Chromosoma (Berl.) 83, 191–197 (1981)

CHROMOSOMA
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Single-Copy DNA Relationships Between Diploid and Tetraploid Teleostean Fish Species

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Abstract. The degree of single-copy DNA relatedness among nine Salmonid, Osmerid, and Clupeid species (teleosts, order Isospondyli) was explored by interspecific DNA hybridization and the determination of the thermal stability of these hybrids. It is shown that the extent of base substitution and the amount of shared sequences is largely consistent with the systematic interrelationship of the species compared. A tentative estimate of the average base substitution rate is about 0.1–0.25% per million years, which is in the range typical for animal and plant nuclear genomes. The results are also discussed in view of the phylogenetically tetraploid state of the Salmonid genomes. A comparison of the amount of intra-genomic and inter-genomic divergence in the tetraploids suggests that a polyploidization event occurred recently in Salmonid evolution.

Introduction

A striking feature of Salmonid genomes is ancient polyploidy. A comparison of nuclear DNA content, the chromosome number and number of expressed structural genes coding for various proteins reveals that members of the salmonid sub-families Salmoninae. Coregoninae and Thymallinae have undergone tetraploidization in their evolutionary past, whereas the closely related Osmeridae and Clupeidae, which belong to the same teleostan order (Isospondyli), have remained diploid (for references see Engel and Schmidtke, 1976). Cytogenetic findings suggest that species of the subfamily Salmoninae are engaged in the process of diploidization since, in meiotic metaphase figures bivalents predominate and only few multivalents can be found (Ohno et al., 1965; Nygren et al., 1968, 1971; Davisson et al., 1973). Also the fact that only about half of the isozyme genes appear to be expressed in duplicate in the tetraploids (Allendorf et al., 1975; Engel et al., 1975) may be interpreted as the result of functional gene silencing during diploidization. On principle such a finding could also indicate that tetraploidization in these species occured so recently that sufficient diversification of many hitherto identical gene products had not

0009-5915/81/0083/0191/$01.40

yet taken place in order to be detectable by the electrophoretical methods used. We have recently presented evidence in support of the latter view (Schmidtke et al., 1979): Measurements of DNA reassociation kinetics and of the thermal stability of single-copy DNA show that very little intra-genomic diversification has occured in *Salmo irideus, Salvelinus fontinalis* (Salmoninae and *Coregonus lavaretus* (Coregoninae) but to a large extent in *Thymallus thymallus* (Thymallinae). Paleontological findings indicate that *Salmo* originated at least some 25 million years (myr) ago and the first fossile records of *Coregonus* and *Thymallus* date back about 20 and 60 myr, respectively (Obruchev, 1967). If these species had derived from some common tetraploid ancestor, intragenomic sequence divergence should have been much larger than that observed. Therefore, it was assumed that polyploidy occured independently in each line, unless the rate of nucleotide substitution is unusually low in these tetraploids or the relatively scarce paleontological data are misleading.

In the present investigation we have determined the extent of interspecific nucleotide sequence differences in hybrid single-copy DNA of Salmonid, Osmerid and Clupeid species. We can show, first, that the degree of nucleotide substitution and the amount of reactable sequences parallel the systematic interrelationship of these species. Secondly, the amount of inter-specific sequence divergence is compatible with the paleontological record; an estimate of the average nucleotide substitution rate is about 0.1–0.25% per myr, which is in the range typical for animal and plant genomes. It is discussed that the degree of sequence divergence appears to be higher between rather than within the tetraploid genomes.

Materials and Methods

Provenance of the Animals. Specimens of *Salmo irideus, Salmo trutta, Salmo salar* and *Salvelinus fontinalis* were obtained from trout hatcheries in the Black Forest and the Odenwald, West Germany. *Thymallus thymallus* was caught wild in the Rhine, Switzerland. *Coregonus lavaretus* and *Coregonus fera* were caught in the Lake of Constance, and *Sprattus sprattus* and *Osmerus esperlanus* were captured in the North Sea, near the Isle of Helgoland.

DNA Preparations. DNA was isolated from erythrocyte nuclei, fragmented and sized as described before (Schmidtke et al., 1979). The single strand fragment length of total nuclear DNA preparations ranged between 220 and 530 nucleotides. From *Salmo irideus* DNA a radioactively labelled single-copy enriched DNA was prepared by gap translation in the following manner: Total nuclear DNA, dissolved in 0.12 M phosphate buffer (PB), was sheared to a single strand fragment length of 440 nucleotides, incubated at 60° C to a C_0t of 200, and passed over hydroxyapatite (HAP) equilibrated with 0.12 PB at 60° C. The HAP column was eluted with 0.12 PB and the peak fractions, which are strongly enriched in single-copy sequences (Schmidtke et al., 1979), were precipitated with 2.5 vol. ethanol at −20° C. The precipitate was dissolved in 0.12 PB, dialysed against 0.55 PB, and incubated at 65° C to an equivalent (E) C_0t (Britten et al., 1974) of 10,000. After passage over HAP, and washings with 0.12 PB, the reassociated fragments were then recovered with 0.4 PB, followed by another cycle of HAP chromatography. Material from the peak fraction of the last 0.4 PB elution was introduced into the gap translation assay consisting of 8.6 µg DNA, 68 µCi ^{3}H-dCTP (Amersham-Buchler), 50 units DNA-polymerase from *E. coli* (Boehringer), 55 µM of each dATP, dGTP, and dTTP, 47.4 mM Tris, 9.3 mM $MgCl_2$, 9.4 mM dithiothreitol, and 10 µg bovine serum albumin (Behringwerke, Marburg, West Germany), pH 7.9, in a final volume of 200 µl. The mixture was incubated at 12° C for 19 h, and thereafter applied to a Sephadex-G-100 column and eluted with H_2O. The specific activity of the preparation was 2×10^6 cpm/µg. The

peak fraction of the Sephadex chromatography was adjusted to 0.55 PB, incubated to an EC_0t of 2, in order to remove contaminating palindromic and repetitive sequences, and then passed over HAP as above. The non-reassociated material, now referred to as "*Salmo irideus* single-copy DNA", had a final single strand fragment length of 190 nucleotides, as determined by alkaline sucrose density centrifugation.

DNA Hybridization and Melting Experiments. 4,000 cpm of the *Salmo irideus* single-copy DNA (tracer) were driven with a vast excess of unlabelled total nuclear DNA (2.4–5.2 mM nucleotides) of each of the nine species to an EC_0t of 10,000 (0.55 M PB, 65° C, EC_0t not corrected for the retardation of reassociation due to mispairing). The incubations were terminated by chilling in an ice bath, and the samples were frozen at −20° C until further processing. After thawing and adjustment to 0.12 PB, the samples were adsorbed to HAP equilibrated in the same buffer at 60° C. After two washings at 60° C the temperature of the column was raised by increments of 5° C, and DNA rendered completely single-stranded was eluted with 0.12 PB at each temperature step. The effective temperature was measured directly in the HAP bed using a Pt-100 temperature meter equipped with a digital thermometer. The temperature sensor was introduced into the column through a lateral boring coated with a silicone gasket. Aliquots of the eluates were mixed with a suitable scintillation cocktail and counted in a liquid scintillation spectrometer.

Results

Thermal denaturation profiles of hybrid single-copy DNA's are presented in Figs. 1 and 2. In each figure the melting curve of *Salmo irideus* single-copy DNA hybridized with *Salmo irideus* total nuclear DNA is included for comparison. The melting point (the temperature at which 50% of the hybrid DNA are rendered completely single stranded, T_m) of this homologous hybrid is 76.3° C. This is almost 11° C below the T_m of long native *Salmo irideus* DNA (Schmidtke et al., 1979), and is largely attributable to the short tracer fragment length expected after long incubation periods (Britten et al., 1974; Angerer et al., 1976). The heterologous hybrids melt at temperatures between 3.3 and

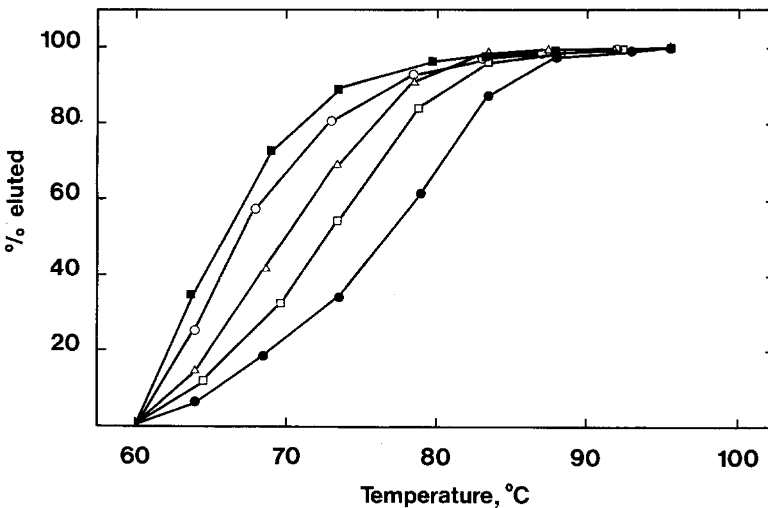

Fig. 1. Thermal elution profiles of *Salmo irideus* single-copy tracer DNA hybridized with excess total sheared nuclear DNA of *Salmo irideus* (●), *Salmo salar* (□), *Coregonus fera* (△), *Osmerus esperlanus* (○), and *Sprattus sprattus* (■)

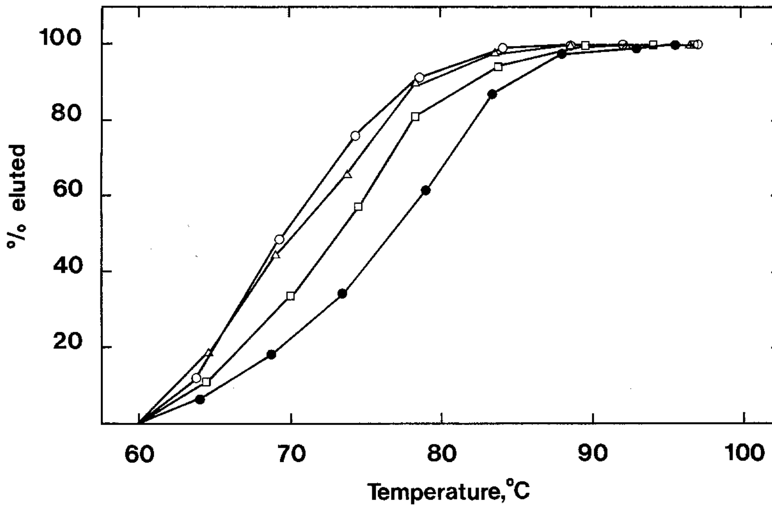

Fig. 2. Thermal elution profiles of *Salmo irideus* single-copy tracer DNA hybridized with excess total sheared nuclear DNA of *Salmo irideus* (●), *Salvelinus fontinalis* (□), (the melting curve of *Salmo trutta* is almost identical with that of *Salvelinus fontinalis* and was omitted for clarity in the figure), *Coregonus lavaretus* (△), and *Thymallus thymallus* (○)

Table 1. Single-copy DNA relationships between species of the order Isospondyli[a]

Driver DNA species	% homologous to S. irideus tracer DNA[b]	T_m of tracer × driver hybrid DNA [°C]	ΔT_m [°C][c]	Average melting temperature [°C][d]	Average sequence divergence [%]
Salmo irideus	100.0	76.3 ± 0.3	0	76.3 ± 0.3	0
S. trutta	80.4 ± 2.6	72.8 ± 0.2	3.5	70.4 ± 0.4	5.9
S. salar	74.8 ± 3.1	72.6 ± 0.4	3.7	69.4 ± 0.2	6.9
Salvelinus fontinalis	80.5 ± 2.7	73.0 ± 0.1	3.3	70.6 ± 0.3	5.7
Coregonus fera	71.2 ± 0.6	70.0 ± 0.0	6.3	66.5 ± 0.2	9.8
C. lavaretus	72.9 ± 0.8	70.0 ± 0.0	6.3	66.7 ± 0.1	9.6
Thymallus thymallus	63.7 ± 0.7	69.5 ± 0.0	6.8	65.1 ± 0.1	11.2
Osmerus esperlanus	22.4 ± 2.8	68.1 ± 0.9	8.2	–	–
Sprattus sprattus	10.1 ± 0.4	65.9 ± 0.1	10.4	–	–

[a] All experiments were done in duplicate; the values in the table indicate the mean and the range

[b] The percentage reassociation of the radioactively labelled *Salmo irideus* single-copy tracer DNA with total *Salmo irideus* nuclear DNA was set equal to 100

[c] The ΔT_m values indicate the differences between the mean melting points of the homologous and the heterologous reactions. They are equivalent to the percentage of nucleotide differences between shared DNA sequences

[d] For explanation see text

10.4° C below the T_m of the homologous melt. These results are detailed in Table 1. The reactivity of the *Salmo irideus* tracer in the homologous reassociation was 64.6 ± 0.7% (mean and range of duplicate determination). Percentage reassociation in the heterologous renaturation is calculated as the hybridized percentage of the tracer reactable in the homologous reaction. These estimates

range between 10.1 and 80.5% and are also detailed in Table 1. A convenient measure of DNA sequence divergence, which combines both the percentage of reacted sequences and their thermal stability, is the *average divergence* (Kohne et al., 1972). This measure is derived from the temperature at which 50% of the total reactable radioactive DNA is in a hybrid form (average melting temperature). These figures are also given in Table 1, for those cases in which more than 50% of the reactable tracer is included in the hybrid.

Discussion

The degree of single-copy DNA relatedness among nine species of the teleostan order Isospondyli was measured in this study by determining (1) the extent of hybridisability of a radioactively labelled single-copy DNA tracer of one of these species (*Salmo irideus*) with the DNA of *Salmo trutta, Salmo salar, Salvelinus fontinalis, Coregonus fera, Coregonus lavaretus, Thymallus thymallus, Osmerus esperlanus*, and *Sprattus sprattus*, and (2) the thermal stability of the hybrid DNAs.

As can be inferred from Fig. 1 and 2 and Table 1, both the thermal stability of the reacted sequences and the extent of hybridization are largely consistent with the systematic relationship established within this teleostean order. It is noteworthy that in the sub-family Salmoninae no significant differences of thermal stability between interspecific and intergeneric DNA hybrids were seen; *Salmo irideus* × *Salmo trutta* and *Salmo irideus* × *Salmo salar* DNA hybrids melt at about the same temperature as the *Salmo irideus* × *Salvelinus fontinalis* DNA hybrid. This result suggests that *Salvelinus* could be phylogenetically derived from the genus *Salmo*.

Using the data of Obruchev (1967) on the first fossile record of the Salmonid species, maximum base substitution rates can be estimated. It is emphasized, however, that due to the inaccuracy of the fossile data, these estimates are only very crude. It is assumed here, that a depression of 1° C in the melting point reflects 1% nucleotide exchange (Bonner et al., 1973). If *Salmo* and *Salvelinus* diverged about 25 myr ago, the rate of nucleotide replacement is about 0.1% per myr [5.7/(25 × 2)]. For *Salmo* and *Coregonus* this rate is 0.25%, and for *Salmo* and *Thymallus* 0.1%. Such rates are similar to those observed by Kohne (1970) in Primates, Angerer et al. (1976) and Harpold and Craig (1978) in sea urchins, Galau et al. (1976) in *Xenopus*, and Stein et al. (1979) in one plant genus.

A comparison of our previous kinetic and thermal results on intragenomic sequence divergence in the Salmonids (Schmidtke et al., 1979) with the results presented here, suggests that the amount of nucleotide replacement appears to be smaller *within* the tetraploid genomes of *Salmo, Salvelinus* and *Coregonus* than *between* these genomes. Since most of the single-copy sequences do not diverge at a grossly retarded rate between these species (compared with other animal model groups) the low amount of intraspecific divergence is interpreted to reflect that only a short period of time elapsed since a tetraploidization event, rather than selective constraints on the duplicated sequences. Furthermore polyploidization should have occurred independently in these genera, and possibly even in species of the same genus. Estimates of the time of occurrence

of a polyploidization event in a given taxon are of importance when the rate of functional silencing of structural genes is evaluated in terms of mutation and genetic drift (Takahata and Maruyama, 1979). Our previous results suggest that even if all members of a particular systematic group are tetraploid, the polyploidization event ought not necessarily to have occurred in a common ancestor sepcies, and therefore the time of polyploidization must not be identical with the phylogenetic age of the taxon.

Apparently in conflict with our suggestion of recent polyploidization in Salmonid evolution are the results of structural gene divergence, which point to a more ancient polyploidization event, 80–100 myr ago (Lim et al., 1975; Lim and Bailey, 1977). It should be noted, however, that the "tetraploid" Salmonid genomes must have experienced a complicated history of DNA additions, possibly including more than one round of polyploidization and/or extensive regionally confined duplications. This is reflected by their DNA contents, which are 3–5 times larger compared with their diploid relatives (see Schmidtke et al., 1976). At present, however, it is not possible to reconcile the conflicting data satisfactorily.

Acknowledgements. This work was supported by the Deutsche Forschungsgemeinschaft. We are grateful to the Biologische Anstalt, Helgoland, for providing material and laboratory facilities.

We thank Dr. M. Schmid for a critical review of the manuscript, and Mrs. H. Tuczynski and Mr. T.S. Jackson for their editorial help.

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Received October 27, 1980–February 4, 1981 / Accepted by W. Beermann
Ready for press February 14, 1981