Chromosoma (Berl.) 75, 185–198 (1979)

CHROMOSOMA
© by Springer-Verlag 1979

Non-repetitive DNA Sequence Divergence in Phylogenetically Diploid and Tetraploid Teleostean Species of the Family Cyprinidae and the Order Isospondyli

Jörg Schmidtke, Eberhard Schmitt, Elisabeth Matzke and Wolfgang Engel

Institut für Humangenetik der Universität, D-3400 Göttingen, Nikolausberger Weg 5a, Federal Republic of Germany

Abstract. Non-repetitive DNA of anciently tetraploid teleostean species was analysed for the presence of duplicated sequences. Closely related diploid species were investigated in comparison. From the reassociation kinetics of total nuclear DNA, rate constants and fraction sizes of classes of repetitive and non-repetitive sequences were determined. DNA fractions enriched in the slowest renaturing sequence class were prepared and subjected to reassociation. The rate constants of these reactions were compared with the values expected for single-copy DNA from analytical genome size determinations. From reassociated DNA enriched in non-repetitive sequences also the melting temperatures were determined as a measure of internal base sequence heterogeneity. It has been shown that the two ancient tetraploids *Cyprinus carpio* and *Thymallus thymallus* are, with regard to the thermal stability of reassociated non-repetitive DNA, and with regard to the correspondence of reaction rates with the values expected for single copy DNA, indistinguishable from diploid controls (*Rutilus rutilus, Clupea harengus* and *Sprattus sprattus*). The tetraploid species *Salmo irideus*, *Salvelinus fontinalis* and *Coregonus lavaretus* appear as very recent tetraploids with regard to these criteria. The significance of the results for estimating the time of occurence of polyploidisation events in these taxa is discussed.

Introduction

Polyploidy is a powerful tool of evolution. At redundant genes mutations may accumulate and eventually lead to modified or previously unknown functions (Huxley, 1942; Ohno, 1970). During this process of exploitation of gene redundancy a tetraploid organism, originating from a diploid one, gradually reestablishes a diploid state of the genome, both by DNA sequence divergence and chromosomal rearrangement. Among vertebrates a number of model systems suitable for the study of this evolutionary process of diploidisation of the tetraploid have been described with respect to karyotype, genome size, and patterns

0009-5915/79/0075/0185/S02.80

of gene expression, e.g. Ceratophrydidae (Beçak et al., 1966, 1968; Beçak and Pueyo, 1970; Beçak and Goissis, 1971; Schmidtke et al., 1976; Schwantes et al., 1977); Catastomidae (Uyeno and Smith, 1972; Ferris and Whitt, 1977a, 1978); Cobitidae (Ferris and Whitt, 1977b); Cyprinidae (Ohno et al., 1967; Wolf et al., 1969; Engel et al., 1975; Schmidtke et al., 1975, 1976a; Schmidtke and Engel, 1975, 1976); Isospondyli (Klose et al., 1968; Engel et al., 1975; Allendorf et al., 1975; Schmidtke et al., 1975a, 1976a, 1976b).

Where karyotypic diversification has occurred to any large extent, a presumptive tetraploid state of a genome is not easily verified. Comparisons of the DNA content of such a genome with that of a presumptive diploid relative is weak evidence of tetraploidy, because genome size may also vary due to regionally confined sequence ampflification. Comparing the number of expressed structural gene loci coding for a given polypeptide in presumptive diploid and tetraploid organisms may likewise be misleading, because gene duplication can occur in tandem in a diploid genome, and the duplicates existing in a tetraploid may not be resolvable due to lack of sequence divergence or due to functional loss. Determinations of the kinetic complexity of non-repetitive DNA, however, may, by comparison with the analytical complexity, resolve the question of ploidy, even in karyotypically or otherwise obscure cases. Such an approach is applicable only where the extent of sequence divergence during diploidisation is low enough to permit the detection of previously fully homologous sequences by the method used. As a test of polyploidy, DNA renaturation techniques have been utilized by several authors (Straus, 1971; Mitra and Bhatia, 1973; Smith and Flavell, 1975; Ordahl, 1977; Zimmerman and Goldberg, 1977). No attempt has been made hitherto to relate the results of renaturation experiments of anciently polyploid DNA to species age. By a combination of these two experimental procedures, namely, (1) the estimation of the fraction of non-repetitive sequences present in duplicate, and (2) the determination of the amount of base substitution between strands capable of annealment, two problems may be solved alternatively: If the time elapsed since the polyploidisation event is known (by combining paleontological, taxonomical and biochemical results), the rate by which base exchanges have been fixed within a tetraploid genome can be determined. If, however, such results are to be used to "time" the polyploidisation event, the amount of sequence divergence in a genome can be related to the rate of sequence change per unit of time determined from other sources. For example, Britten and co-workers (1976, 1978) and Harpold and Craig (1978) have estimated the rate of single-copy DNA sequence divergence between different sea urchin species with 0.2–0.3% per million years (myr). These estimates correspond to the mean rate observed in several mammalian species pairs by Kohne et al. (1972)[1]. A similar rate of change is also observed in the genus *Xenopus* (Galau et al., 1976). It appears that in several diverse lineages the rate of nucleotide substitution in the non-repeated DNA

[1] The large standard error of the mean of Kohne's results would considerably decrease if a smaller divergence time between man and chimpanzee, and man and gibbon were used and if the exceedingly high estimate of nucleotide differences between mouse and rat were replaced by McCarthy and Farquhar's more moderate estimate (1972), without any marked effect on the mean value

is roughly linear with time and amounts to about 0.3% per myr, and that this figure may be used as an "evolutionary clock".

In the present study the genomic DNA of presumably ancient tetraploid species of the teleostean family *Cyprinidae* and of the teleostean order *Isospondyli*, and, for comparison, of closely related species, which appear not to have undergone polyploidisation, was analysed for the following properties: (1) the amount of repeated and non-repeated sequences, (2) the rate of reassociation of the non-repetitive genome fraction, (3) the thermal stability of reassociated non-repetitive DNA. From these results the amount of sequences retained in duplicate and the extent of diversification was to be estimated. These species were chosen, because, according to previous findings, they should represent a sample of diverse phylogenetical age. Using the rate estimate of non-repetitive sequence divergence from other sources (see above) we can show that the findings in *Cyprinus carpio (Cyprinidae)* and *Thymallus thymallus (Isospondyli)* are compatible with the view that these species are very old tetraploids. Sequence diversification has proceeded to such an extent that their DNA is indistinguishable from diploid DNA with respect to kinetic and thermal properties. *Salmo irideus*, *Salvelinus fontinalis*, and *Coregonus lavaretus (Isospondyli)* on the other hand appear to be very recent tetraploids judged by the same criteria.

Materials and Methods

1. Origin of the Animals. Specimens of *Cyprinus carpio*, *Salmo irideus*, and *Salvelinus fontinalis* were purchased from fish hatcheries. *Rutilus rutilus* and *Thymallus thymallus* were caught wild from the Rhein river, and *Coregonus lavaretus* was caught in the Lake of Konstanz. Specimens of *Clupea harengus* and *Sprattus sprattus* were captured in the North Sea, near Helgoland.

2. Preparation of DNA. Blood was collected into 0.65% NaCl–0.1% heparine. Erythrocytes were wash-centrifuged with 0.65% NaCl and lysed with 4%. Triton-X-100 (Serva, West Germany). Nuclei were pelletted, lysed with water, and incubated for 1 h at 37° C with 0.25 mg/ml pre-self-digested Pronase E (Merck, West Germany) in 5 mM $CaCl_2$-1 M LiCl (final concentrations). Subsequently, the lysate was treated with 0.5% sodium dodecyl sulfate for 1 h at 37° C. After at least three extractions with equal volumes of phenol-cresol-8-hydroxyquinoline (100 g phenol + 14 ml m-cresol + 0.1 g 8-hydroxyquinoline + 25 ml H_2O) and chloroform – 1% isoamylalcohol at room temperature, DNA was precipitated with 0.6 vol. of 2-propanol, transferred to 75% ethanol – 0.1 M NaCl, air dried, dissolved in 0.1 SSC (0.015 M NaCl–0.0015 M Na-citrate, pH 7.0), reprecitated twice and finally dissolved in 0.55 M phosphate buffer, pH 6.7 (PB). DNA was fragmented by ultrasonic treatment (Branson sonifier B 12 cell discruptor) to lengths of 0.2–0.4 kilobases (kb). Sizing was performed by neutral and alkaline sucrose gradient centrifugation as described previously (Epplen et al., 1978), using tritium-labelled 4S-RNA and electron-microscopically sized E. coli-DNA as markers.

3. Reassociation and Hydroxyapatite Fractionation. DNA samples were heat-denatured at 90–100° C for 5–10 min and were reassociated in 0.04–0.55 M PB at 60–66° C, corresponding to 20–25° C below the melting temperatures under these buffer conditions. All C_0t-values are corrected to 0.18 M Na^+ (equivalent C_0t, Britten et al., 1974). Separation of single-stranded fragments from fragments containing duplex regions was performed by hydroxyapatite chromatography assay using stepwise elutions with 0.12 M PB and 0.4 M PB at 60° C. A water-jacketted chromatography column equipped with a filtration apparatus was used (Britten et al., 1974). Reassociation rates were calculated by a computer-aided non-linear least squares regression procedure.

4. Fractionation of the Genome. Genome fractions enriched in the slowly renaturing DNA sequences were prepared by incubating fragmented total genomic DNA (referred to as "whole DNA") to a C_0t where the repeated sequences had reannealed and the non-repeated were still single-stranded, as judged by the renaturation kinetics of whole DNA. The DNA was passed over hydroxyapatite and was fractionated with 0.12 M PB and 0.4 M PB. The 0.12 M PB fraction was adjusted to 0.55 M PB and concentrated by ultrafiltration (Amicon 8 MC-cell equipped with PM-10 ultrafiltration membranes). This preparation was examined for kinetic properties as described above. After reassociation fragment sizes were 0.2–0.3 kb, as determined from alkaline sucrose-density centrifugation. For the measurement of thermal stability of the reassociation products at high C_0t samples were fractionated over HAP, adjusted to 0.12 M PB, and concentrated by ultrafiltration.

5. Melting Experiments. DNA prepared as described above and native DNA, both in 0.12 M PB, were melted in a Unicam SP 1800 spectrophotometer using water-jacketted cuvettes. Change in absorbance, as a function of increased temperature, was registered by using a Unicam AR 25 linear recorder, while the temperature was monitored by a Lauda digital thermometer having a Pt-100 resistance temperature meter.

Results

As may be inferred from Figures 1–8, in which the reassociation kinetics of whole unfractionated DNA are depicted together with the reassociation kinetics of DNA preparations, enriched in the slow kinetic fraction, the genomes of the species under comparison contained highly variable amounts of repeated and non-repeated sequences. While the genome of *Sprattus sprattus* contains 61% unique sequences, the genome of *Salmo irideus* consists mainly of repetitive DNA (84%). These values were corrected for the fraction of DNA not reannealing even at the highest C_0t values, probably made up of highly degraded material. These two species represent both extremes of DNA sequence composition, the remaining species ranging in between. Where the non-repetitive portion makes up only a small fraction of the whole DNA, the reassociation constants which, theoretically, would already provide a basis for the calculation of the complexity of this fraction, can be determined only with minimal accuracy. Therefore, preparations enriched in the slowly reacting sequences were made and then subjected to kinetic analysis; for ease of comparison this was done with material from all species. The results of these experiments are depicted in the inserts of Figures 1–8. These preparations also served as material for the determination of thermal stability of reassociated sequences.

1. Reassociation of Whole DNA

The least squares solutions of the reassociation of whole genomic DNA depicted in Figures 1–8 and summarized in Table 1 were calculated with the BMDX 85 non-linear least squares computer program. Best estimates (minimizing the root mean square error (RMSE)) were obtained providing three or four kinetic fractions. The reassociation-rate constants of the slowest kinetic fractions were fixed on the values expected for single- copy, or two-copy DNA, respectively. These values were calculated on the basis of analytical genome size determinations of the species investigated here (Ohno and Atkin, 1966; Klose et al., 1968;

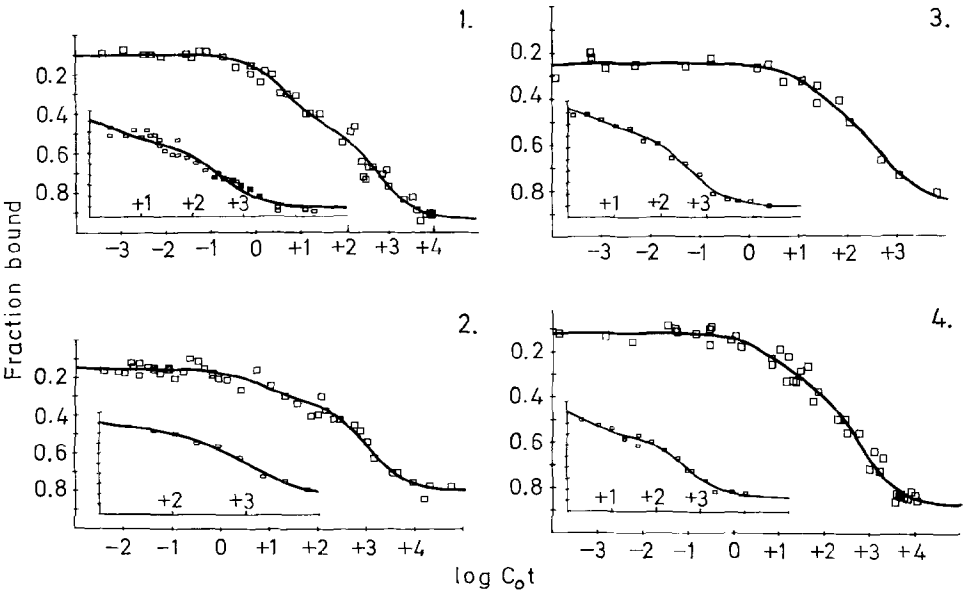

Fig. 1. Reassociation of *Rutilus rutilus* whole DNA and a DNA preparation enriched in non-repetitive sequences (insert). The fraction of fragments bound to hydroxyapatite is plotted as a function of C_0t. The upper curve represents a least squares solution for three second order components, namely 10.1% with a $K > 10^4$, 36.9% with a K of 0.213 $M^{-1}s^{-1}$, and 46.6% with a K of 0.00178 $M^{-1}s^{-1}$. For this solution the rate of the non-repetitive component was fixed at the value expected for unique DNA from the analytical genome size estimate. The RMSE value of the solution is 0.035. The curve in the insert represents a least squares solution for two second order components, namely 30.2% with a K of 0.474 $M^{-1}s^{-1}$ and 62.4% with a K of 0.00305 $M^{-1}s^{-1}$. The RMSE value of the solution is 0.050

Fig. 2. Reassociation of *Cyprinus carpio* whole DNA and a DNA preparation enriched in non-repetitive sequences (insert). The fraction of fragments bound to hydroxyapatite is plotted as a function of C_0t. The upper curve represents a least squares analysis for three second order components, namely 15.1% with a $K > 10^3$ $M^{-1}s^{-1}$, 16.7% with a K of 0.156 $M^{-1}s^{-1}$, and 48.5% with a K of 0.00096 $M^{-1}s^{-1}$. For this solution the rate of the non-repetitive component was fixed at the value expected for unique DNA from the analytical genome size estimate. The RMSE value of the solution is 0.035. The curve in the insert represents a least squares solution for two second order components, namely 17.1% with a K of 0.118 $M^{-1}s^{-1}$ and 68.5% with a K of 0.00093 $M^{-1}s^{-1}$. The RMSE value of the solution is 0.030

Fig. 3. Reassociation of *Clupea harengus* whole DNA and a DNA preparation enriched in non-repetitive sequences (insert). The fraction of fragments bound to hydroxyapatite is plotted as a function of C_0t. The upper curve represents a least squares solution for three second order components, namely 24.8% with a $K > 10^4$ $M^{-1}s^{-1}$, 22.7% with a K of 0.0375 $M^{-1}s^{-1}$, and 38.3% with a K of 0.00176%. For this solution the rate of the non-repetitive component was fixed at the value expected for unique DNA from the analytical genome size estimate. The RMSE value of this solution is 0.036. The curve in the insert represents a least squares solution for two second order components, namely 25.7% with a K of 0.369 $M^{-1}s^{-1}$ and 65.3% with a K of 0.00262 $M^{-1}s^{-1}$. The RMSE value of the solution is 0.030

Fig. 4. Reassociation of *Sprattus sprattus* whole DNA and a DNA preparation enriched in non-repetitive sequences (insert). The fraction of fragments bound to hydroxyapatite is plotted as a function of C_0t. The upper curve represents a least squares solution for three second order components, namely 11.8% with a $K > 10^4$ $M^{-1}s^{-1}$, 22.4% with a K of 0.114 $M^{-1}s^{-1}$, and 53.6% with a K of 0.0016 $M^{-1}s^{-1}$. The rate of the non-repetitive component was fixed at the value expected for unique DNA from the analytical genome size determination. The RMSE value of the solution is 0.041. The curve in the insert is a least squares solution for two second order components, namely 31.9% with a K of 0.470 $M^{-1}s^{-1}$, and 55.6% with a K of 0.00204 $M^{-1}s^{-1}$. The RMSE value of the solution is 0.035

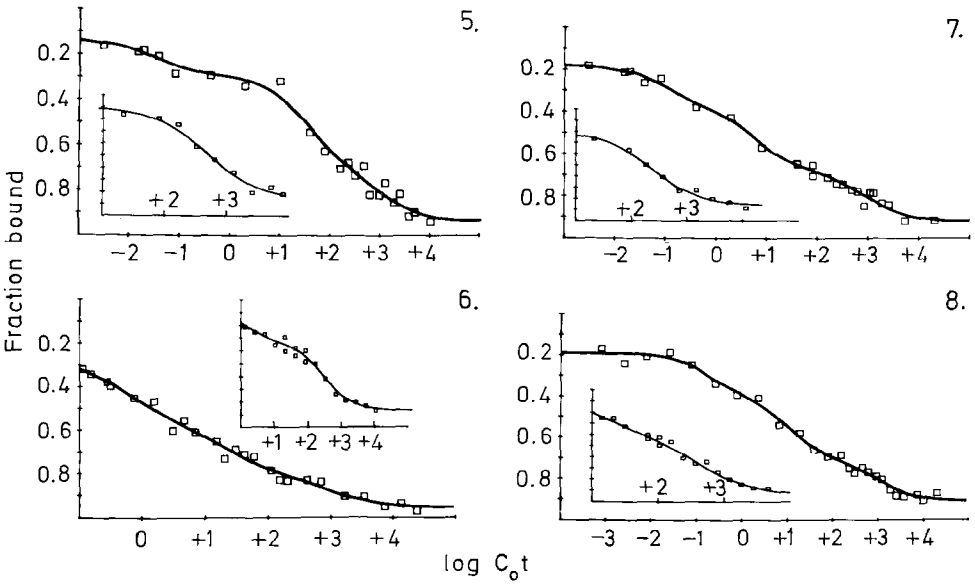

Fig. 5. Reassociation of *Thymallus thymallus* whole DNA and a DNA preparation enriched in non-repetitive sequences (insert). The fraction of fragments bound to hydroxyapatite is plotted as a function of C_0t. The upper curve represents a least squares solution for four second order components. These are: 13.9% with a $K > 10^3\ M^{-1}s^{-1}$, 15.3% with a K of $31.5\ M^{-1}s^{-1}$, 42.3% with a K of $0.289\ M^{-1}s^{-1}$, and 22.7% with a K of $0.00083\ M^{-1}s^{-1}$. The rate of the non-repetitive component was fixed at the value expected for unique DNA from the analytical genome size determination. The RMSE value of this solution is 0.041. The curve in the insert is a least squares solution for two second order components, namely 10.3% with a $K > 10^{-1}\ M^{-1}s^{-1}$, and 76.8% with a K of $0.0021\ M^{-1}s^{-1}$. The RMSE value of the solution is 0.044

Fig. 6. Reassociation of *Salmo irideus* whole DNA and a DNA preparation enriched in non-repetitive sequences (insert). The fraction of fragments bound to hydroxyapatite is plotted as a function of C_0t. The lower curve represents a least squares solution for four second order components. In this case repeated sequences reassociating prior to $C_0t\ 10^{-1}$ have not been studied. The components are: 28% with a $K > 10^1\ M^{-1}s^{-1}$, 29.4% with a K of $1.66\ M^{-1}s^{-1}$, 22.5% with a K of $0.0488\ M^{-1}s^{-1}$, and 15.6% with a K of $0.00124\ M^{-1}s^{-1}$. The rate of the slowest component was fixed on the value expected for two-copy DNA from the analytical genome size determination. The RMSE value of the solution is 0.027. The curve in the insert is a least squares solution for two second order components, namely 33.5% with a K of $1.23\ M^{-1}s^{-1}$, and 54.0% with a K of $0.00354\ M^{-1}s^{-1}$. The RMSE value of this solution is 0.034

Fig. 7. Reassociation of *Coregonus lavaretus* whole DNA and a preparation enriched in non-repetitive sequences (insert). The fraction of fragments bound to hydroxyapatite is plotted as a function of C_0t. The lower curve depicts a least squares solution for four second order components, namely 18.0% with a $K > 10^3\ M^{-1}s^{-1}$, 20.5% with a K of $9.32\ M^{-1}s^{-1}$, 29.8% with a K of $0.158\ M^{-1}s^{-1}$, and 23.8% with a K of 0.0011. For this solution the rate constant of the slowest component was fixed at the value expected for two copy DNA from the analytical genome size estimate. The RMSE value of the solution is 0.026. The curve in the insert is a least squares solution for two second order components, namely 21.5% with a $K > 10^{-1}\ M^{-1}s^{-1}$, and 65.6% with a K of $0.00421\ M^{-1}s^{-1}$. The RMSE value of this solution is 0.033

Fig. 8. Reassociation of *Salvelinus fontinalis* whole DNA and a preparation enriched in non-repetitive sequences (insert). The fraction of fragments bound to hydroxyapatite is plotted as a function of C_0t. The lower curve depicts a least squares solution for four second order components, namely 19% with a $K > 10^3\ M^{-1}s^{-1}$, 20.8% with a K of $5.42\ M^{-1}s^{-1}$, 30.8% with a K of $0.0911\ M^{-1}s^{-1}$, and 20.6% with a K of $0.00099\ M^{-1}s^{-1}$. The rate constant of the slow component was fixed at the value expected for two-copy DNA from the analytical genome size estimate. The RMSE value of the solution is 0.027. The curve in the insert is a least squares solution for two second order components, namely 39.2% with a K of $0.0949\ M^{-1}s^{-1}$ and 55.4% with a K of $0.00241\ M^{-1}s^{-1}$. The RMSE value of this solution is 0.032

Table 1. Kinetic analysis of whole DNA of species of the family Cyprinidae and of the order Isospondyli

Species	f_1 (%)	K_1 ($M^{-1}s^{-1}$)	f_2 (%)	K_2 ($M^{-1}s^{-1}$)	f_3 (%)	K_3 ($M^{-1}s^{-1}$)	f_4 (%)	K_4 ($M^{-1}s^{-1}$)
Rutilus rutilus	0.101	$>10^4$	0.369	0.213	0.466	0.00178		
Cyrinus carpio	0.151	$>10^3$	0.167	0.156	0.485	0.00096		
Clupea harengus	0.248	$>10^4$	0.227	0.0375	0.383	0.00176		
Sprattus sprattus	0.118	$>10^4$	0.224	0.114	0.536	0.0016		
Thymallus thymallus	0.139	$>10^3$	0.153	31.5	0.423	0.289	0.227	0.00083
Salmo irideus	0.28	$>10^1$	0.294	1.66	0.225	0.0488	0.156	0.00124
Coregonus lavaretus	0.18	$>10^3$	0.205	9.32	0.298	0.158	0.238	0.0011
Salvelinus fontinalis	0.19	$>10^3$	0.208	5.42	0.308	0.0911	0.206	0.00099

f_1–f_4 denote the relative sizes of the kinetic fractions, the rate constants (moles nucleotides $\times$ liter^{-1} $\times s^{-1}$) of which are designated by K_1–K_4. The rate constants of the slow kinetic components were fixed on the value expected for single- or two-copy DNA (see text)

Wolf et al., 1969; Ohno, 1974; Schmidtke et al., 1975a) and of *E. coli* (Cairns, 1963), used as a kinetic standard, the $C_0t_{1/2}$ of which was 2.6 M $\times$ s under our own standard conditions. It should be emphasized that any estimate as to the *size* of the slow fraction is hardly affected, whether the rate constant of two-copy or of single-copy DNA is provided. The difference amounts to a negligible percentage. The rate constants employed for the calculations are always derived from the results of the experiments with slow fraction enriched DNA (see below).

2. *Reassociation of Enriched Non-repetitive DNA*

Since, during the preparation of DNA samples enriched in non-repetitive sequences only one renaturation step was provided to remove repeated DNA, there was still a contaminating residual repeat fraction present, the relative size of which was comparable with the extent of purification as reached by others using the same technique (cf. Smith and Flavell, 1975; Murray et al., 1978; Goldberg, 1978). The solutions of the reassociation kinetics given in the inserts of Figures 1–8 are based on providing two kinetic components, one representing the residual repeat, and the other representing the non-repetitive fractions. Table 2 lists the results of this analysis. If the rate constant of the non-repetitive fraction is corrected for 100% purity a value referred to as "K_{pure}" is obtained. Due to enrichment of the sequences in the slow component during their fractionation from whole DNA, the reassociation rate of the self-annealing kinetics of the non-repetitive sequences is expected to increase. In whole DNA, therefore, these sequences would react more slowly by the same factor that these sequences are a fraction of the whole genomic DNA ("F_{whole}"). A comparison of the rate constant expected for single-copy DNA with the experimental value "$K_{pure} \times F_{whole}$" yields an estimate of repetition frequency in the slow component. The results of these calculations are presented in Table 3. As expected, the slowest renaturing genome fractions of the three phylogenetically

Table 2. Kinetic analysis of DNA enriched for non-repetitive sequences

Species	Residual repeat		Non-repetitive	
	Fraction size (%)	Rate constant ($M^{-1}s^{-1}$)	Fraction size (%)	Rate constant ($M^{-1}s^{-1}$)
Rutilus rutilus	0.302	0.474	0.624	0.00305
Cyprinus carpio	0.171	0.118	0.685	0.00093
Clupea harengus	0.257	0.369	0.653	0.00262
Sprattus sprattus	0.319	0.470	0.556	0.00204
Thymallus thymallus	0.103	$>10^{-1}$	0.768	0.0021
Salmo irideus	0.335	1.23	0.540	0.00354
Coregonus lavaretus	0.215	$>10^{-1}$	0.656	0.00421
Salvelinus fontinalis	0.392	0.0949	0.554	0.00241

Table 3. Calculation of repetition frequency in the slow kinetic DNA fraction

	K_{pure} (rate constant of enriched slow component)[a]	K_{whole} (rate constant expected for single copy DNA)[b]	Repetition frequency in the slow component ($K_{pure} \times F_{whole}/K_{whole}$)[c]
Rutilus rutilus	0.00397	0.00178	1.0
Cyprinus carpio	0.00134	0.00096	0.7
Clupea harengus	0.00401	0.00176	0.9
Sprattus sprattus	0.00367	0.0016	1.2
Thymallus thymallus	0.00273	0.00083	0.7
Salmo irideus	0.00656	0.00062	1.7
Coregonus lavaretus	0.00642	0.00055	2.8
Salvelinus fontinalis	0.00435	0.00050	1.8

[a] K_{pure} is the reassociation rate constant of the non-repetitive sequences in the fractionated DNA preparation, corrected for 100% purity

[b] K_{whole} is calculated from the equation $K_{whole} = G_{std} \times K_{std}/G$, where G denotes genome size,and where E. coli DNA was taken as the single-copy DNA standard ($K_{std} = 0.38\ M^{-1}s^{-1}$, $G_{std} = 0.0047$ pg)

[c] The values for F_{whole} are given in Table 1

diploid species *Rutilus rutilus*, *Clupea harengus*, and *Sprattus sprattus* consist of sequences present only once per haploid genome, based on analytical genome size determinations (lit. cit.), and the kinetic complexity as determined in this study. The two ancient tetraploids *Thymallus thymallus* and *Cyprinus carpio* exhibit a mean repetition frequency of 0.7 in the non-repetitive DNA fractions. This value is probably not appreciably different from 1, i.e., these two tetraploids must have experienced large scale diversification of homologous sequences after (if autotetraploid) and before (if allotetraploid) the polyploidisation event. In order to test the possibility that the reassociation rate in these DNAs is retarded, compared with tetraploid expectation values due to base mismatch of partially diverged sequences (Bonner et al., 1973), the thermal stability of the reassociation products was determined (see below).

Kinetic analysis of the genomes of *Salmo irideus*, *Salvelinus fontinalis* und *Coregonus lavaretus* revealed the existence of extensive conservation of duplicated sequences, capable of reannealment under the conditions employed. Mathemati-

Table 4. Thermal stability of reassociated DNA enriched for the slow kinetic component

	T_m[°C][a] (reassociated slow DNA)	T_m[°C] (of native DNA)	ΔT_n[°C]
Rutilus rutilus	84.5	85.7	1.2
Cyprinus carpio	84.5	85.6	1.1
Clupea harengus	84.9	88.5	3.6
Sprattus sprattus	86.3	88.1	1.8
Thymallus thymallus	85.1	87.7	2.6
Salmo irideus	86.6	87.2	0.6
Coregonus lavaretus	85.3	87.8	2.5
Salvelinus fontinalis	84.2	86.4	2.2

[a] The melting point (T_m) is defined as the temperature of half-denaturation. All values are corrected for fragment length. All determinations were performed in 0.12 M phosphate buffer (pH 6.7). Absorbance values have not been corrected for water expansion

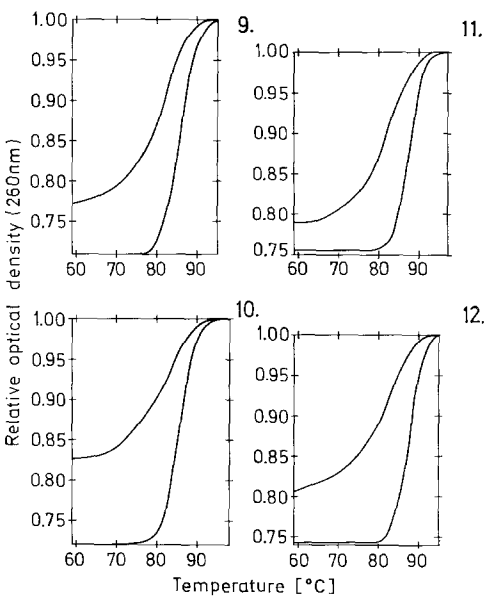

Fig. 9. *Rutilus rutilus.* Melting profile of native DNA and a non-repetitive-sequence-enriched DNA preparation of the reassociation history: C_0t 10 unbound, C_0t 15,000 bound. Meltings were performed in 0.12 M PB in the spectrophotometer

Fig. 10. *Cyprinus carpio.* Melting profile of native DNA and a non-repetitive-sequence-enriched DNA preparation of the reassociation history: C_0t 100 unbound, C_0t 6,400 bound. Meltings were performed in 0.12 M PB in the spectrophotometer

Fig. 11. *Clupea harengus.* Melting profile of native DNA and a non-repetitive-sequence-enriched DNA preparation of the reassociation history: C_0t 10 unbound, C_0t 21,000 bound. Meltings were performed in 0.12 M PB in the spectrophotometer

Fig. 12. *Sprattus sprattus.* Melting profile of native DNA and a non-repetitive-sequence-enriched DNA preparation of the reassociation history: C_0t 12 unbound, C_0t 5,100 bound. Meltings were performed in 0.12 M PB in the spectrophotometer

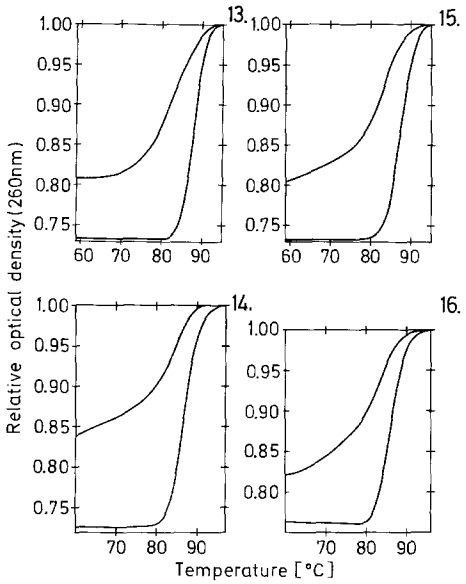

Fig. 13. *Thymallus thymallus.* Melting profile of native DNA and a non-repetitive-sequence-enriched DNA preparation of the reassociation history: C_0t 300 unbound, C_0t 2,400 bound. Meltings were performed in 0.12 M PB in the spectrophotometer

Fig. 14. *Salmo irideus.* Melting profile of native DNA and a non-repetitive-sequence-enriched DNA preparation of the reassociation history: C_0t 200 unbound, C_0t 10,000 bound. Meltings were performed in 0.12 M PB in the spectrophotometer

Fig. 15. *Coregonus lavaretus.* Melting profile of native DNA and a non-repetitive-sequence-enriched DNA preparation of the reassociation history: C_0t 100 unbound, C_0t 2,800 bound. Meltings were performed in 0.12 M PB in the spectrophotometer

Fig. 16. *Salvelinus fontinalis.* Melting profile of native DNA and a non-repetitive-sequence-enriched DNA preparation of the reassociation history: C_0t 100 unbound, C_0t 1,800 bound. Meltings were performed in 0.12 M PB in the spectrophotometer

cally, the repetition frequencies of the slowly renaturing components of these phylogenetically tetraploid genomes range between 1.7 and 2.8. Also, in these cases it was interesting to determine the thermal stability of reassociated non-repetitive sequences in order to detect indications of base sequence divergence. These experiments are reported below.

3. Melting Experiments

The main purpose of these experiments was to detect differences in the thermal stability of native and reassociated non-repetitive DNA, which would indicate the extent of base-sequence divergence of formerly homologous sequences still capable of reannealment under the criterion employed. All meltings were performed in 0.12 M PB. A comparison of the melting points (temperature of half-maximal increase in optical density, T_m) of native and reassociated non-repetitive DNA is given in Table 4. In this table the melting points of reassociated

Table 5. Guanine plus cytosine (GC) content in the DNA of species of the family Cyprinidae and of the order Isospondyli

Species	GC (%)
Rutilus rutilus	40.4
Cyprinus carpio	40.2
Clupea harengus	47.2
Sprattus sprattus	46.3
Thymallus thymallus	45.3
Salmo irideus	44.1
Coregonus lavaretus	45.5
Salvelinus fontinalis	42.1

The guanine plus cytosine contents were estimated from the melting points of native DNA dissolved in 0.12 M phosphate buffer (0.18 M Na^+) according to the formula given by Mandel and Marmur (1968): $GC = 2.44 (T_m - 81.5 - 16.6 \log M\ Na^+)$

DNA are corrected for the effect of paired sequence length on T_m (estimated from hyperchromicity) (Britten et al., 1974). As can be seen from the table, there are only minor T_m-differences between DNA melted in the native or in the reassociated state. The differences observed in the ancient tetraploid genomes range in between those seen among the diploid species. The differences noted are comparable with the findings of other authors using DNA probes of haploid or diploid organisms such as *E. coli* and man (1.5–3.7° C difference in native sheared and reassociated sheared DNA (Britten and Kohne, 1968; Schmid and Deininger, 1975)).

In Figures 9–16 typical melting curves of reassociated DNA enriched in non-repetitive sequences are shown together with native DNA controls. It is evident, when comparing the shapes of these curves, that there is sequence mismatching in the reassociated samples. However, the close resemblance of the situations as seen in diploid DNAs and phylogenetically tetraploid DNAs suggests that sequence divergence during tetraploid evolution has contributed little to the sequence mismatch during reassociation. Part of the sequence mispairing probably results from the residual repeat contamination in these probes.

Table 5 lists the guanine plus cytosine contents calculated from the melting points of native DNA according to the formula of Mandel and Marmur (1968).

Discussion

This study has demonstrated that by means of DNA renaturation methods and melting experiments the genomic DNA of phylogenetically tetraploid species of the teleostean order *Isospondyli* can be distinguished kinetically from that of diploid relatives. These tetraploid species, namely *Salmo irideus*, *Salvelinus fontinalis* and *Coregonus lavaretus* appear to be recent tetraploids on the basis of our experimental results. It is conceded that still more refined methods such as performing renaturation kinetics under differently stringent criteria could reveal otherwise undetected sequence divergence within an ancient tetraploid genome, but the extent of base exchange cannot be substantial. On the other hand, the two ancient tetraploids *Thymallus thymallus (Isospondyli)* and *Cyprinus carpio (Cyprinidae)* do not seem to be distinguishable from diploid controls with respect to DNA kinetics. How are these findings related to species age?

According to paleontological findings (Obruchev, 1967) the first record of the genus *Salmo* dates back some 25 myr, *Coregonus* is about 20 myr old, and both *Thymallus* and *Cyprinus carpio* date back to about 60 myr. Assuming that polyploidy occurred early in the species histories, the time elapsed during evolution of the latter two is sufficient to permit sequence change so extensive that the divergent sets of previously homologous sequences are no longer capable of reannealment in the reassociation assay. If these species were allotetraploids much of the sequence divergence may have occurred even prior to polyploidisation. The age reported for the remaining tetraploids (*Salmo, Coregonus*) likewise should suffice to permit sequence divergence well recognizable by the techniques used, allowing for a rate of nucleotide substitution of 0.3% per myr (see Introduction). Since this is not the case, the conclusion may be drawn that, unless the rate of sequence divergence is grossly lowered in these tetraploids, the event of polyploidisation must have occurred very recently in the history of these genera; also polyploidy must have happened independently in the lines leading to the close relatives *Salmo irideus* and *Salvelinus fontinalis*.

Chromosomally, the genomes of the latter two tetraploids are largely rearranged towards a diploid state. Only very few chromosomes are capable of forming multivalents during the meiotic metaphase I, whereas most chromosomes form bivalents (Ohno et al., 1965; Davisson et al., 1973). Chromosomal diploidisation, the prerequisite of the establishment of functional diversification of duplicated loci, must have proceeded with a fast rate in these genomes.

The question arises, whether the proposition that the Salmonids are recent polyploids is compatible with previous findings as to the numbers of structural genes expressed as distinct duplicates in these tetraploid species. A very recent autotetraploid disposes of four identical copies of each gene coding for a certain polypeptide and hence gene duplication is not manifest at the protein level. During evolution of the tetraploid the duplicated genes diversificate, either retaining or losing their original function; i.e., during the process of diploidisation they acquire the function of genes coding for isoproteins or become functionally silenced. Ferris and Whitt (1977b, 1978), using the theoretical formulations of Nei and Chakraborty (1973) and their own experimental results (1977), have shown that the 50% duplicate expression of structural genes in *Cyprinus carpio* is the result to be expected from a roughly 60 myr history after polyploidisation. In the Salmonids also about 50% of the structural genes appear to be expressed in duplicate (Allendorf et al., 1975; Engel et al., 1975), but 60 myr seems to be too long to account for their species history. However, as Ferris and Whitt (1978) have pointed out, 50% duplicate gene expression is also expected after only 3 myr after polyploidy, at a time when practically all genes not expressed in duplicate, are *not yet* diversified by mutation. The estimate of a 3 myr history after polyploidisation is very well compatible with the results of the kinetic DNA analysis presented here.

Acknowledgements. This work was supported by the Deutsche Forschungsgemeinschaft. We are grateful to the Biologische Anstalt, Helgoland, as well as to Dr. Deufel of the Institut für Seenforschung, Langenargen, Lake Konstanz, for providing material and laboratory facilities. We thank Ms. Sonja Pätzold for her technical assistance and Ms. I. Schmidt and Mr. J.S. Jackson for their editorial help.

References

Allendorf, F.W., Utter, R.M., May, B.P.: Gene duplication within the family Salmonidae. II. Detection and determination of the genetic control of duplicate loci through inheritance studies and the examination of populations. In: Isozymes (C.L. Markert, ed.), Vol. IV, pp. 415–432, New York: Academic Press 1975

Beçak, M.L., Beçak, W., Rabello, M.N.: Cytological evidence of constant tetraploidy in the bisexual South American frog Odontophrynus americanus. Chromosoma (Berl.) **19**, 188–193 (1966)

Beçak, W., Goissis, L.: DNA and RNA content in diploid and tetraploid amphibians. Experientia (Basel) **27**, 345–346 (1971)

Beçak, W., Pueyo, M.T.: Gene regulation in the polyploid amphibian Odontophrynus americanus. Exp. Cell. Res. **63**, 448–451 (1970)

Beçak, W., Schwantes, A.R., Beçak, M.L.: Polymorphism of albumin-like proteins in the South American tetraploid frog Odontophrynus americanus (Salientia: Ceratophrydidae). J. exp. Zool. **168**, 473–476 (1968)

Bonner, T.I., Brenner, D.J., Neufeld, B.R., Britten, R.J.: Reduction in the rate of DNA reassociation by sequence divergence. J. molec. Biol. **81**, 123–135 (1973)

Britten, R.J., Cetta, A., Davidson, E.H.: The single-copy DNA sequence polymorphism of the sea urchin Strongylocentrotus purpuratus. Cell **15**, 1175–1186 (1978)

Britten, R.J., Graham, D.E., Neufeld, B.R.: Analysis of repeating DNA sequences by reassociation. In: Methods in enzymology (L. Grossman and K. Moldave, eds.), Vol. 29E, pp. 363–418. New York: Academic Press 1974

Britten, R.J., Kohne D.E.: Repeated sequences in DNA. Science **161**, 529–540 (1968)

Cairns, J.: The chromosome of E. coli. Cold Spr. Harb. Symp. quant. Biol. **28**, 43–46 (1963)

Davisson, M.T., Wright, J.E., Atherton, L.M.: Cytogenetic analysis of pseudolinkage of LDH loci in the teleost genus Salvelinus. Genetics **73**, 645–658 (1973)

Engel, W., Schmidtke, J., Wolf, U.: Diploid-tetraploid relationships in teleostean fishes. In: Isozymes (L. Markert, ed.), Vol. IV, pp. 449–462. New York: Academic Press 1975

Epplen, J.T., Leipoldt, M., Engel, W., Schmidtke, J.: DNA sequence organisation in avian genomes. Chromosoma (Berl.) **69**, 307–321 (1978)

Ferris, S.D., Whitt, G.S.: Loss of duplicate gene expression after polyploidisation. Nature (Lond.) **265**, 258–260 (1977a)

Ferris, S.D., Whitt, G.S.: Duplicate gene expression in diploid and tetraploid loaches (Cypriniformes, Cobitidae). Biochem. Genet. **15**, 1097–1112 (1977b)

Ferris, S.D., Whitt, G.S.: The evolution of duplicate gene expression in the carp (Cyprinus carpio). Experientia (Basel) **33**, 1299–1301 (1977c)

Ferris, S.D., Whitt, G.S.: Phylogeny of tetraploid catastomid fishes based on the loss of duplicate gene expression. Syst. Zool. **27**, 189–206 (1978)

Galau, G.A., Chamberlin, M.E., Hough, B.R., Britten, R.J., Davidson, E.H.: Evolution of repetitive and non-repetitive DNA in two species of Xenopus. In: Molecular evolution (F.J. Ayala, ed.), p. 200. Sunderland, Mass.: Sinauer Press 1976

Goldberg, R.B.: DNA sequence organisation in the soybean plant. Biochem. Genet. **16**, 45–68 (1978)

Harpold, M.M., Craig, S.P.: The evolution of nonrepetitive DNA in sea urchins. Differentiation 10, 7–11 (1978)

Huxley, J.: Evolution: The modern synthesis. Allen and Unwin: London 1942

Klose, J., Wolf, U., Hitzeroth, H., Ritter, H., Atkin, N.B., Ohno, S.: Duplication of the LDH gene loci by polyploidisation in the fish order Clupeiformes. Humangenetik **5**, 190–196 (1968)

Kohne, D.E., Chiscon, J.A., Hoyer, B.H.: Evolution of mammalian DNA. Proc. 6th Berk. Symp. Math. Stat. Prob. (L.M. LeCame, J. Neyman and E.L. Scott, eds.) Vol. V, pp. 193–209. Los Angeles: University of California Press 1972

Mandel, M., Marmur, J.: Use of ultraviolet absorbance – temperature profile for determining the guanine plus cytosine content of DNA. In: Methods in enzymology (L. Grossman and K. Moldave, eds.), Vol. 12B, pp. 195–206. New York: Academic Press 1968

McCarthy, B.J., Farquhar, M.N.: The rate of change of DNA in evolution. Brookhaven Symp. Biol. **23**, 1–43 (1972)

Mitra, R., Bhatia, C.R.: Repeated and non-repeated nucleotide sequences in diploid and polyploid wheat species. Heredity **31**, 251–262 (1973)

Murray, M.G., Cuellar, R.E., Thompson, W.F.: DNA sequence organisation in the pea genome. Biochemistry **17**, 5781–5790 (1978)

Nei, M., Chakraborty, R.: Genetic distance and electrophoretic identity between taxa. J. molec. Evol. **2**, 323–328 (1973)

Obruchev, D.V.: Fundamentals of Paleontology, Vol. XI, Agnatha, Pisces. Jerusalem: Israel Program for Scientific Translations 1967

Ohno, S.: Evolution by gene duplication. Berlin, Heidelberg, New York: Springer 1970

Ohno, S.: Protocordata, Cyclostomata and Pisces. In: Animal Cytogenetics (B. John, ed.) 4. Chordata 1. Berlin-Stuttgart: Gebrüder Borntraeger 1974

Ohno, S., Atkin, N.B.: Comparative DNA values and chromosome complements in eight species of fishes. Chromosoma (Berl.) **18**, 455–466 (1966)

Ohno, S., Muramoto, J., Christian, L., Atkin, N.B.: Diploid-tetraploid relationship among Old-World members of the fish family Cyprinidae. Chromosoma (Berl.) **23**, 1–9 (1967)

Ohno, S., Stenius, C., Faisst, E., Zenzes, M.T.: Postzygotic chromosomal rearrangements in rainbow trout (Salmo irideus Gibbons). Cytogenetics **4**, 119–129 (1965)

Ordahl, C.P.: Reassociation kinetics of polyploid hepatocyte DNA. Biochim. biophys. Acta (Amst.) **474**, 17–29 (1977)

Schmid, C.W., Deininger, P.L.: Sequence organisation of the human genome. Cell **6**, 345–358 (1975)

Schmidtke, J., Atkin, N.B., Engel, W.: Gene action in fish of tetraploid origin. II. Cellular and biochemical parameters in Clupeoid and Salmonoid fish. Biochem. Genet. **13**, 301–309 (1975a)

Schmidtke, J., Beçak, W., Engel, W.: The reduction of genic activity in the tetraploid amphibian Odontophrynus americanus is not due to loss of ribosomal DNA. Experientia (Basel) **32**, 27–28 (1976)

Schmidtke, J., Engel, W.: Gene action in fish of tetraploid origin. I. Cellular and biochemical parameters in Cyprinid fish. Biochem. Genet. **13**, 45–51 (1975)

Schmidtke, J., Engel, W.: Gene action in fish of tetraploid origin. III. Ribosomal DNA amount in Cyprinid fish. Biochem. Genet. **14**, 19–26 (1976)

Schmidtke, J., Schulte, B., Kuhl, P., Engel, W.: Gene action in fish of tetraploid origin. V. Cellular DNA and protein content and enzyme activities in Cyprinid, Clupeoid, and Salmonoid species. Biochem. Genet. **14**, 975–980 (1976a)

Schmidtke, J., Zenzes, M.T., Dittes, H., Engel, W.: Regulation of cell size in fish of tetraploid origin. Nature (Lond.) **254**, 426–427 (1975)

Schmidtke, J., Zenzes, M.T., Weiler, C., Bross, K., Engel, W.: Gene action in fish of tetraploid origin. IV. Ribosomal DNA amount in Clupeoid and Salmonoid fish. Biochem. Genet. **14** (1976b)

Schwantes, M.L.B., Schwantes, A.R., Beçak, W.: Electrophoretic studies on polyploid amphibians. I. 6-phosphogluconate dehydrogenase (6-PGD). Comp. biochem. Physiol. **56**B, 393–396 (1977)

Smith, D.B., Flavell, R.B.: Characterisation of the wheat genome by renaturation kinetics. Chromosoma (Berl.) **50**, 223–242 (1975)

Straus, N.A.: Comparative DNA renaturation kinetics in amphibians. Proc. nat. Acad. Sci. (Wash.) **68**, 799–802 (1971)

Uyeno, T., Smith, G.R.: Tetraploid origin of the karyotype of Catastomid fishes. Science **175**, 644–646 (1972)

Wolf, U., Ritter, H., Atkin, N.B., Ohno, S.: Polyploidisation in the fish family Cyprinidae, order Cypriniformes. I. DNA content and chromosome sets in various species of Cyprinidae. Humangenetik 7, 240–244 (1969)

Zimmerman, J.L., Goldberg, R.B.: DNA sequence organisation in the genome of Nicotiana tabacum. Chromosoma (Berl.) **59**, 227–252 (1977)

Received May 18, 1979/Accepted May 31, 1979 by W. Beermann
Ready for press June 5, 1979