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Size class homogeneity of repeat lengths and evolutionary divergence of ribosomal RNA genes in fishes as studied by restriction fragment length analysis

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Abstract. Fish ribosomal RNA genes (rDNA) have been compared by restriction endonuclease digestion followed by Southern hybridization using rRNA or cloned rRNA genes as labelled probes. In several species belonging to the orders Cypriniformes and Perciformes, the simple restriction patterns revealed a high degree of size class homogeneity among the rDNA repeats and similar restriction map within a species. Different species have different restriction patterns and fragment lengths arising mostly out of different length of the nontranscribed spacer. Polymorphic restriction sites are present in some species. The species-specific differences in fragment lengths produced in rDNA by some restriction enzymes can thus be used to study interspecific fish hybrids.

Keywords. Restriction fragment length polymorphism; Cypriniformes; Perciformes; rRNA gene; rDNA; Pisces.

1. Introduction

The ribosomal RNA genes (rDNA) of higher eukaryotes exist as multiple copies arranged in tandem repeats clustered at the nucleolar organiser regions (NORs) on specific chromosomes. Each repeating unit consists of a transcribed region and a nontranscribed spacer (NTS) region (Long and Dawid 1980; Mandal 1984). The rDNA coding region of vertebrate species is transcribed to produce a precursor RNA which is processed into mature 18S, 5·8S and 28S rRNA molecules. The fact that ribosomal RNA genes consist of two well-defined regions, transcribed and nontranscribed whose evolution differs in that the transcribed region is more conserved, make this highly repeated gene family a useful system for studying differential evolution of various regions in one genetic unit (Fedoroff 1979).

The transcribed regions are highly conserved because 18S, 5·8S and 28S rRNA contains a high degree of secondary structure (the most important parameter for their function) and therefore the genes must have a very precise primary structure. In contrast it is difficult to assign the parameters responsible for the evolution of spacer region. The spacer regions have changed during evolution much more rapidly. In case of vertebrates, the spacer region appears to differ in length and sequence not only between related species, but also within a single individual (Popodi *et al.* 1985; Dasgupta *et al.* 1989). The differing number of small repetitive elements in the nontranscribed spacer region is the cause for length heterogeneity of rDNA repeat unit.

The evolutionary relationship of ribosomal DNA sequences has been studied in invertebrates, amphibians, birds and mammals (Mandal 1984). Fishes comprise the

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second largest evolutionary class of animals next to insects, more ancient than amphibians in the evolution of vertebrate animals. But the studies of evolutionary relatedness in terms of ribosomal DNA are scanty in fishes. The organization of rRNA genes of three Salmonid species has been studied (Popodi *et al.* 1985). The organization, size class homogeneity and cloning of rRNA genes from catfish *Heteropneustes fossilis* have been previously reported from our laboratory (Dasgupta and Mandal 1988; Dasgupta *et al.* 1989).

In this study, we examined the organization of rDNA repeats in several species of fish belonging to the orders Cypriniformes and Perciformes. The results indicated a high degree of size class homogeneity of rDNA repeats within species while the spacer length varied. Several restriction sites in rRNA coding regions were found to be conserved. The differences of restriction enzyme sites and fragment lengths observed between species were mostly due to the variation in the nontranscribed spacer. The differences in the restriction fragment lengths between species could be used to distinguish some intergeneric hybrids in Cyprinid fish.

2. Materials and methods

2.1 *Fishes*

The species of fishes used are listed in table 1. Most of the living specimens were collected from commercial fisheries supplying pure stocks from induced spawning. For the carps the hybrid fishes were produced by induced breeding (Jhingram 1990). The male and female fishes were given HCG (Human Chorionic Gonadotrophin) hormone injection (1000 I.U./kg of body weight) intramuscularly and released in breeding cages suspended in tanks. The hatchlings were collected and reared in the nursery pond and fingerlings were transferred to the rearing pond until needed. The interfamily hybrid of two catfishes were produced as described (Choudhuri and Mandal 1979).

Table 1. Fish species used in this study.

Common name	Scientific name	Family	Order	Source
Carp (Rohu)	*Labeo rohita*	Cyprinidae	Cypriniformes	Naihati Fish Farm
Carp (Calbasu)	*Labeo calbasu*			
Carp (Mrigala)	*Cirrhinus mrigala*			
Carp (Catla)	*Catla catla*			
Silver carp	*Hypopthalmichthys molitrix*			
Common carp	*Cyprinus carpio*			
Cat fish	*Clarias batrachus*	Claridae		Local fish market
Stinging cat fish	*Heteropneustes fossilis*	Heteropneustidae		
Striped dwarf cat fish	*Mystus vittatus*	Bagridae		
Climbing perch	*Anabus testudineus*	Anabantidae	Perciformes	
Tilapia	*Tilapia mosambica*	Cichlidae		

2.2 *DNA and RNA isolation*

Live fishes were sacrificed and DNA was isolated from their blood by some modification of the method of Marmur (1961). DNA was further purified by digestion successively with DNase-free RNase and Proteinase K, extraction with phenol, phenol–chloroform and chloroform followed by precipitation with ethanol. Plasmid DNA was isolated by the method of Mukhopadhyay and Mandal (1983). Ribosomes and ribosomal RNA was isolated from fish liver as described before (Dasgupta *et al.* 1989).

2.3 *Labelling of RNA and DNA probes*

RNA was fragmented by incubation at alkaline pH and labelled by polynucleotide kinase and [γ-^{32}P] ATP (specific activity 3000 Ci/m mole; Bhabha Atomic Research Centre, Bombay) as described by Gergen *et al.* (1979). DNA was labelled by nick translation after Rigby *et al.* (1977) using [α-^{32}P] dCTP (specific activity 3000 Ci/m mole; Bhabha Atomic Research Centre, Bombay). Unincorporated nucleotides were removed by Sephadex G-50 gel filtration chromatography. All enzymes were purchased from Bethesda Research Laboratories, USA and were used under conditions prescribed by the supplier.

2.4 *Southern blot hybridization*

Genomic DNA of different fish species were digested with appropriate restriction enzymes and fractionated on 0·8% agarose gel by constant voltage electrophoresis. The DNA was transferred to nitrocellulose or nylon membranes (Schleicher and Schuell, Germany) and the filters were hybridized and washed according to the manual of Maniatis *et al.* (1982) in presence of 50% formamide at 37°C for 14–24 h. The entire plasmid or gel purified rDNA fragments or rRNA were used in different experiments as the labelled probe. After hybridization and washing, the filters were autoradiographed using Agfa or ORWO X-ray films. The molecular weight of the hybridized fragments were determined in reference to HindIII digested and end-labelled lambda phage DNA samples run on each gel.

3. Results

Nuclear DNA was isolated from erythrocytes of nine species of order Cypriniformes, two species of order Perciformes and hybrid fishes and digested with the restriction endonucleases which recognize six base sequences within the DNA. Digested DNA after Southern transfer was hybridized using either pXlr101 plasmid, or fragments of 18S and 28S rDNA generated from pXlr101 digested with BamHI as the labelled probe. Restriction sites were positioned by comparing the restriction map of rDNA repeat unit of *Xenopus laevis* (Pruitt and Reeder 1984), and *H. fossilis* (Dasgupta *et al.* 1989) with the autoradiographic bands obtained by complete restriction enzyme digests of fish genomic DNA.

Digestion of genomic DNA with HindIII enabled us to estimate the repeat length of a ribosomal DNA unit (figure 1). In all the cases HindIII cut once in a repeat

unit. The same hybridization pattern was obtained when either 18S or 28S rRNA subunit specific fragments were used as probes (results not presented). Results from EcoRI and BamHI digests of genomic DNA as presented below confirmed the repeat length. The generation of more than one band in HindIII digests of *M. vittatus*, *A. testudineus*, Silver carp, *C. catla* is due to the length heterogeneity in rDNA repeat units. Comparing with the HindIII restriction map of *H. fossilis* and *X. laevis* the single HindIII site is positioned in the spacer region of rDNA repeat unit.

The autoradiographic results of EcoRI digests show a common band of about 5 kb size in all fish species examined (figure 2). This same band lights up when either 18S or 28S rDNA BamHI fragment of rDNA repeat unit of *Xenopus laevis* (Dasgupta and Mandal 1989) is used as a probe (figure 3). Hybridization of this 5 kb band with the both 18S and 28S specific probe confirms the two EcoRI sites positioned near the 3′ end of 18S and 28S rRNA genes as in *X. laevis*, *H. fossilis*, and Salmonid rDNA repeat unit. The higher intensity of the 5 kb band compared to the bands for larger fragments in the cat fishes *C. batrachus*, *M. vittatus* and *A. testudineus* enabled us to consider the 5 kb band as a doublet as it appears in case of *H. fossilis* (Dasgupta and Mandal 1988). This 5 kb doublet is generated due to the third EcoRI site positioned at the NTS region along with that of two other sites at the 3′ end of 18S and 28S rRNA genes. This third EcoRI site also generates smaller fragments (2 to 4·5 kb) in the three cat fishes. It must be noted that in figure 2, apart from the 5 kb and lower bands (visible only after longer exposure), there are some larger bands in *H. molitrix*, *C. carpio*, *C. batrachus* and *A. testudineus*. The uppermost band in *C. carpio* is due to uncut DNA. Other minor bands larger than 5 kb might be due to partial digestion. In *C. carpio*, *C. batrachus* and *A. testudineus*, the bands of around 8–9 kb in length are quite reproducible, indicating heterogeneity and polymorphism in some of the repeats. Due to the rather small amount of DNA being loaded on the gel the bands in lane Lr (*Labeo rohita*) are hardly visible in the photograph, though there were 5 kb and 6 kb bands in the autoradiogram. These polymorphic EcoRI sites have been used to identify the parental rRNA genes in at least two cases of intergeneric hybrids of Cyprinid fish (figure 4 a,b).

It is evident that in the intergeneric hybrid of the cat-fishes *C. batrachus* and *H. fossilis*, the rRNA genes from both the parents are inherited (figure 4a). Similarly, in the hybrid of two major carps (*L. rohita* and *C. mrigala*) also, both the parental genes are inherited (figure 4b). These results clearly demonstrate that restriction fragment length polymorphism of rRNA genes can be used to identify fish hybrids. Similarly, BamHI, PstI and BglII also cut once in all fish rDNA repeats excepting *H. fossilis* (Dasgupta and Mandal 1988), *T. mossambica* and *H. molitrix*, which have two sites each. Some of the results are presented in figure 5a,b. Figure 6 depicts the conserved restriction site map of rRNA gene repeats of the fish species studied. The boundaries of the 18S and 28S rRNA coding region and the spacers depicted are only approximate and positioned in comparison with *H. fossilis* and *X. laevis* rDNA studied in detail.

4. Discussion

Our results agree with the hypothesis that the rRNA genes of vertebrates have remained conserved throughout evolution. Three of the mapped restriction sites are

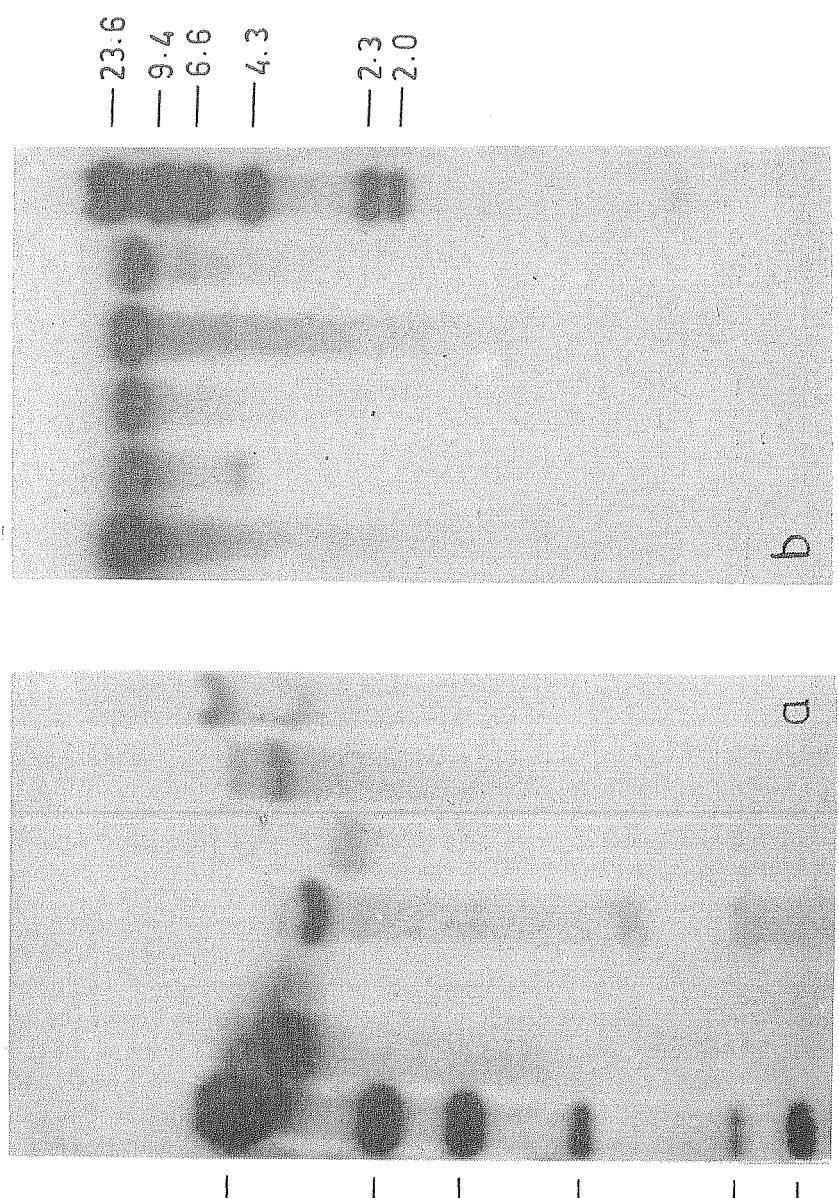

Figure 1. Southern blot of HindIII digests of fish DNA. Nuclear DNA was digested with HindIII and electrophoresed on 0·8% agarose gel. DNA was transferred to Nytran membrane and hybridized with ^{32}P-labelled pXlr101A. Lane M contains HindIII-digested lambda DNA marker run on the same gel and hybridized with ^{32}P-labelled lambda DNA in the same hybridization solution. (**a**) Cb: *C. batrachus*; Tm: *T. mosambica*; At: *A. testudineus*; Lr: *L. rohita*; Cc: *C. carpio*; Ct: *C. catla*. (**b**) Lc: *L. calbasu*; Mv: *M. vittatus*; Cm: *C. mrigala*; Cb: *C. batrachus*; Hm: *H. molitrix*.

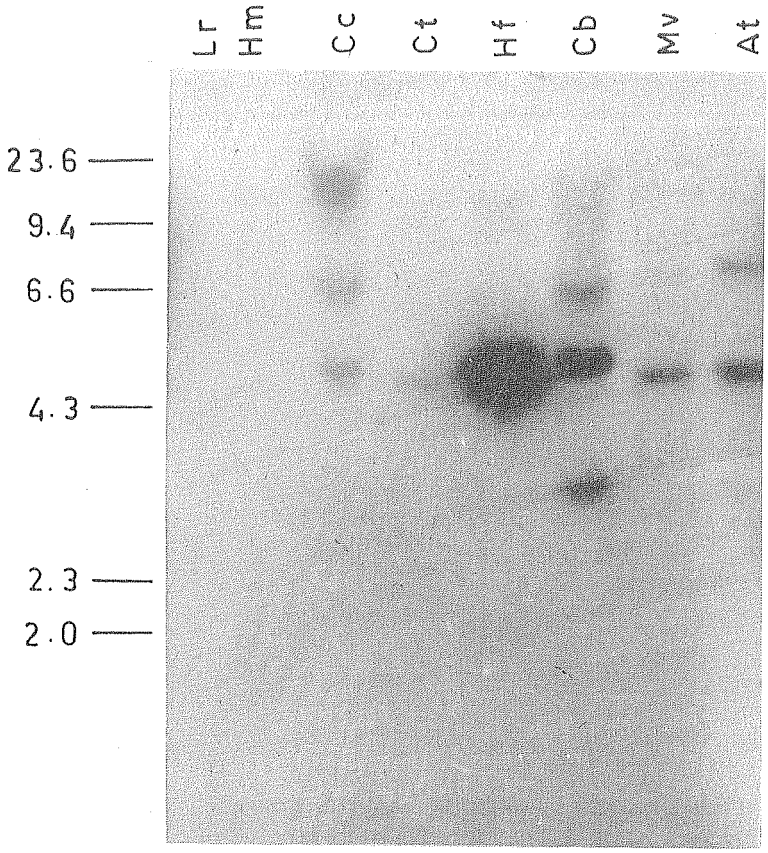

Figure 2. Southern blot of EcoRI digests of fish DNA. Nuclear DNA was digested with EcoRI and electrophoresed on 0·8% agarose gel. DNA was transferred to Nytran membrane and hybridized with ^{32}P-labelled *H. fossilis* rRNA. The position of λ/HindIII size markers in kb are indicated. Lr: *L. rohita*; Hm: *H. molitrix*; Cc: *C. carpio*; Ct: *C. catla*; Hf: *H. fossilis*; Cb: *C. batrachus*; Mv: *M. vittatus*; At: *A. testudineus*.

present at the same position in all fish species examined. Others are more variable. The restriction sites which are highly conserved throughout evolution usually fall in areas where a specific secondary structure has been predicted. The EcoRI site at the 3′ end of the 18S gene is present in all of the species surveyed (Mandal 1984). The EcoRI site is present at a highly conserved stemloop junction of smaller rRNA sequences of *E. coli* as well as in unicellular to multicellular vertebrates. The second EcoRI site near the 3′ end of 28S rRNA gene, though not conserved in all organisms, is conserved in all the fishes examined. This region is also important in the function of the larger subunit RNA and has conserved nucleotide homology (Gourse and Gerbi 1980; Mandal 1984). A conserved BamHI site is located between the highly conserved BglII and EcoRI sites in 28S rRNA genes of all warm-blooded animals. But in lower vertebrates this BamHI site is absent and another BamHI site is present instead near the 5′ end of the 28S gene (Tanhauser *et al.* 1986). Our results correspond to the BamHI restriction map of lower vertebrates. Again in the evolution of vertebrates, an EcoRI site at the 3′ end and a BglII site in the middle

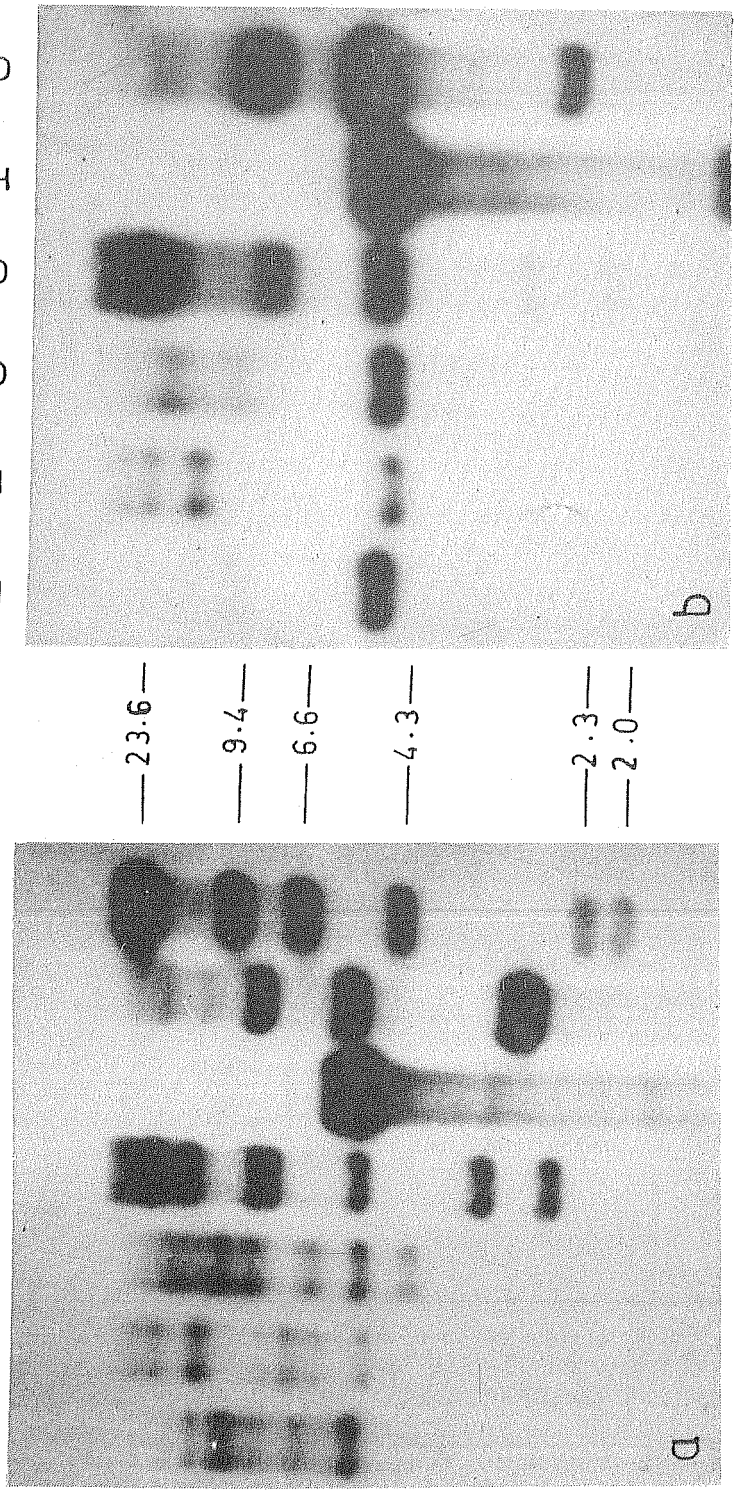

Figure 3. Southern blots of EcoRI-digested fish DNA hybridized with subunit-specific probes. (**a**) Probed with 4·4 kb BamHI (18S subunit) rDNA fragment of pXlr101A. (b) The same blot reprobed with 3·4 kb BamHI (28S subunit) rDNA fragment of pXlr101A. Lane M shows HindIII-digested lambda DNA marker run on the same gel and hybridized with ^{32}P-labelled lambda DNA in the same hybridization solution. Mv: *M. vittatus*; Hm: *H. molitrix*; Ct: *C. catla*; Cc: *C. carpio*; Hf: *H. fossilis*; Cb: *C. batrachus*.

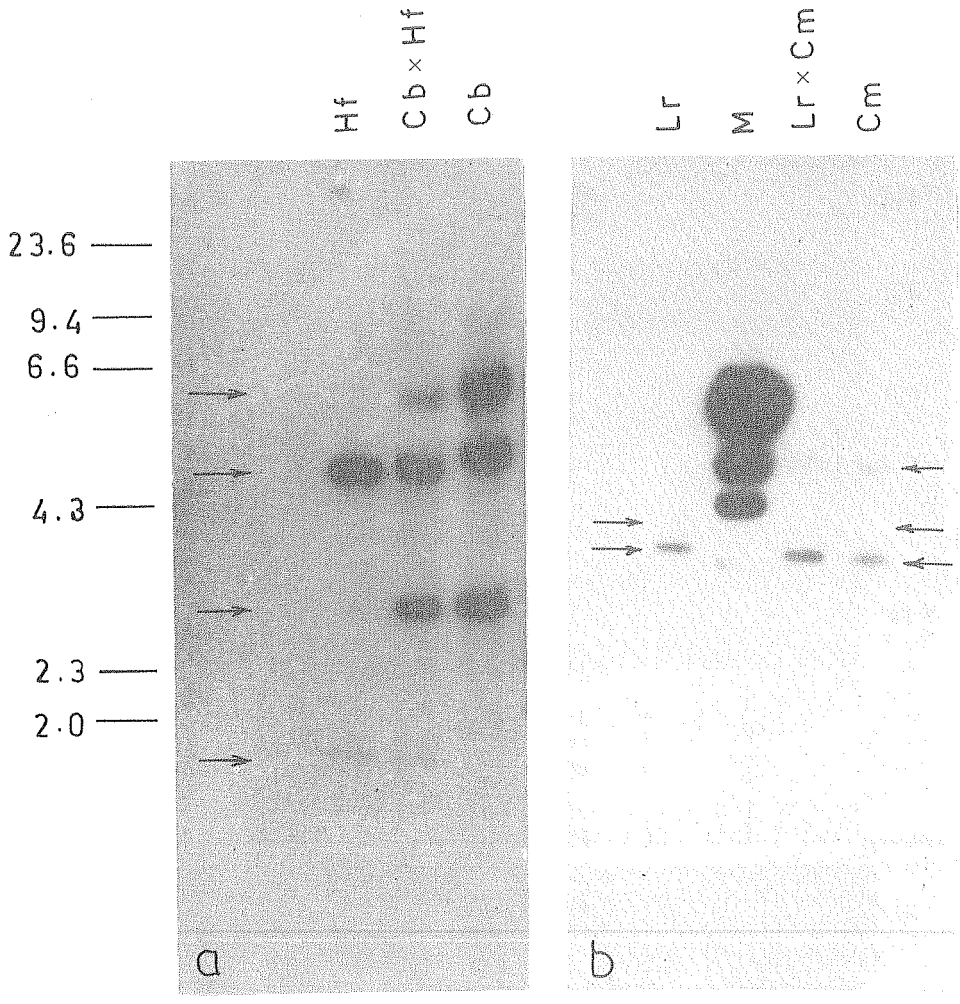

Figure 4. Southern blots of EcoRI-digested fish DNA probed with labelled rDNA fragment from pXlr101A. **(a)** Hf: *H. molitrix*; Cb: *C. batrachus*; Cb × Hf: hybrid of *C. batrachus* (♂) × *H. fossilis* (♀). **(b)** Lr: *L. rohita*; Cm: *C. mrigala*; Lr × Cm: hybrid of *L. rohita* (♂) × *C. mrigala* (♀). M stands for λ/HindIII marker DNA. Arrows indicate the position of the hybridized fragments in the parental and hybrid DNA. The size markers of λ/HindIII DNA (in kb) are indicated by lines.

of the 28S rRNA gene is highly conserved. Our observation is consistent with this highly conserved site.

The present study also shows that the repeat lengths of different fish species vary widely, probably due to differing lengths of the spacer region. The repeat lengths fall within certain size ranges like 12 kb (*H. fossilis*), 16 kb (*C. catla*, *M. vittatus*) and 20 kb (*T. mosambica*, *A. testudineus*). Thus the repeat lengths lie within the size of rDNA of *Xenopus* (12 kb) and that of Salmonid fishes (26 kb), which are much smaller than the 44 kb of mammals. The limited heterogeneity with respect to some polymorphic restriction sites within a species can arise by point mutation and are of

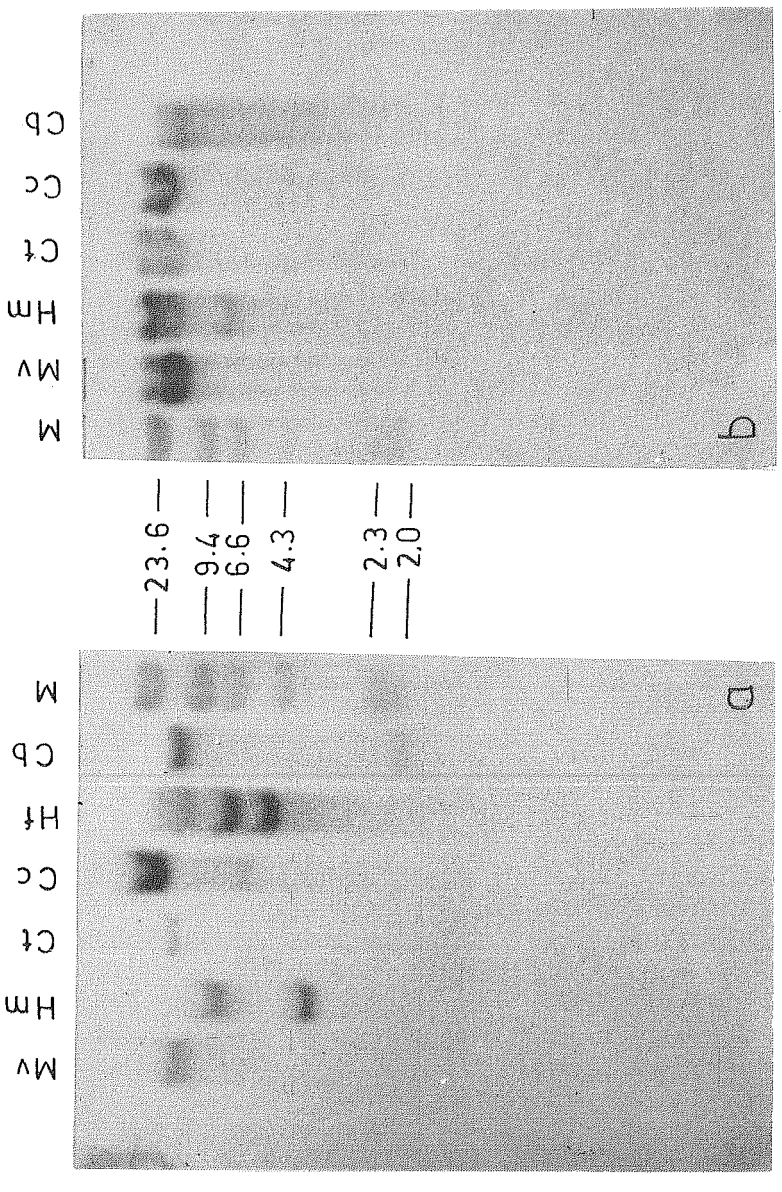

Figure 5. Southern blot of (**a**) BamHI and (**b**) BglII digests of fish DNA, probed with labelled rDNA fragment from pXlr101A. Mv: *M. vittatus*; Hm: *H. molitrix*; Ct: *C. catla*; Cc: *C. carpio*; Cb: *C. batrachus*; Hf: *H. fossilis*. M stands for λ/HindIII marker DNA.

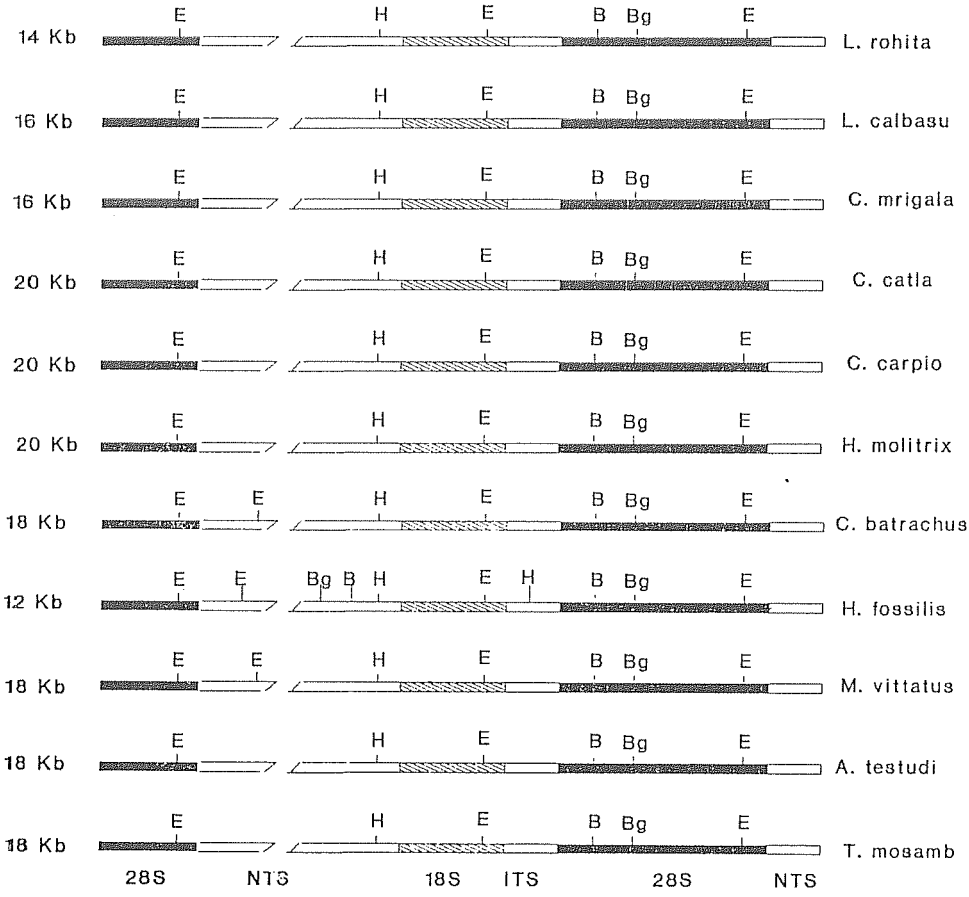

Figure 6. Conserved restriction sites in genomic rDNA of fishes. B: BamHI; Bg: BglII; E: EcoRI; H: HindIII. The boundaries of the 18S, 28S rRNA coding regions and the spacers are approximately positioned. NTS: Non transcribing spacer; ITS: Internal transcribed spacer.

general occurrence (Mandal and Dawid 1981; Cortadas and Pavan 1982). There could also be heterogeneity in restriction sites in the spacers which might have escaped our detection, as nonconserved spacer fragments will not hybridize with heterologous probes. The nonconserved restriction sites of rDNA repeats can be profitably used for genetic studies of intergeneric hybrids of fishes which can be produced easily and also for identification of cultured stock produced by the common practice of mixed spawning.

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