; Ueda, Hara, and Gorbman, 1967) or some other possible premating mechanisms.

Transplanting of stocks, however, is likely to have had some influence on gene frequency, but the degree of this influence cannot be determinee by the results of the present investigation. This problem could probably be solved by introducing progeny with known gene frequencies which differ markedly from the frequency in the recipient river (Møller and Naevdal, 1968), by the identification and use of other genetic systems, or by an extensive sampling and analysis of transferrin patterns, particularly in localities where no introduction has taken place.

The second question raised by the present results concerns maintenance of a polymorphic character in small populations. It has been postulated that genetic drift, "random fluctuations in gene frequencies in effectively small populations," will result in variation in the gene frequency from one generation to another which tends towards the homozygous state (Dobzhansky, 1951). If the migration of salmon individuals is severely restricted between localities, as the present results suggest, the number of breeding individuals must be low in most salmon populations and homozygosity, therefore, a common occurrence. From this viewpoint, therefore, the kind of genetic diversity revealed in this study is unexpected; both alleles are well represented in all samples.

The existence of genetic diversity in Atlantic salmon cannot be denied; the investigation confirms the existence of populations which are clearly different genetically. However, it is assumed that interchanges of stocks may have affected the gene frequencies of the original populations, but they have not led to a general breakdown of the natural isolating mechanism. Genetically distinct groups of Atlantic salmon still exist.

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Table 1. Survey of collected samples in 1969.

Sample No.	Locality	Date of catching/ sampling	Gear	Type of animals	Number of specimens
1	Sand Hill R., Labrador	July 23 -29	Counting fence	Grilse	130
2	Adies Stream, Nfld.	Aug. 1	Counting fence	Grilse	112
3	Carleton, P.Q.	June 1-25	Trap-net	Large salmon	120
4	Restigouche R., N.B.	Sept. 2	Seine	Grilse/large salmon	120
5	Millbank, N.B.	July 24-29	Trap-net	Grilse/large salmon	59
6	NW Miramichi R., N.B.	May	Counting fence	Smolt	93
7	NW Miramichi R., N.B.	July 3-6	Counting fence	Grilse	117
8	NW Miramichi R., N.B.	July 17-29	Counting fence	Grilse	146
9	SW Miramichi R., N.B.	Sept.-Oct.	Trap-net	Grilse/large salmon	117
10	R. Philip, N.S.	July-Sept.	Fishway trap	Grilse/large salmon	120
11	Margaree R., N.S.	July-Sept.	Seine	Grilse/large salmon	95
12	St. John R., N.B.	June 10-July 2	Fishway trap	Grilse/large salmon	105
13	St. John R., N.B.	July 5-Oct. 3*	Fishway trap	Grilse/large salmon	142
14	St. John R., N.B.	Sept. 16-Nov. 13*	Fishway trap	Grilse/large salmon	91
15	Machias R., Maine	June-Sept.	Counting fence	Grilse/large salmon	32
16	Narraguagus R., Maine	June-Sept.	Counting fence	Grilse/large salmon	24
17	Margaree Hatchery, N.S.	Dec. 10		Pre-smolt	100
18	Margaree Hatchery, N.S.	Dec. 10		Pre-smolt	100
19	Margaree Hatchery, N.S.	Dec. 10		Pre-smolt	102

*Separation of fall-and summer-run fish during late September was based on differences in coloration.

Table 2. Observed distributions of the transferrin patterns in samples of offspring compared to expected distributions in offspring of known parentage.

Sample	Observed distributions of transferrin patterns Tf AA	Tf AC	Tf CC	Probable parents	Expected distributions from probable parents Tf^A/Tf^A homozygotes	Tf^ATf^C heterozygotes	Tf^C/Tf^C homozygotes
17	51	49		$Tf^A/Tf^A \times Tf^A/Tf^C$	50	50	
18	1	99		$Tf^A/Tf^A \times Tf^C/Tf^C$		100	
19	29	52	21	$Tf^A/Tf^C \times Tf^A/Tf^C$	25.5	51	25.5

Table 3. Transferrin polymorphism of salmon samples; genotypic composition compared with the Hardy-Weinberg distribution.

Sample	Tf^A/Tf^A homozygotes obs.	exp.	Tf^A/Tf^C heterozygotes obs.	exp.	Tf^C/Tf^C homozygotes obs.	exp.	X^2	Probability of worse fit	Frequency of the Tf^A allele (q^A)
1	2	0.93	18	20.14	110	108.94			.0846
2	1	0.57	14	14.85	97	96.57			.0714
3	22	20.42	55	58.16	43	41.42			.4125
4	21	20.01	56	57.98	43	42.01			.4083
5	7	4.90	20	24.20	32	29.90			.2881
6	11	11.36	43	42.29	39	39.35			.3495
7	22	15.08	40	53.84	55	48.07	7.7522	0.01-0.005	.3590
8	28	22.25	58	69.50	60	54.25	3.9983	0.05-0.025	.3904
6-8	61	48.58	141	165.86	154	141.57	7.9728	0.01-0.005	.3694
9	10	5.78	32	40.44	75	70.78	5.0941	0.025-0.01	.2222
10	12	12.68	54	52.65	54	54.67			.3250
11	19	18.57	46	46.86	30	29.57			.4421
12	8	5.49	32	37.03	65	62.48			.2286
13	14	10.71	50	56.57	78	74.72			.2746
14	10	7.72	33	37.57	48	45.72			.2912
12-14	32	23.70	115	131.60	191	182.70	5.3777	0.025-0.01	.2648
15	10	11.28	18	15.44	4	5.28			.5938
16	9	8.76	11	11.48	4	3.76			.6042
15-16	19	20.04	29	26.92	8	9.04			.5982

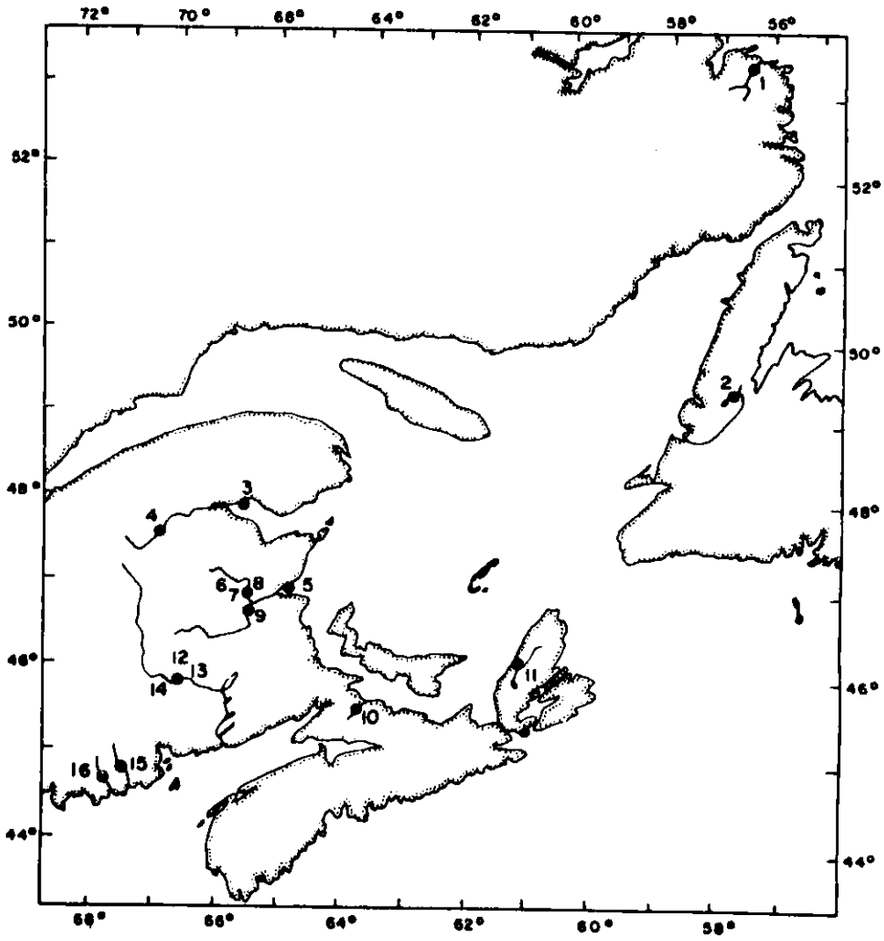

Fig. 1. The location of capture of the 16 wild samples.

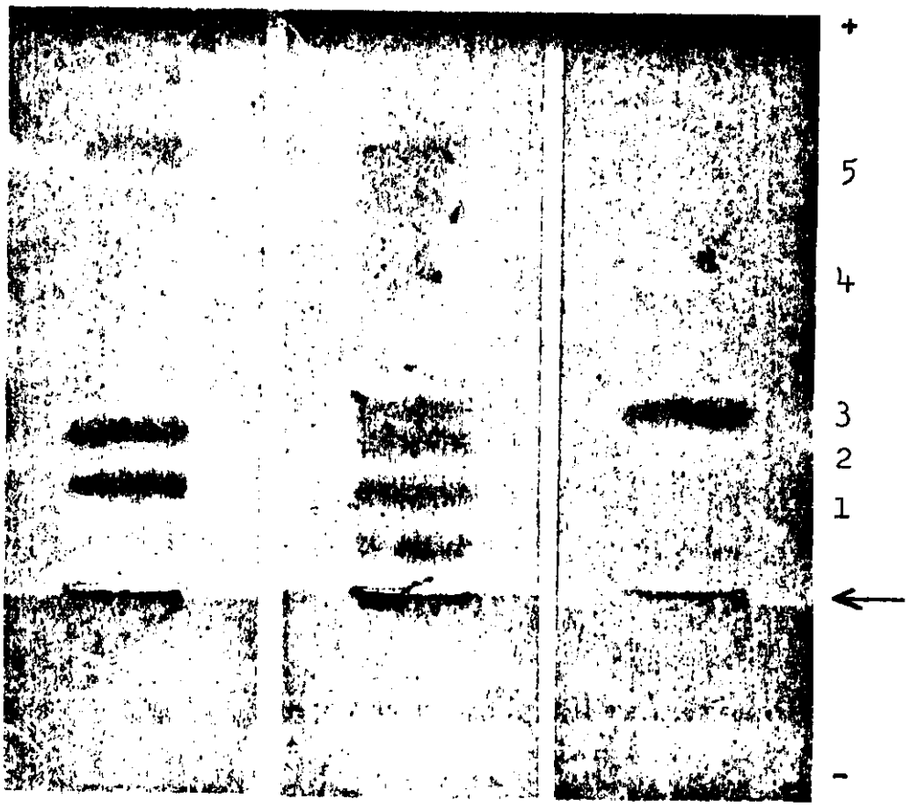

Fig. 2. Main Atlantic salmon transferrin patterns obtained by starch-agar electrophoresis on microscopic slides. Band 1, 4, and 5 are unidentified molecules; 2 and 3 transferrins. Arrow indicates the site of application.

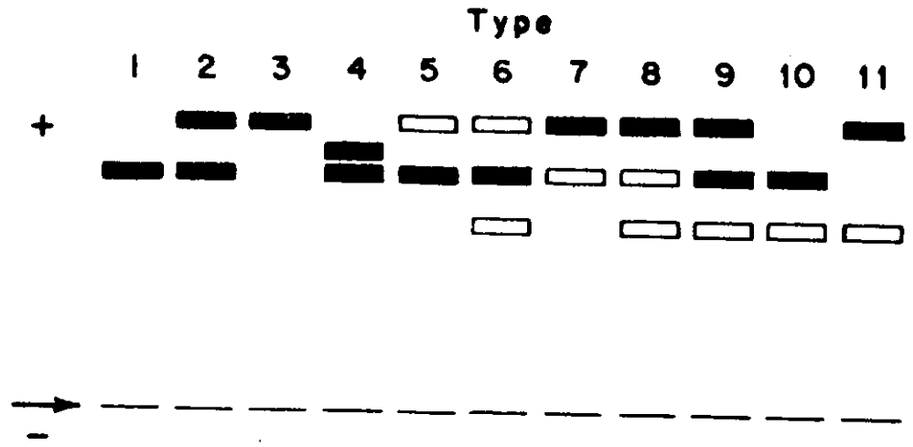

Fig. 3. Patterns in the transferrin region after starch-agar electrophoresis of Atlantic salmon sera. Type 1 to 3 main patterns, type 4 to 11 rare patterns. Filled in bars: bands with high intensity; open bars: faint bands. Arrow indicates the site of application.

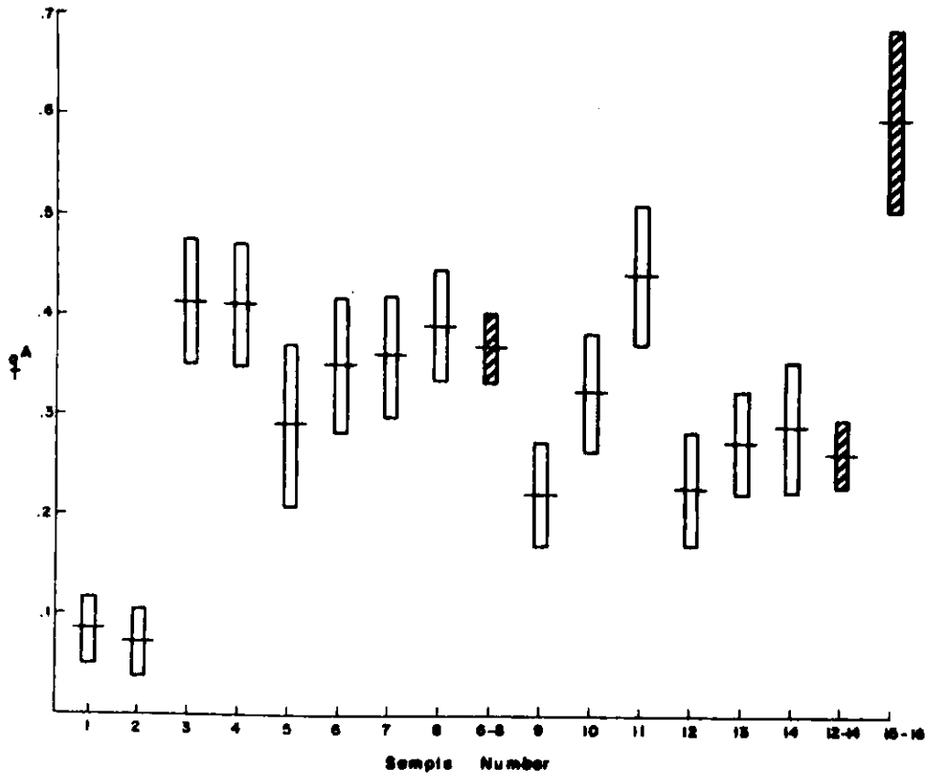

Fig. 4. Confidence intervals of the gene frequency of $\underline{Tf}^A$ (q^A) in samples of wild Atlantic salmon. The horizontal lines indicate the observed mean frequencies, and the vertical ranges of the bars give the 95 % confidence limits. Cross-hatched bars represent values calculated from sub-totals of samples. Sample 1: Sand Hill; 2: Adies Str.; 3: Bay of Chaleur; 4: Restigouche; 5: Miramichi Estuary; 6-8: NW Miramichi; 9: SW Miramichi; 10: R. Philip; 11: Margaree; 12-14: St. John; 15-16: Machias and Narraguagus in Maine.