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A study of euteleost phylogeny based on the genomic diversity of ribosomal DNA as a function of euteleost evolution

Jahangir, Z. M. G. Sarwar, Ph.D.

City University of New York, 1995

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A

A STUDY OF EUTELEOST PHYLOGENY BASED ON THE GENOMIC DIVERSITY OF RIBOSOMAL DNA AS A FUNCTION OF EUTELEOST EVOLUTION

by

Z.M.G. SARWAR JAHANGIR

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City university of New York
1995

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Preface

"" *...concepts which have proved useful for ordering things easily assume so great an authority over us, that we forget their terrestrial origin and accept them as unalterable facts. They then become labelled as "conceptual necessities," "a priori situations," etc. The road to scientific progress is frequently blocked for long periods by such errors. It is therefore is not an idle game to exercise our ability to analyze familiar concepts, and to demonstrate the conditions on which their justification and usefulness depend, and the way in which these developed, little by little, from the data of experience. In this way they are deprived of their excessive authority. Albert Einstein.*" Sober, 1988: ix.

DEDICATED TO MY PARENTS
FOR THEIR LOVE
TO
EDUCATION AND KNOWLEDGE

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LIST OF ABBREVIATIONS

ci	consistency index
CsCl	cesium chloride
ITS	internal transcribe spacer
mtDNA	mitochondrial DNA
mtrDNA	mitochondrial ribosomal DNA
nrDNA	nuclear ribosomal DNA
NTR	non-transcribed region
rDNA	ribosomal DNA
RE	restriction endonuclease
ri	retention index
SSC	standard saline citrate
TR	transcribed region

INTRODUCTION

A. The Diversity of Fishes:

Fishes represent a paraphyletic assemblage of vertebrates based on their symplesiomorphic (*earlier ancestral*) characters that include agnathans (hagfishes and lampreys), chondrichthyans (rays, skates and sharks), sarcopterygians (the coelacanth and lungfishes) and actinopterygians (from bichirs\sturgeons to perches) (Lauder and Liem, 1983; Nelson, 1984; Maisey, 1986; Nelson, 1989). Although the presence of a lateral-line system originating from the otic placode is a possible synapomorphy (*shared acquired character*) of fishes, no cladistic or parsimony analysis support their monophyletic origin (Maisey, 1986). The phylogenetic relationships of major groups of fishes based on morphology are subjected to extensive revisions (Patterson, 1982; Lauder and Liem, 1983; Maisey, 1986; Nelson, 1989). Based on morphological data from various sources, Nelson (1989) recently summarized the possible interrelationships of major groups of fishes, which will be used as a point of reference in this thesis (Figure 1). However, Stock *et al.* (1991b) pointed out the existence of disagreements with Nelson (1989) and presented a relationship of major fish groups as a compromise with Peterson (1982), Lauder and Liem (1983), Forey (1984, 1988), Maisey (1986), and Nelson (1989).

The vast majority of the extant fishes (about 23,000) belong to an inclusive taxa, Actinopterygii (Figure 1; also Lauder and Liem, 1983; Nelson, 1984; Maisey, 1986; Gardiner and Schaeffer, 1989; Nelson, 1989). Fossil records support the origin of actinopterygian fishes during the Devonian Period of the Paleozoic Era on the geological time scale (Lagler *et al.*, 1977; Pearson and Westoll, 1979) which was about 350 million years ago. Over this long period of time, actinopterygian fishes went through tremendous radiations that resulted in extensive variations in their morphology, ethology and ecology (Lauder and Liem, 1983). This evolution is unparallel to any other vertebrate, and gave rise to a diverge assemblage of members (Rosen *et al.*, 1981; Lauder and Liem, 1983; Nelson, 1984; Maisey, 1986).

A large number of these actinopterygians, representing 96% of the extant fishes, constitute a natural monophyletic group, Teleostei (Patterson and Rosen, 1977; Patterson, 1982; Rosen, 1982; Lauder and Liem, 1983; Nelson, 1984; Normark *et al.*, *et al.*, 1991). The teleost fishes probably originated 100 million years ago in the Cretaceous Period of the Mesozoic Era and they differ from their sister group, Halecomorphi, by the presence of synapomorphic (i) uroneurals, (ii) unpaired basibranchial toothplates, (iii) a mobile premaxilla and (iv) internal carotid foramen in the parasphenoid (Lauder and Liem, 1983). The uroneurals are the neural spines located at the dorsal part of the ural (caudal) vertebrae (Rosen, 1974), the basibranchials refer to the median part of the gill arch elements (Nelson, 1969), while the parasphenoid is a large flat bone the makes up most of the roof of the oral cavity (Lagler, *et al.*, 1977; Fink and Weitzman, 1982).

A large majority (>94%) of the extant teleosts form the Euteleostei clade (Lauder and Liem, 1983; Fink, 1984; Nelson, 1984; Rosen, 1985; Parenti, 1986; Nelson, 1989). They probably originated during the last 70-100 million years in the Late Mesozoic and Early Cenozoic Era (Lauder and Liem, 1983; Nelson, 1989). However, the interrelationships of higher teleosts (euteleosts) are still unresolved (Rosen, 1982; Fink, 1984; Rosen, 1985; Parenti, 1986; Begle, 1991a,b, 1992; Stiassny and More, 1992; Stiassny, 1993; Johnson and Patterson, 1993; Nelson, 1994). Since, there exists ambiguity and disagreements in the relationships of these and other fishes, Nelson, J. (1987), Nelson, G. (1989) and Stock *et al.* (1991b) emphasized the need for molecular studies in order to resolve decisively the continued controversy of fish phylogeny. Johnson (1993) stated that the conventional morphological analysis to fish phylogeny may approach a limitation in their resolution, and many of these problems will be addressed with molecular data in the near future. However, the success of the molecular approach is yet to be seen (Johnson, 1993).

B. Problems in Euteleost Phylogeny:

The euteleostean fishes includes Ostariophysi, Protacanthopterygi and Neoteleostei, that form a group with diverse characters, and little or no convincing evidence in support of their monophyletic origin is known (Lauder and Liem, 1983; Nelson, 1984; Rosen, 1985). The characteristics that corroborate euteleostean phylogeny are (i) the presence of an adipose fin posterior to the dorsal fin, (ii) nuptial breeding tubercles on the head and the body, and (iii) an anterior membranous component to the 1st uroneural (Lauder and Liem, 1983). However, these characters are observed in primitive euteleosts and are lost in the derived forms which puts the monophyly of Euteleostei into question (Lauder and Liem, 1983; Fink, 1984; Rosen, 1985). Although, some of the euteleostean clades are known to be monophyletic, however, their phylogeny remains controversial due to homoplasy of characters (Lauder and Liem, 1983; Nelson, 1984; Rosen, 1985; Begle, 1991a,b).

B.1. Relationships of Ostariophysi with Other Euteleosts are Arbitrary:

Ostariophysi, an euteleostean clade, is a monophyletic group based on several characters including unique air-bladder morphology, specialization of the vertebrae, adductor mandibulae anatomy, and absence of dermopalatine (Lauder and Liem, 1983; Fink, 1884). Examination of ostariophysan relationships to other euteleosts began with Rosen and Greenwood (1970). Later, Rosen (1973) described Ostariophysi and Neognathi (Protacanthopterygii plus Neoteleostei) as sister groups, and this is supported later by Lauder and Liem (1983). The Protacanthopterygii included Esocoidei (Esocae), Salmonoidae and Osmeroidae (Rosen, 1974). Fink and Weitzman (1982) separated Esocoidei from the rest of the protacanthopterygians, and proposed Esocoidei as the sister group of the rest of the protacanthopterygians and Ostariophysi, while the Protacanthopterygii plus Ostariophysi together form the sister group to Neoteleostei (Fink and Weitzman, 1982).

In the meantime, Rosen (1982) emphasized the importance of structure function relationships in resolving phylogeny of teleosts. However, with the new approach, involving locomotor and feeding mechanisms, still the Ostariophysi and Protacanthopterygii relationships remained unresolved (Rosen, 1982). Later, Fink (1984) further separated the Salmonidae from the above Protacanthopterygii-Ostariophysi group and proposed Argentinoidei, Osmeroidei and Ostariophysi together as the sister group to Salmonidae and *Lepidogalaxias.* Again, Rosen (1985), based on comparison of the neurocranial joints with the first vertebra, rostral cartilage and other anatomical features separated the osmeroids from the Protacanthopterygii-Ostariophysi group and proposed argentinoids and ostariophysans together to form the sister group to salmonoids. These frequent reshuffling of the Ostariophysi relationships with other euteleosts could not be resolved beyond controversy, and in 1991, Begle (1991b) proposed that the ostariophysan and salmonids relationships need to be worked out again.

B.2. Protacanthopterygii and Their Relationships with Other Euteleosts:

In 1966, Greenwood and co-authors combined several groups of fishes together into a clade, Protacanthopterygii, which included, the ostariophysans, salmoniforms, stomiiforms, alepocephaloids, myctophids, and neoscopelids (Greenwood *et al.* 1966). However, later one of the above authors, Rosen (1973), reexamined their morphological characters, and found this group to be very arbitrary, and decided to remove the ostariophysans, stomiiforms, alepocephaloids, myctophids, and neoscopelids from this clade. Subsequently, Rosen (1974) redefined the Protacanthopterygii to contain Esocoidei (Esocidae, Umbridae, and Lepidogalaxiidae), Salmonoidea (Salmonidae and Galaxiidae), and Osmeroidea (Osmeridae, Salangidae, Plecoglossidae and Retropinnidae) as a monophyletic group, based on two presumptive synapomorphic characters: (1) fusion of the third gill arch toothplate with the third pharyngobranchial, and (2) enlarged toothplate associated to the fourth pharyngobranchial without fusion. Sometime later, Fink and Weitzman (1982) pointed out that, fusion of toothplates with endochondral gill elements is a primitive feature present in clupeocephalan, and also present in ostariophysans, stomiiforms and in many eurypterygians. Hence, this character does not support the monophyly of this clade with euteleosts (Fink and Weitzman, 1982).

B.2.1. Is Esocoidei a Euteleost Clade?

The relationships of esocoids which included Esocidae and Umbridae with other euteleosts are under continuous debate. Rosen (1974) proposed equivocally that argentinoids or galaxiids are the primitive Protacanthopterygii (Salmoniform) based on caudal skeletal anatomy. Simultaneously, Rosen (1974) also demonstrated the esocoids as the most primitive Protacanthopterygii based on hypobranchial anatomy. Since, the latter relationship requires only one assumption as opposed to multiple assumptions needed for the former relationship, Rosen (1974) adopted the latter relationship. Again, Fink and Weitzman (1982) identified the presence of dentigerous toothplates on the fourth basibranchial in osteoglossiforms, elopiforms, a clupeiform (*Chirocentrus*), and in Esocoidei including *Lepidogalaxias*, while absent in primitive euteleosts. Since, the Esocoidei lacks adipose fin, and possesses toothplates on the 4th basibranchial (absent in ostariophysans and non-esocoid euteleosts), Fink and Weitzman (1982) found Esocoidei to be separated from the rest of the protacanthopterygians. Hence, they proposed Esocoidei as *sedis mutabilis*, or to be removed from the Protacanthopterygii, and placed as the sister group to all euteleosts (Fink and Weitzman, 1982; Fink, 1984). Soon, Rosen (1985), based on synapomorphic characters of (1) articular bone in the lower jaw, (2) retroarticular in lower jaw, (3) outgrowth of the first uroneural, (4) neural arches, and (5) fusion of toothplates, proposed clupeomorphs and esocoids to form a group and, hence, esocoids are not considered as euteleosts by Rosen. Further, interrelationships between the Esocidae/Umbridae with Euteleostei and thus their interrelationships are still needs clarification (Wilson and Veilleux, 1982; Wilson, 1984; Wilson, 1992).

B.2.1.1. Relationships of Lepidogalaxias with Other Euteleosts are Unknown:

Another problem exists with the position of *Lepidogalaxias;* while Rosen (1974) included this clade with esocoids, Fink and Weitzman (1982) proposed this clade to be related to osmeroids instead of Esocoidei since it possesses mesopterygoid teeth. Later, although both McDowall and Pusey (1983) and Fink

(1984) independently confirmed the absence of mesopterygoid teeth in *Lepidogalaxias*, Fink (1984) proposed it to be the sister group of Neoteleostei due to the presence of retractor dorsalis and tripartite occipital condyle in this genus.

B.2.1.2. Relationships of Umbridae with Other Euteleosts are Arbitrary:

Five species of fishes, *Umbra pygmaea, Umbra limi, Umbra krameri, Novumbra hubbsi,* and *Dallia pectoralis* representing three genera, constitute umbrid family (Rosen, 1974). Rosen (1974) examined the epibranchial and caudal skeletons of umbrid fishes in comparison to *Esox* and other salmonoid fishes and proposed Umbridae as the sister group of Esocidae and included them under Esocoidei. However, the interrelationships of the *Umbra, Novumbra* and *Dallia,* remained unresolved, in spite of the information available from their skeletal characters (Rosen, 1974). Later, Wilson and Veilleux (1982) also analyzed the situation based on several osteological characters and supported that *Dallia* is more related to *Umbra* than *Novambra* and supported the sister group relationships of Umbridae and Esocidae. They, Wilson and Veilleux (1982), also summarized that the Umbridae could be strictly monophyletic based on osteological characters. However, their relationships with other euteleosts remain unresolved.

B.2.2. Relationships of Osmeroidei with Other Euteleosts are Also Arbitrary:

As mentioned before, Osmeroidea includes Osmeridae, Salangidae, Plecoglossidae and Retropinnidae (Rosen, 1974). However, the constitution of this group and their relationships with other euteleosts are still uncertain (Rosen, 1973; Rosen, 1974; Fink and Weitzman, 1982; Lauder and Liem, 1983; Fink, 1984; Rosen, 1985; Begle, 1991a,b, 1992).

In 1973, Rosen included the Osmeroidea within the Protacanthopterygii (Salmoniformes), and proposed Protacanthopterygii as the sister group to Neoteleostei. In 1974, Rosen again projected two separate phylogenies of protacanthopterygian clades based on either caudal anatomy or hyobranchial anatomy with osmeroids as the most derived form in either phylogeny scheme. In 1982, Fink and Weitzman proposed Osmeroidei to form a group including Ostariophysi, Argentinoidei and Salmonidae, and this group to be the sister to Neoteleostei (Fink and Weitzman, 1982). However, Lauder and Liem (1983) proposed a group containing Ostariophysi, Argentinoidei and Osmeroidei, based on the loss of dentigerous toothplate on the fourth basibranchial, to be the sister group to Salmonidae, while the latter is the sister group to Neoteleostei. Fink (1984) supported the above phylogeny with the addition of *Lepidogalaxias* to Salmonidae to form the sister group of Neoteleostei.

However, Rosen (1985) based on three synapomorphic characters, (1) acellular endoskeletal bone, (2) toothed alveolar process on the premaxilla, and (3) caudal skeleton, proposed osmeroids alone to be the sister group of neoteleosts. Begle (1991a) analyzed eighty four morphological characters and reconstructed the interrelationships of the osmeroids. However, in this analysis, the salmonoids and neoteleosts form the sister taxa based on the presence of tripartite occipital condyle (Begle, 1991a) and Osmerae (osmeroids and argentinoids) form a sister taxa to salmonoids and neoteleosts together (Begle, 1991a). Recent evidence on the posterior extension of premaxilla ventrally beyond the maxilla disqualified any single euteleostean clade alone to be a sister group of Neoteleostei (Begle, 1991b). Based on this new information, Begle (1991b) proposed that the euteleosts to belong to six different clades: esocoids, ostariophysans, salmonids, osmeroids, argentinoids and neoteleosts and that their relationships need to be worked out. However, recently Begle (1992) proposed that the osmeroids are closer to Neoteleostei than the salmonids based on the presence of alveolar process of the premaxilla and reduced laminar bone of hyomandibula in the osmeroids and neoteleosts.

B.2.3. Relationships of Salmonoids with Other Euteleosts are Arbitrary too:

Although the salmonoids were proposed to contain Salmonidae, and Galaxiidae by Rosen (1974), the latter was removed from the salmonoids and replaced with osmeroids based on five synapomorphic characters by Begle (1991a). However, the relationships of salmonoids with ostariophysans, esocoids, osmeroids and neoteleosts were frequently shuttled as already discussed (Rosen, 1973; Rosen, 1974; Fink and Weitzman, 1982; Fink, 1984; Rosen 1985; Begle 1991a,b; Roberts, 1993). Fink and Weitzman (1982) produced suggestive evidence of the salmonoid neoteleostean sister group relationships, based on the presence of paired cartilages anterior to ethmoid and exoccipital forming a part of occipital condyle. On the other hand, Rosen (1985) considered salmonoids as the sister group to osmeroids based on paired stegural outgrowth of the first uroneural and presence of partly acellular bone; while the osmeroids form the neoteleostean sister group.

Since, the galaxiids are considered to be osmeroids, the salmonoids only contain Coregoninae, Thymallinae, and Salmoninae clades (Nelson, 1984; Begle, 1991a). The exoccipital forms a part of the condyle in salmonines, *Thymallus* and *Prosopium*, but absent in other coregonines (Fink and Weitzman, 1982). Fink (1984) reported the presence of maxillary teeth in early forms of *Prosopium*, a coregonine, although coregonins are known to lack maxillary teeth. Information on the interrelationships of coregonine, thymalline, and salmonine are still vague (Fink, 1984).

B.3. Neoteleostei has Unresolved Phylogeny:

Euteleosts with further derived characters are grouped together as a monophyletic group, Neoteleostei, with the development of an important synapomorphic muscle character, the *retractor arcus branchialium* or *retractor dorsalis* (RD) (Rosen, 1973; Winterbottom, 1974; Rosen, 1985). Although the RD also evolved independently in *Lepisosteus, Amia, Pantodon,* and *Lepidogalaxius,* the neoteleosts are considered to possess RD proper (Lauder and Wainwright, 1992). This muscle originates posteriorly from any of the first to sixteenth vertebrae and inserts anteriorly into the gill-arch elements called, pharyngobranchials (Rosen, 1973 and Winterbottom, 1974). Liem (1970 and 1978) showed that the RD plays a key role in manipulation, trituration and swallowing of prey in neoteleost fishes. Lauder (1983) reported that, in absence of the RD, the levatores externi 1 and 2, and levatores externi 3 and 4 produce posterior and protraction movements, respectively, in the feeding mechanism in a primitive protacanthopterygian euteleost, *Esox*.

The following synapomorphic characters support the monophyly of Neoteleostei: (i) the unique functional design and structure of the RD, (ii) a rostral cartilage, (iii) type 4 tooth attachment mode, (iv) exoccipital and basioccipital bones articulating with the vertebral column, and (v) insertion of the third internal levator on the fifth upper pharyngeal toothplate (Fink and Weitzman, 1982; Lauder and Liem, 1983; Johnson, 1993). However, Hartel and Stiassny (1986) questioned the synapomorphy of rostral cartilage among Neoteleostei, except acanthomorphs.

Several authors (Fink and Weitzman, 1982; Lauder and Liem, 1983; Rosen 1985) separately proposed phylogeny of the major groups of neoteleosts with Stomiiformes as the primitive and Acanthopterygii as the derived form. The acanthopterygians represent a monophyletic group based on (i) RD inserts principally the 3rd pharyngobranchial, (ii) 2nd and 3rd epibranchials enlarged, (iii) that supports the upper pharyngeal jaws (Rosen, 1973), and (iv) pelvic girdle complex (Stiassny and Moore, 1992).

B.3.1. The Phylogeny of Acanthopterygians Needs to be Worked Out:

Acanthopterygians are divided into (i) Atherinomorpha, and (ii) highly derived and poorly defined Percomorpha. Until recently, Percomorpha was accepted to be a polyphyletic group representing more than 10,000 species of fish belonging to 13 orders (Lauder and Liem, 1983; Nelson, 1984). However, Stiassny (1990) supported the monophyly of percomorph based on the pelvic girdle morphology where the two pelvic bones unite centrally displacing ventral anteromedial processes. Later, Stiassny and Moore (1992) made a review of the pelvic girdle of the percomorphs and further supported its monophyly excluding beryciforms, one of the percomorph orders, based on the central union of pelvic bones and interpelvic ligaments. Recently, Roberts (1993) described the transforming ctenoid scales as the synapomorphic characters diagnosing the percomorphs which excludes beryciforms, gasterosteiforms, atherinomorphs and several others. On the contrary, Johnson and Patterson (1993) examined a wide variety of morphological characters and argued that the Percomorpha of Rosen (1973) is polyphyletic and exclusion of some Rosen's percomorphs and inclusion of some Rosen's non-percomorphs will make it monophyletic. Johnson and Patterson (1993) finds defaults with the monophyletic percomorphs proposed by Stiassny (1990 and 1993), and Roberts (1993). To that end, Johnson and Patterson (1993) proposed a monophyletic percomorph that includes atherinomorphs and smegmamorphs (includes gasterosteiforms) and excludes beryciforms and zeiforms. This emphasizes the need for reinvestigation of percomorph monophyly.

A percomorph ordial group, Perciformes, represents 7,800 species and includes highly diverse percomorphs. Rosen and Greenwood (1976) proposed the interarcual cartilage to be a synapomorphy of perciforms, however, Travers (1981) found it to be present also outside the perciforms. At this juncture, it is important to obtain more character information on the perciforms to reveal their relationships with other euteleosts.

C. Evolutionary Changes are Rooted at the Deoxyribonucleic Acid (DNA) Level:

In the early 60's, Zuckerkandl and Pauling (1965) suggested nucleic acids to contain more evolutionary history inscribed than any other molecules that occur in living organisms, and defined them as primary semantophores. The primary semantophores are the molecules that carry the highest degree of evolutionary history of organisms (Zuckerkandl and Pauling, 1965). Recently, Alberts *et al.* (1989) and McDonald (1990), restated that all evolutionary changes must be rooted at the DNA level and much of the evolutionary history of organisms are recorded in their genome which can be deciphered from a careful analysis of their DNA sequences.

The DNA molecules carry the heredity information of all living organisms, with the exception of ribonucleic acids (RNA) in RNA viruses and possibly proteins in prions (Griffith, 1928; Avery *et al.*, 1944; Alberts *et al.*, 1989; Li and

Graur, 1991). These DNA molecules of an organism are faithfully copied under stringent regulations with extraordinary fidelity and passed on to the next generation to effect inheritance of characters without any distortion (Alberts *et al.*, 1989). However, the DNA molecules are also subject to changes spontaneously or due to various environmental assaults like thermal collisions, ultraviolet light, reactive metabolites, and carcinogens among others, that cause deletions and substitutions in the DNA molecules (Alberts *et al.*, 1989). As a result, 5,000 purine bases are lost per day from the DNA of a human cell, and 100 molecules of cytosine are deaminated to uracil in the DNA of a cell per day (Alberts *et al.*, 1989). Thus, in every cell generation, one base pair out of every 10^9 base pairs of DNA molecules changes stably (Alberts *et al.*, 1989). Despite these random assaults causing extreme changes to a DNA molecule, as mentioned above, only a few changes become stable while the majority are repaired by the DNA repair mechanisms of the cell (Alberts *et al.*, 1989). In addition, during DNA replication, due to unequal crossing over, replication slippage or activity of transposons deletions, insertions, inversions, and transpositions occur in the DNA sequences (Li and Graur, 1991; Lewin, 1994).

The DNA molecules in the germ plasm are also subject to similar changes and they are passed on to the next generations. Such mutations progressively increase their frequency in a population and subsequently may become selected under the selection pressure of nature causing organismic evolution (Lewontin, 1986; Li and Graur, 1991).

Recently, we have observed that the cDNAs of β-gonadotropin genes from carp (*Cyprinus carpio*) and Pacific chinook salmon (*Oncorhynchus tschawytscha*) do not recognize each other, when hybridized following standard Southern hybridization techniques, nor do they recognize the β-gonadotropin gene(s) from five other euteleost fishes studied: brook trout (*Salvelinus fontinalis*), yellowfin tuna (*Thunnus albacarus*), rohu (*Labeo rohita*), bluefish (*Pomatomus saltatrix*) and winter flounder (*Pseudopleuronectes americanus*) (Jahangir and Eckhardt, unpublished). This was further confirmed by Drs. Xiong and Hew (University of Toronto; personal communication) who also observed that the cDNA of β-gonadotropin from chinook salmon does not hybridize with the β-gonadotropin gene(s) of other salmonid fishes. These observations reflect extreme molecular divergence within the β-subunits of the gonadotropin genes among these euteleosts representing relatively close phylogenetic groups (Lauder and Liem, 1983).

On the other hand, another molecule, the nuclear ribosomal DNA (rDNA) is universal to all organisms and contains both rapidly evolving and highly conserved domains which has been demonstrated to be useful in determining phylogeny of closely to distantly related organisms (Wilson *et al.*, 1984; Hillis and Davis, 1986; Hillis and Davis, 1988; Hillis and Dixon, 1989; Wheeler, 1989). In this realm, the present study was undertaken to characterize the rDNA of euteleost fishes in order to determine the genomic diversity of their rDNA as well as their phylogeny based on the rDNA characteristics.

D. Current Approach to Resolve Problems in Phylogeny:

D.1. From Morphological to Molecular Approach:

The establishment of distant phylogenetic relationships among animals based on comparative morphology, anatomy, and embryology remain speculative due to: (1) lack of character traits to unify phyla into larger units, (2) often uncertain homology of similar features among phyla, and (3) lack of fossil records to establish historical continuity among phyla (introductions, Field *et al.*, 1988; Wheeler, 1989: Hendriks *et al.*, 1990). Also among closely related species, for example, most species (about 300) of *Rana* possess limited morphological divergence (Hillis and Davis, 1986). As a result, the phylogenetic relationships of *Rana* were poorly understood based on morphological characters (Hillis and Davis, 1986). Similarly, evidence from the fossil bird, *Archaeopteryx,* support close relationships of birds with crocodilians, however, the presence of single aortic trunk, folded cerebellum, scroll-like turbinals, loop of Henle, adventitious cartilage, and endothermy support the closeness of birds to mammals rather than to crocodilians (Hedges, 1994). These circumstances support the need for an additional approach in order to resolve the phylogeny of organisms.

With the knowledge of the molecular basis of inheritance, biological macromolecules (e.g. proteins and nucleic acids) have acquired an important role in resolving some of the unanswered questions on phylogenetic relationships between organisms (Hillis and Davis, 1986; Hillis, 1987; Field *et al.*, 1988; Dowling and Brown, 1989; Patterson, 1989; Hedges *et al.*, 1990; Hendriks *et al.*, 1990; Lake, 1990; , see Moritz and Hillis, 1990; Hedges, 1994; Wada and Satoh, 1994). Earlier application of molecular studies on systematics mostly involves characterization of proteins using immunological approaches, histochemical staining, isozyme electrophoresis or amino acid sequencing (Lowenstein, 1985; Moritz and Hillis, 1990). Diversity in chromosome numbers and structures also were used as genetic markers to differentiate between species as well as to determine their genealogies (Moritz and Hillis, 1990).

Since it became known that all evolutionary changes must be rooted at the DNA level (for review, see Alberts *et al.*, 1989; McDonald, 1990), the advancement of nucleic acid analysis and manipulation techniques during the last decade has led to the wide use of molecular genetics to resolve some of the unresolved questions left from the traditionally derived phylogenies between closely as well as distantly related organisms (for closely related organisms, see Brownell *et al.*, 1983; Ferris *et al.*, 1983; Wilson *et al.*, 1984; Hixson and Brown, 1986; Suzuki *et al.*, 1986; Holmquist *et al.*, 1988b; Wheeler, 1989; Wilson *et al.*, 1989; for distantly related organisms, see Field *et al.*, 1988; Patterson, 1989; Lake, 1990; Hendriks *et al.*, 1990; Hedges *et al.*, 1990; Hedges, 1994; Wada and Satoh, 1994; for a review on plants, see Zurawski and Clegg, 1987). These studies used either DNA-DNA/RNA hybridization, restriction enzyme analysis, or sequencing nucleic acids of mitochondrial, chloroplast, or nuclear origin (Zurawski, and Clegg, 1987; Hedges *et al.*, 1990; Hedges, 1994; Hendriks *et al.*, 1990; Moritz and Hillis, 1990; Wada and Satoh, 1994;). There are reports on the flow of mitochondrial DNA (mtDNA) across species, for example, in between *Mus musculus* and *Mus domesticus* (Ferris *et al.,* 1983), which put the use of mtDNA in determining interspecies phylogeny into question. However, due to the rapid rate of evolution, and maternal inheritance of mtDNA, this technique may be useful to study relationships of intraspecific populations (Hillis and Davis, 1986; Wilson *et al.,* 1989). On the other hand, although chloroplast DNA is much conserved as compared to the mtDNA, it is obviously limited to plants and protists (Hillis and Davis, 1986; Zurawski and Clegg, 1987).

However, recently, many workers pointed out as well as demonstrated the importance of nuclear rDNA, due to its rapidly evolving and highly conserved domains, in determining phylogenetic relationships between both closely as well as distantly related organisms (Brownell *et al.*, 1983; Wilson *et al.*, 1984; Hillis and Davis, 1986; Suzuki *et al.*, 1986; Field *et al.*, 1988; Hillis and Davis, 1988; Hillis and Dixon, 1989; Patterson 1989; Wheeler 1989; Hendriks *et al.*, 1990; Lake, 1990; Wada and Satoh, 1994).

D.2. Rationale Behind the Choice of Nuclear rDNA:

D.2.1. Nuclear rDNA is Tandemly Repeated in Eukaryotes:

In eukaryotes, the nuclear rDNA is composed of a tandemly repeated unit that includes a nontranscribed spacer (NTS) plus a transcribed region containing pre-ribosomal RNA (rRNA) genes (Figure 2). In *Xenopus laevis,* the pre-rRNA genes include one 18S, 5.8S and 28S RNA genes and transcribed spacers as shown in Figure 2. This arrangement of an rDNA repeating unit is universal in all eukaryotes studied with minor variations and exceptions (Dover, 1988). In some dipteran insects, the 28S rRNA gene is interrupted. The rDNA repeats are always in a head-to-tail configuration but exist in a head-to-head and tail-to-tail configuration in *Acetabularia exigua*. In the extrachromosomal rDNA of *Tetrahymena*, the units are repeated invertedly in alternate positions and thus two adjacent units form a palindrome (for a review, see Long and Dawid, 1980). Unlike other repetitive DNA segments, rDNA is divided into two domains as shown before in Figure 2, the NTS and the transcribed region (TR), that evolve at different rates (Hillis and David, 1986; Williams *et al.*, 1988).

D.2.2. Stretches of rDNA are Conserved Among Distant Phylogenetic Groups:

The transcribed regions of the rDNA are identical or nearly identical within a species in all species studied (for a review, Long and Dawid, 1980; Hillis and Davis, 1986). While the NTS of the rDNA in different species of *Xenopus* are found to diverge widely, certain sequences of their rRNA genes are homologous to some sequences in the rDNA of *Escherichia coli* (Wilson *et al.*, 1984). Similarly, certain sequences of human rRNA gene show considerable similarity with that of *Xenopus* (Wilson *et al.*, 1984). Schmidtke and Engel (1976) and Schmidtke *et al.* (1976) were also able to hybridize rDNAs from several teleost including clupeiforms, cypriniforms and salmoniforms, with human HeLa cell rRNAs. The highly conserved nature of the rDNA even between human and goldfish was demonstrated by Pedersen (1971), and Schmidtke and Engel (1976), by saturation hybridization.

Popodi *et al.* (1985) also found that the rRNA probes containing mouse 28S (clone I-19) and 18S (clone 25-46) RNA sequences specifically hybridizes with rDNAs of three species of trouts: lake trout (*Salvelinus namaycush*), brook trout (*Salvelinus fontinalis*), and rainbow trout (*Oncorhynchus mykiss*). Phillips *et al.* (1989) also used clones I-19 (28S mouse rDNA) and PEB-4 (18S Chinese hamster rDNA) for specific hybridization with rDNAs from brook trout, lake trout, rainbow trout, Arctic charr (*Salvelinus alpinus*), Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*). Recently, both Huang (1990) and Kehagias (1990) independently demonstrated that the probes containing *X. laevis* rDNA hybridizes with the rDNAs from several species of euteleost fishes: winter flounder (*Pseudopleuoronectus americanus*), window pane flounder (*Scophthalmus aquosus*), summer flounder (*Paralichthys dentatus*), and bluefin tuna (*Thunnus thynnus*). Halstead *et al.* (1993) demonstrated that mouse rRNA clones (28S, clone I-19; 18S, clone 25-46) strongly hybridizes with *Xenopus laevis* rDNA (pXlr101A) under high stringency. The above observations support the occurrence of highly conserved segments of rRNA genes among fishes, *Xenopus*, and mammals including human. This conservation of the transcribed region of the rDNA is an indication of a selection pressure on this segment of the rDNA. As a result, rDNA clones are useful in screening libraries of distantly related species (Williams *et al.*, 1988). Further, the copy number of the tandemly repeated rDNA per genome is high which ranges from 200 in human to more than a thousand in some teleosts to 4800 in a lungfish, *Neoceratodus forsteri*, (for a review, see Long and Dawid, 1980). Additionally, mature rRNAs constitute a large part of the cellular RNAs and this is more so in some of the specialized cells, for example, ova, which can be easily used as probes to hybridize with the rDNA cistrons. This phenomenon makes rDNA relatively easy for both cloning and probing across species (for reviews, see Williams *et al.*, 1988; Hillis and Dixon, 1989).

D.2.3. The rDNA Evolves in a Concerted Fashion:

Again, the numerous copies of rDNA evolve in a concerted fashion, and therefore, intraspecies variation in the rDNA sequences is minimum (Dover, 1982; see Hillis, 1987). This observation supports that an rDNA sample from a single individual is sufficient to correctly represent a taxa for phylogenetic analysis (Hillis and Davis, 1986 and 1988; Hillis and Dixon, 1989). The above conclusion has been demonstrated amply to be true as in the 33 species (out of 300) of frogs of the genus *Rana* and in the plant taxa *Lisianthius skinneri* (for a review, see Hillis and Davis, 1988).

D.2.4. Summary:

The above unique characters of rDNA, (1) universal occurrence, (2) similarity in function, (3) organization as tandemly repeated units, (4) rapidly evolving and highly conserved domains, and (5) concerted evolution, make it an attractive molecule to study phylogenetic relationships between both closely as well as distantly related organisms (Wilson *et al.*, 1984; Hillis and Davis, 1986; Hillis and Davis, 1988; Hillis and Dixon, 1989; Wheeler, 1989).

D.4. Use of 18S and/or 28S rRNA/rDNA Sequences in Determining Phylogeny:

Since, the length of a rDNA repeat is very large (9 Kb in *Saccharomyces cerevisiae* to 44 Kb in *Mus musculus*, see Lewin, 1994), sequencing the entire length for phylogeny analysis is cumbersome, if not impossible. Hence, stretches of rDNA, e.g., 18S, 28S, and externally transcribed rDNA spacers, are sequenced for phylogeny reconstruction (Field *et al.*, 1988; Hedges *et al.*, 1990; Hendriks *et al.*, 1990; Hillis *et al.*, 1991; Wada and Satoh, 1994).

Field *et al.* (1988) reconstructed phylogenetic relationships of metazoans based on their 18S rRNA sequences representing 22 classes belonging to 10 phyla, using the distance matrix method. Later, both Patterson (1989) using a parsimony analysis and Lake (1990), by applying the "Evolutionary Parsimony Method" on the same rRNA sequences used previously by Field *et al.* (1988), separately demonstrated the monophyletic origin of the metazoans studied. This finding is consistent with traditional views. Soon after, Hendriks *et al.* (1990) reported the complete sequence of 18S rRNA of the sea anemone, *Anemonia sulcata*, and compared that with 39 other known eukaryotic small rRNA sequences using the "Matrix Optimization Method" for determining their phylogenetic relationships. They once again demonstrated the monophyletic origin of all the metazoans studied (Hendriks *et al.*, 1990).

The brachyuran crabs represent both morphologically and ecologically an extremely divergent group of about 6,000 marine, freshwater and terrestrial species, which are distributed from tropical mountains to deep-sea hydrothermal vents (Spears *et al.*, 1992). Their extensive convergence in adult morphology plagued any attempt in resolving their phylogeny (Spears *et al.*, 1992). However, analysis of their 18S rRNA and rDNA sequences supported: (1) the monophyly of Brachyura excluding Dromiidae, (2) Raninidae as the primitive Brachyura, (3) both Dromiidae and Oxystomata are not monophyletic, (4) the rDNA phylogeny to be concordant with phylogenies based on spermatozoan ultrastructure or larval morphology (Spears *et al.*, 1992).

Similarly, the ancestry of arrowworm (chaetognaths) is highly controversial with deuterostome like ontogeny, pseudocoelomate like morphology and protostome like nervous system (Ghirardelli, 1981; Rehkemper and Welsch, 1985; Willmer, 1990). Telford and Holland (1993) using 18S rDNA sequences demonstrated the chaetognaths not to be related to deuterostomes. Wada and Satoh (1994) using 1,369 unambiguously aligned 18S rDNA sequences from two chaetognaths, one mollusc, one ascidian, one arthropod, five echinoderms, one hemichordate, two urochordates, one cephalochordate and three vertebrates demonstrated the monophyly of deuterostomes. This is in agreement with the generally accepted view (Wallace *et al.*, 1991) and in disagreement with Løvtrup (1975) who supported the existence of a common ancestor to mollusc and vertebrates, different from echinoderms. Further, Wada and Satoh (1994) found the chaetognaths unrelated to deuterostomes which is in agreement with Rehkemper and Welsch (1985) and Telford and Holland (1993).

The potential for 28S ribosomal RNA gene in elucidating vertebrate phylogeny has been demonstrated by Hillis and Davis (1987) and Hillis and Dixon (1989). Recently, Hedges *et al.* (1990) reconfirmed the crocodilian-bird common ancestry based on 28S rRNA sequences, analyzed with the Neighbor-Joining method. Hillis *et al.* (1991) sequenced 2,000 base long 28S rDNA from eight species of vertebrates, and upon analysis, could demonstrate that the coelacanth belongs to the sarcopterygians. Again, Hedges *et al.* (1990) used sequences from both 18S and 28S rRNAs to reconstruct the phylogeny of amniotes.

The above reports demonstrate the use of 18S and/or 28S rRNA/rDNA in reconstructing phylogenetic relationships among distant animal phyla.

D.4. Use of Restriction Endonuclease Maps of rDNA to Determine Phylogeny:

Wilson *et al.* (1984) characterized a 16Kb region from human rDNA surrounding the transcription initiation site immediately upstream to the 18S rRNA gene by restriction enzyme mapping. They took conservative segments of this region and used them separately as probes to locate complementary segments in rDNAs from five different primates. Genomic DNA from each of the primates was digested either with *Bam*HI, or *Sal*I, or *Hinc*II, and each digest was hybridized against either of the probes separately or as one probe followed by another (Wilson *et al.*, 1984). From the above, a restriction enzyme map of the rDNA of each of the primates was constructed. The maps were analyzed using computer assisted parsimony analysis to construct a primate phylogenetic tree (Wilson *et al.*, 1984). This analysis demonstrated that the chimpanzee is more closely related to human than is the gorilla. This conclusion also received support later from Diamond (1988) and Holmquist *et al.* (1988a, b). Similarly, Suzuki *et al.* (1986) used a 0.7Kb rDNA probe comprising the 5′-end of the 28S rDNA and the following nontranscribed spacer from mouse (*Mus musculus*). Using this, they determined unique patterns of organization of the segments complementary to the probe in the rDNA from more than seven subspecies of wild mice collected from 31 locations in four continents (Suzuki *et al.*, 1986). Based on the information obtained from the above experiment, they constructed restriction enzyme maps of the rDNA for each of the subspecies for comparison (Suzuki *et al.*, 1986). Thus, they were able to merge several subspecies together into one and reclassify these wild mice into three subspecies (Suzuki *et al.*, 1986). Recently, Suzuki *et al.* (1994) were also able to demonstrate differences in the RE recognition sites between field mouse nuclear rDNA, *Apodemus speciosus*, populations in the main and small islands of Japan, which may have phylogenetic implications.

The restriction endonuclease (RE) recognition site characters of rDNA also are currently applied in determining the phylogeny, population identification, and species differentiation among many plants and fungi (Crisci *et al.*, 1990; Gardes *et al.*, 1990; Bobola *et al.*, 1992; Govindaraju *et al.*, 1992; Harvey, 1992; Crawford *et al.*, 1993; Bernier *et al.*, 1994; Shen, *et al.*, 1994). Crisci *et al.* (1990) used the rDNA RE recognition site characters of flowering plants belonging to the family Onagraceae family to determine their phylogeny, and thus were able to demonstrate the monophyletic origin of the genus *Epilobium*. Similarly, Harvey (1992) using both nuclear and chloroplast rDNA of several varieties of bulb onion, *Allium cepa*, and wild species *Allium phyllodolon*, was able to demonstrate their origins. On the other hand, Govindaraju *et al.* (1992), Crawford *et al.* (1993), Bernier *et al.* (1994), and Shen *et al.* (1994) used nuclear rDNA of ten species of pines (*Pinus*), rDNA from 41 populations of five angiosperm species belonging to *Robinsonia*, nuclear rDNA from 26 varieties of *Gremmeniella* and one variety of *Ascolyx* fungi, and small rDNA from 15 species of *Kluyveromyces* fungus, respectively, to determine their phylogenies.

There are limited morphological differences between the species of *Rana* to determine their phylogeny. Hillis and Davis (1986) mapped the rDNAs from 32 species of *Rana* with restriction endonucleases. Then they constructed the phylogeny of these frogs based on the differences in restriction endonuclease sites, as well as the internal spacer lengths of their rDNAs (Hillis and Davis, 1986).

Similarly, due to a lack of sufficient evidence on the morphological traits, there were disagreements for a long time concerning the phylogeny of three insect groups: Paleoptera, Eumetabola and Holometabola (Wheeler, 1989). However, Wheeler (1989) selected eleven species of insects representing these taxa and mapped their rDNAs with recognition sites for nine different restriction endonucleases. Analyzing these data using Swofford's PAUP program, Wheeler (1989) was able to construct a phylogenetic tree with monophyletic groups at several levels of the insects studied.

The Phylogenetic relationships of the New World leaf-nosed bats of the family Phyllostomidae is a subject of continuous reshuffling. The phyllostomids form the most diverse chiropteran taxon containing 140 species in 46 genera (Smith and Hood, 1984). In a review, Baker *et al.* (1989) enlisted their enormous morphological, ecological, chromosomal and biochemical diversity. Van Den Bussche (1991; 1992) analyzed the restriction endonuclease sites of rDNAs from 45 bats belonging to 42 genera and demonstrated that the phyllostomids and the desmodontids form a monophyletic group.

D.5. Summary on the Use of rDNA in Determining Phylogeny:

D.5.1. Use of Highly Conserved Versus Rapidly Evolving Segments of rDNA

In the studies mentioned before, both 18S and 28S rRNA/rDNA sequences were used for determining distant phylogenetic relationships among the animal phyla (Hillis and Davis, 1987; Field *et al.*, 1988; and Hillis and Dixon, 1989; Patterson, 1989; Hedges *et al.*, 1990; Lake, 1990; Hendriks *et al.*, 1990; Hillis *et al.*, 1991). Wheeler (1989) on the other hand mapped the entire coding regions of rDNAs of the eleven insect species by restriction enzyme analysis to determine their phylogenetic relationships. Hillis and Davis (1986) also mapped the conserved segments of the rDNAs (18S and 28S) from 32 species of *Rana* and determined their phylogenetic relationships. The rapidly evolving segments of rDNA, on the other hand, were used to trace closer phylogenetic relationships among primates (Wilson *et al.*, 1984) and wild mice (Suzuki *et al.*, 1986). These observations support that the conserved segments of the rDNA may be used for determining distant phylogeny while the rapidly evolving segments of the rDNA may be used for determining closer phylogeny, even at the species level.

D.5.2. Restriction Enzyme Mapping in Determining Phylogeny:

Both restriction enzyme mapping and sequencing have been recently applied to determine phylogenetic relationships (Hillis and Moritz, 1990). Neither of the techniques can be superior to the other under all circumstances, although one may be preferred over the other to answer a specific question (Hillis and Moritz, 1990). Field *et al.* (1988), Patterson (1989), Lake (1990) and Hendriks *et al.* (1990) used the nucleotide sequences of 18S rRNAs or small rRNAs from different representative organisms for phylogenetic analysis. Wilson *et al.* (1984), Hillis and Davis (1986), Suzuki *et al.* (1986), Phillips *et al.* (1989) and Wheeler (1989), instead of sequencing the rDNAs, used restriction enzyme maps to determine the phylogenetic relationships between the organisms studied in each case.

Recently, Diamond (1988) pointed out that in the DNA sequencing method, only a small fragment of the genome is used to find the phylogenetic relationships. Hence, there exists a greater probability for error resulting from small numbers of mismatches (Diamond, 1988). At the same time Wheeler (1989) pointed out the advantages on the use of restriction enzyme maps in comparison to DNA sequencing to determine phylogenetic relationships. Wheeler (1989) mentioned that the restriction enzyme analysis considers a larger segment of the genome than could readily be sequenced. As a result, a piece of DNA insertion and deletion, small or large, which may go unnoticed while using the DNA sequencing method, will be more easily recognized in restriction endonuclease mapping to determine phylogenetic relationships (Wheeler, 1989).

D.6. Mathematical Analysis of Characters in Establishing Phylogeny:

The mathematical tools currently used to analyze phylogeny based on morphology are also widely used in molecular systematics. They are of two types: Parsimony analysis and Distance Matrix Method. In most of the studies on molecular phylogenetics, as mentioned before, used either of the methods (e.g. Wilson *et al.*, 1984; Hillis and Davis, 1986; Field *et al.*, 1988; Patterson, 1989; Hendriks *et al.*, 1990; Lake, 1990). These tools mostly apply the parsimony analysis, in addition to others, which is based on cladistics. They are available as computer software packages, for example, PHYLIP, PAUP, *Hennig86*, MacClade and MALIGN (see Sanderson, 1990; Swofford and Olsen, 1990; Wheeler and Gladstein, 1991). All these programs differs from each other on the methods of analysis (e.g. parsimony) and the algorithm in use (Sanderson, 1990; Swofford and Olsen, 1990). PHYLIP, PAUP and *Hennig86* use "exact" algorithms that guarantees to find the minimal tree(s) (Sanderson, 1990). It has been stated that equally parsimonious trees were generated by using both PAUP and *Hennig86* on a couple of sets of data, however, with different efficiency (Sanderson, 1990). There are also limits on the number of taxa that can be resolved with efficiency for phylogenetic relationships using this software (Sanderson, 1990). For an example, in a complex set of data (>40 taxa), *Hennig86* has been stated to be significantly faster than the PAUP (Sanderson, 1990). However, while analyzing a complex set of data in order to resolve phylogenetic relationships, application of a combination of these software packages may be useful (Sanderson, 1990).

Use of these mathematical tools to analyze the (nucleic acid) data in order to determine the phylogenetic relationships are not without potential for error (for a review, see Holmquist *et al.*, 1988a). The Parsimony method is used to minimize the number of events in resolving phylogenetic relationships although one does not have to believe that evolution must follow the parsimony path (Fitch, 1977; Holmquist *et al.*, 1988a). Felsenstein (1988) discussed the extent of the assumptions needed behind some of the mathematical analyses and raised doubts as to the applicability of the conclusions to evolution. Similarly, Lake (1990) explains that even the extensive algorithms used in these methods may fail to find correct phylogenetic relationships when rates of nucleotide substitutions are frequent but unequal in juxtaposed branches. Many of the shortcomings inherent in the mathematical analysis of finding phylogenetic relationships were eliminated by Lake's new methods of phylogenetic invarients and operator matrix (for a review, Holmquist *et al.*, 1988a).

However, Penny (1988) after discussing the assumptions needed in the use of these tools, supported the use of available mathematical methods to build phylogenetic trees instead of publishing the DNA sequences alone. Similarly, Hixon and Brown (1986), Wheeler (1989), Normark *et al.* (1991) used PAUP version 3.0 to analyze the restriction fragment characters of the rDNAs to construct phylogenetic trees. Wilson *et al.* (1984) used computer assisted parsimony analysis described by Fitch (1977) to analyze the restriction enzyme data of the rDNA of primates to determine their phylogeny. Hendriks *et al.* (1990) used the Matrix optimization Method to obtain tree topology among the eukaryotes using 18S rDNA sequences. Field *et al.* (1988) used Distance Matrix Method, Patterson (1989) used parsimony analysis and Lake (1990) applied rate independent techniques of Evolutionary Parsimony to analyze the genealogies of the metazoans.

The best program for phylogeny analysis depends on the complexity of the set of data and the flexibility needed. For a small set of data PHYLIP, PAUP, or *Hennig86* will be able to generate the shortest trees, although PHYLIP will be slower as compared to the others (Sanderson, 1990).

E. Use of Molecular Genetics in Tracing the Phylogeny of Fishes:

E.1. Introduction:

Literature on molecular phylogeny of fishes is scanty and it was more so when the original proposal for this thesis was written in 1991 (for a review, see

Stock *et al.*, 1991b). There are a few reports where molecular data were used to determine fish phylogeny (e.g., hemoglobin sequences by Goodman *et al.*, 1987; cytochrome *b* and 12S mitochondrial (mt) rRNA by Meyer and Wilson, 1990; mtDNA by Dowling and Brown, 1989; cytochrome *b*, cytochrome oxidase I and II from the mtDNA by Normark *et al.*, 1991; 18S rRNA by Joss *et al.*, 1991 and Stock *et al.*, 1991a; 28S rRNA by Le *et al.*, 1989; Hillis and Dixon, 1989; Hillis *et al.*, 1991; rDNA restriction maps by Popodi *et al.*, 1985; and Phillips *et al.*, 1989; 1992). In a number of cases, molecular data of representatives from major fish groups were utilized to address the phylogeny of other organisms (Maeda *et al.*, 1984; Goodman *et al.*, 1988; Hedges *et al.*, 1990). Recently, Phillips *et al.* (1992), Bernardi *et al.* (1993) and Le *et al.* 1993) reconstructed the phylogenetic relationships of salmonids, bony fishes and gnathostomes, based on rDNA restriction endonuclease recognition characteristics, growth hormone amino acid and rRNA sequences, respectively.

E.2. Use of Polypeptides:

In 1984, Maeda *et al.* (1984) examined α parvalbumin sequence from one elasmobranch, seven teleosts, a coelacanth, and four tetrapods as well as β parvalbumins from a teleost, a coelacanth and three tetrapods to determine the relationships of lower vertebrates. However, in the above study, it was not possible for the authors to demonstrate monophyly of Amphibia and Amniotes. Further, the relationships within the Teleostei thus generated were in gross disagreement with the currently accepted ordinal relationships (Maeda *et al.*, 1984, also for a review, Stock *et al.*, 1991b). For example, the data on parvalbumins diversity, demonstrated that boa (*Boa constrictor*) to cluster with teleosts in the most parsimonious β parvalbumin lineage (Maeda *et al.*, 1984). Similarly, the most parsimonious trees based on both α and β parvalbumins support an actinopterygian instead of sarcopterygian to be the ancestor of tetrapods (Maeda, *et al.*, 1984). Based on these anomalies, the authors concluded that the parvalbumins are unsuitable for determining relationships among vertebrates due to their uneven rates of evolution, homoplasy and gene duplication in gnathostomes (Maeda *et al.*, 1984).

Again, Goodman *et al.* (1987) compared both α and β hemoglobin sequences of some invertebrates, cyclostomes, a shark, a lungfish, and a few teleosts with about 200 tetrapods. The tree based on β hemoglobin sequences shows shark as the primitive gnathostome with lungfish as the sister group of teleosts and tetrapods. On the other hand, the tree based on α hemoglobin sequences shows lungfish as the primitive gnathostome with shark as the sister group of teleosts and tetrapods. They subsequently included globin genes, α crystallin, cytochrome *c*, as available, along with α and β hemoglobin sequences and generated a tree identical to the tree based on β hemoglobin sequences. Based on this large set of data, Goodman *et al.* (1987) only concluded that their data supported a sister group relationship between teleosts and tetrapods. However, the anomalous relationships of sharks being closer to bony fishes and tetrapods than lungfishes generated by α hemoglobin puts the reliability of hemoglobin genes into question.

Recently, however, Bernardi *et al.*, 1993, reconstructed the phylogeny of 26 actinopterygian fishes belonging to Chondrostei, Elopomorpha, Ostariophysi, Protacanthopterygii and Acanthopterygii clades based on growth hormone amino acid sequences. Although the phylogeny of major groups are in agreement with Lauder and Liem (1983) and Nelson (1989), the relationships of closer groups and terminal clades remain unresolved. The authors concluded that the growth hormone, a highly conserved protein, may be useful for distant phylogeny while nucleic acid sequences may be more informative for closely related taxa (Bernardi *et al.*, 1993).

E.3. Use of Mitochondrial Genes:

Mitochondrial (mt) genes were also used to determine fish phylogeny. Meyer and Wilson (1990) investigated the relationships of a lungfish, a teleost, the coelacanth and a frog using 664 nucleotides of 12S mtrRNA and cytochrome *b* gene. Their results demonstrated a sister group relationship of the lungfish and tetrapod, however, the number of taxa examined was limited to four in this study (Meyer and Wilson, 1990). However, Sanderson (1989) has demonstrated that levels of conflicts in phylogeny analysis tend to increase with an increase in taxa. If this becomes true for Meyer and Wilson (1990) than addition of taxa may change the relationships as suggested based on mt genes. Hence, this observation could not convincingly demonstrate the reliability of 12S mtrDNA/genes in determining the relationships of major groups of fishes and more so for euteleosts fishes.

The phylogenetic relationships between the minnows (*Notropis*) have been intensely studied using morphological traits but remain unresolved due to their enormous diversity (Dowling and Brown, 1989). In order to resolve the relationships between the minnows, Dowling and Brown (1989) selected four species of minnows (*Notropis chrysocephalus, N. cornutus, N. photogenis* and *N. rubellus*) and generated restriction enzyme maps of their mtDNAs. Side-by-side, they (Dowling and Brown, 1989) also determined 24 gene loci for 14 different allozymes of each of the minnows. The data thus generated were analyzed cladistically with computer softwares PAUP, PHYLIP and BOOT. Further, the data on the restriction enzyme sites and on the allozymes were converted to distances with computer programs developed by Nei and Tajima and Nei and Associates, respectively. An unweighted pair group method (UPGMA) was used for determining their phylogenetic relationships.

On analyzing the data on allozyme loci, it was possible to resolve a monophyletic relationship between *N. chrysocephalus* and *N. cornutus* putting *N. photogenis* and *N. rubellus* separate (Dowling and Brown, 1989). However, analysis of the data on mtDNA could not provide any resolution on the phylogenetic relationships of these minnows. They suggested that the rapid rate of evolution of mtDNA might have reached a level of convergence, and thus, the mtDNAs lost the character traits related to their phylogeny.

Although the monophyly of the Neopterygii fishes is reasonably resolved, the relationships between its three extant taxa, Lepisostidae/Ginglymodi, Amiidae and Teleostei, remained unresolved (Normark *et al.*, 1991). In order to resolve this uncertainty, Normark *et al.* (1991) have sequenced some fragments of three genes: cytochrome *b*, cytochrome oxidase I (COI) and cytochrome oxidase II (COII) from the mtDNA of the representative fishes. In addition, they (Normark *et al.*, 1991) also collected sequence data for similar regions of the mtDNA from some other vertebrates. These nucleic acid sequence data were converted into amino acid sequences and then analyzed using PAUP 3.0 in order to determine their phylogenetic relationships (Normark *et al.*, 1991).

In the above study, the cytochrome *b* sequence along with the majority-rule consensus was able to resolve the relationships between the Neopterygii clades supporting the morphologically supported phylogeny (Normark *et al.*, 1991). However, there was ambiguity rather than any resolution on the phylogenetic relationships of the organisms in question when cytochrome b, COI and COII sequences were considered together (Normark *et al.*, 1991). They suggested that this ambiguity might be the result of rapid evolution of mtDNA and the rate of which again may vary between the loci (Normark *et al.*, 1991).

E.4. Use of rDNA:

The application of nuclear rDNA in determining phylogeny of fish is recent while some are limited to a few taxa or used to generate phylogeny of other organisms, as cited before (Popodi *et al.*, 1985; Hillis and Dixon, 1989; Le *et al.*, 1989; Phillips *et al.*, 1989; Hedges *et al.*, 1990; Hillis *et al.*, 1991; Joss *et al.*, 1991; Stock *et al.*, 1991; Phillips *et al.*, 1992; Le *et al.*, 1993;). I shall briefly summarize some selected key studies below.

E.4.1. Fish rDNA:

As long ago as 1980, Long and Dawid in a review listed the number of rRNA genes that vary from 120 to 1190 among about 20 fishes, as presented in Table 1 (Long and Dawid, 1980). This variation is very wide in salmoniforms, which may be related to their tetraploid genome accompanied by chromosome reduction, and a high degree of rRNA gene duplication (Schmidtke and Engel, 1975). Although there are reports on the reduction of gene expression in tetraploid fishes (Schmidtke and Engel, 1975), the number of rRNA genes maintain a 1:2 ratio between diploid and tetraploid fishes (Schmidtke and Engel, 1976; Schmidtke *et al.*, 1976). The length of each repeat of the teleost rDNAs varies from 20 to 26 Kb in between species and there are reports of the length heterogeneity within a species due to variations in spacer lengths (Kuprijanova *et al.*, 1982; Popodi *et al.*, 1985; Huang, 1990; Kehagias, 1990; Jahangir and others, 1991-1994).

E.4.2. Fish rDNA Hybridizes with rDNA Probes Across Species:

It has been experimentally demonstrated that the rDNAs from representative fishes belonging to Acipenseridae, Anguillidae, Clupeidae, Cyprinidae, Esocidae, Umbridae, Salmonidae, Percichthyidae, Percidae, Cichlidae, Scombridae, Bothidae and Pleuronectidae, contain conserved segments which hybridize with one or more of the probes containing 18S and 28S rRNAs or rDNA from either *X. laevis*, mouse, chinese hamster or human (Schmidtke *et al.*, 1976; Schmidtke and Engel, 1976; Popodi *et al.*, 1985; Phillips *et al.*, 1989; Huang, 1990; Kehagias, 1990; Jahangir and co-workers, 1991-1994). These rDNA probes were labelled with P^{32} or non radioactive labelling containing biotin or digoxigenin for detection after hybridization. Both the radio and non-radio labelled probes hybridized strongly under high stringency in these studies (Huang, 1990; Kehagias, 1990; Jahangir and co-workers, 1991-1994). All of these support the usefulness of the rDNA probes across these species beyond doubt, for detecting rDNA fragments.

E.4.3. Fish rDNA Contains Conserved Restriction Endonuclease Sites:

It has been demonstrated that there is a conserved *Eco*RI site in each of the 18S and 28S rRNA genes in a loach (*Misgurnus fossilis* L.), salmonids, a sea trout (*Cynoscion nebulosus*), representative acipenseroid, anguilloid, clupeoid, cyprinoid, Esocoid, Umbroid, Salmonoid, Percichthyoid, Percoid, and Cichlid as shown by Kuprijanova *et al.* (1982), Popodi *et al.* (1985), Phillips *et al.* (1989), Tanhauser *et* *al.* (1986), and Jahangir and co-workers (1991-1994), respectively. The above observations (by Kuprijanova *et al.*, 1982; Popodi *et at.*, 1985; Tanhauser *et al.*, 1986; Phillips *et al.*, 1989), are in parity with the number of *Eco*RI recognition sites in placental mammals (*Homo sapiens*, man; *Bos taurus*, cow; *Equus caballus*, horse; *Sus scofra*, pig; *Ovis aries*, sheep; *Rattus rattus*, rat), a marsupial (*Didelphis marsupialis*, opossum), a bird (*Gallus domesticus*, chicken), a frog (*X. laevis*), reptile (*Alligator mississipiensis*), and an elasmobranch (*Carcharhinus* species, requiem shark) rDNAs (Bakken *et al.*, 1982; Wilson *et al.*, 1984; Tanhauser *et al.*, 1986). Popodi *et al.* (1985) and Phillips *et al.* (1989) demonstrated the presence of variable restriction endonuclease sites among several salmoniform clades. Kehagias (1990) also observed two bands in the nuclear DNA of either bluefin tuna (*Thunnus thynnus*) or summer flounder (*Paralichthys dentatus*) digested with *Eco*RI followed by hybridization with probes containing *X. laevis* rDNA. This observation indicates the presence of at least one *Eco*RI site that separated the conserved rDNA domain of both bluefin tuna and summer flounder into two segments, each of which contains a part of the conserved domains of the rDNAs. On the other hand, if more than one *Eco*RI sites were present in the conserved domains, then probably some fragments generated by *Eco*RI remained undetected with the probes used.

However, Huang (1990) observed multiple bands of rDNAs, one with a stronger signal, when nuclear DNAs from either window pane (*Scophthalmus aquosus*) or winter flounder (*Pseudopleuronectes americanus*) were digested with

*Eco*RI and then hybridized with probes containing 18S as well as 28S segments of the rDNA from *X. laevis*. This observation does not agree with the number of *Eco*RI restriction sites already known among a wide range of vertebrates including euteleosts, as indicated before (Bakken *et al.*, 1982; Kuprijanova *et al.*, 1982; Wilson *et al.*, 1984; Popodi *et al.*, 1985; Tanhauser *et al.*, 1986; Phillips *et al.*, 1989; Kehagias, 1990). Further, both Huang (1990) as well as Kehagias (1990) observed multiple bands when nuclear DNAs from window pane, winter flounder, summer flounder, and bluefin tuna were digested with *Hin*dIII and then hybridized with probes containing 18S and 28S segments of the rDNA from *X. laevis*. These observations are also inconsistent with that observed in the salmonids (Popodi *et al.*, 1985; Phillips *et al.*, 1989) and *X. laevis* (Bakken *et al.*, 1982). The above disparities, as observed by both Huang (1990) and Kehagias (1990), may be related to incomplete digestion, spacer length polymorphism, species differences or nonspecific cleavage of DNAs during extraction or a combination of some/all of them.

E.4.4. Use of Fish rDNA for Reconstructing Fish Phylogeny:

Since fish rDNAs are amenable to cross-species hybridization and contain both highly conserved and rapidly evolving domains, restriction endonuclease maps of their rDNAs are also used in phylogenetic analysis of a wide range of fishes. Sequencing the entire length (26 Kb) of rDNA is cumbersome, time consuming, expensive. Further, it has been found that the entire length of the rDNA sequences may not be equally informative for phylogeny, only a limited area (300-500 bases in length) is generally sequenced for phylogenetic analysis (Le *et al.*, 1989; 1993). However, in doing so, there is a potential to lose phylogenetically important information by sequencing a limited length of rDNAs. Restriction endonuclease sites, on the other hand, can provide sufficient character information throughout the entire length of the rDNA to be useful for phylogenetic analysis.

E.4.4.1. Use of Fish rDNA Restriction Endonuclease Maps:

Popodi *et al.* (1985) and Phillips *et al.* (1989) reported the construction of restriction endonuclease maps of the rDNAs from lake trout (*Salvelinus namaycush*), brook trout (*Salvelinus fontinalis*), Arctic charr (*Salvelinus alpinus*), brown trout (*Salmo trutta*), Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykis*). Phillips *et al.* (1989), further, constructed a phylogenetic tree of the above mentioned six salmonids based on the restriction enzyme maps of their rDNAs. It was found that the brook trout, lake trout and Arctic charr are closer to each other when compared to either brown trout, rainbow trout or Atlantic salmon (Phillips *et al.*, 1989). Again, brook trout and lake trout are found to be closer to each other when compared to Arctic charr (Phillips *et al.*, 1989). Similarly, the brown trout and Atlantic salmon are found to be closer to each other when compared with rainbow trout (Phillips *et al.*, 1989). However, this result was not compared with their interrelationships based on morphological characters. Later, Phillips *et al.* (1992) further analyzed the relationships of seventeen salmonid fishes based on rDNA restriction endonuclease recognition characters. However, the study was not able to resolve relationships in several cases and generated a bush containing *Salvelinus, Salmo* and *Oncorhynchus* lineages.

E.4.4.2. *Use of Fish rRNA/rDNA Sequence for Phylogeny:*

18S rRNA:

Recently, however, rRNA and rDNA sequences were also analyzed to reconstruct the phylogeny of fishes (Hillis and Dixon, 1989; Le *et al.*, 1989; Hedges *et al.*, 1990; Joss *et al.*, 1991; Hillis *et al.*, 1991; Stock *et al.*, 1991a; Le *et al.*, 1993). Joss *et al.* (1991) used 505 variable sites of 18S rRNA sequences from representative of seven Gnathostome taxa (Holocephali, Cladista, Chondrostei, Holostei, Dipnoi, Anura and Caudata) in order to determine the interrelationships of their major clades. Their data supported that Holocephali and Cladista are monophyletic (Joss *et al.*, 1991) which is in strong disagreement with Maisey (1986) and Nelson (1989) and others, since Holocephali is a chondrichthyan clade and closer to elasmobranchs than to cladists, which are actinopterygians. Similarly, Dipnoi were neither found to be more related to any groups of fish nor to any tetrapod group and remained equidistant from any one of these groups based on these data. This again disagrees with the common ancestry of Dipnoi and tetrapods based on more than 20 synapomorphic morphological characters

(Lauder and Liem, 1983; Maisey, 1986). In overall, this 18S rRNA sequence data could not resolve the relationships of these gnathostomes and generated many controversial groupings (Joss *et al.*, 1991).

Similarly, Stock *et al.* (1991a) also analyzed 1,800 nucleotide long 18S rRNA sequences of six major Gnathostome clades (Actinista, Elasmobranchs, Holocephali, Dipnoi, Amphibia, Amniota, and Actinopterygii) and a petromyzontiform without resolving the relationships of these clades. The authors further concluded that the rate of evolution of 18S rRNA in osteichthyans is rapid and may not be useful for their phylogenetic study (Stock *et al.*, 1991a, b). In another example, phylogenetic analysis of amniotes based on the entire length of 18S rRNA supported birds to be more close to mammalians than to crocodilians which contradicts bird-crocodilian relationship based on fossil records and 28S rRNA, mtrDNA (Hedges *et al.*, 1990, Hedges, 1994). The above studies do not support the independent use of 18S rRNA sequences to be useful in revealing gnathostome phylogeny vis-à-vis fish phylogeny.

28S rRNA/rDNA:

Some 28S rRNA/rDNA sequences appeared to be more promising in resolving fish phylogeny than 18S rRNA sequences. Le *et al.*, (1989) using about 300 nucleotides (150 nucleotides from each of the two domains, D1 and D2) of 28S rRNA from 9 actinopterygians (Nelson, 1989), two elasmobranchs (Nelson, 1984) and one Hyperoartii (Nelson, 1984; Nelson, 1989) reconstructed a phylogenetic tree. The phylogeny of actinopterygians was found to agree well with the classical phylogeny from morphological characters (Lauder and Liem, 1983; Nelson, 1989). However, they have not included any ostariophysan and salmonid taxa in this study. Further, the branch length uniting the tetrapods and actinopterygian in the "distance tree" is very short while in the "consensus tree", tetrapods, actinopterygians and elasmobranchs formed a cluster (Le *et al.*, 1989). The authors suggested rapid radiation of elasmobranchs, tetrapods and actinopterygians may be related to this lack in resolution (Le *et al.*, 1989). However, this may also be related to the short length of the rRNA sequence used for phylogeny analysis.

Hillis and Dixon (1989) investigated 300 base long rDNA from six vertebrate taxa to determine the vertebrate phylogeny with *Drosophila* as an outgroup. Although these rDNA sequences supported the monophyly of sarcopterygians (Maisey, 1986), this conclusion is based on limited samples: (i) no dipnoan was included in this study, (ii) the actinopterygians are represented by an ostariophysan (*Notropis*) without including primitive actinopterygians, (iii) an exceedingly distant outgroup, *Drosophila*, and (iv) only 300 base variable sites were used for the analysis. Subsequently, Hillis *et al.* (1991) examined a longer section of rDNA, a little over 2,000 base in length, still from a small size sample that includes the coelacanth, a teleost, a petromyzon, and a few tetrapods with *Drosophila* as the outgroup. This study strongly supported a closer relationship between the coelacanth and tetrapods than between either of these clades and the teleost (Hillis *et al.*, 1991). There is controversy on the relationships of coelacanth (Actinista), lungfishes (Dipnoi) and tetrapods (Maisey, 1986). However, Lauder and Liem (1983), Maisey (1986), and Nelson (1989) placed Dipnoi to be the sister group of Tetrapoda, and the Actinista to be a sister group to Choanata from which both Dipnoi and Tetrapods probably originated. Since no dipnoan was included in the study, the limitation of these 2,000 base long rDNA sequence in analyzing the above relationship fully assessed. Although this result is promising, whether this phylogenetic topology will remain unchanged in studies where closer outgroups and increased number of fish taxa are included, is not known.

Very recently, Le *et al.* (1993) also extended the length of nucleotide sequence to 500 of the 28S rRNA as well as increased the number of gnathostome taxa to analyze their phylogeny. However, these data could not resolve the relationships of the chondrichthyans, sarcopterygians and actinopterygians (Le *et al.*, 1993). Further, the chondrichthyans, neopterygians and euteleostean clades were found to be robust and show lack of resolution (Le *et al.*, 1993). In a 38-species tree, the clupeomorphs were united with the ostariophysans, and this finding contradicts the monophyly of euteleosteans defined by Patterson and Rosen (1977) and Lauder and Liem (1983). Further, in a 31-species tree, a scorpaeniform, *Trigla lucerna,* instead of a perciform, *Scomber scombrus,* was found to be closer to another perciform, *Perca fluviatilis* (Le *et al.*, 1993). Although the perciform represents a polyphyletic assemblage, the above relationship appears to contradict common acceptance based on morphology (Lauder and Liem, 1983).

Another problem appears in the union of an elopomorph with an osteoglossomorph that contradicts Forey (1973) suggesting that the relationship between elopomorph and osteoglossomorph is unlikely due to the absence of synapomorphic characters (Le *et al.*, 1993). The above cited anomalies among others were explained by the authors to be related to (1) rapid rate of divergence and (2) polyphyletic assemblage in addition to others. However, the problem may also be related to the limited length of rRNA sequence used in this study.

ITS rDNA:

Similar to perciforms, as indicated before, there was no information on the phylogeny of fishes representing Blennioidei, a suborder of perciforms (Nelson, 1984; Rosenblatt, 1984). Among the various groups of blennioids containing 675 species under 127 genera in six families, Rosenblatt (1984) could not find any phylogenetically informative character. This generated a lot of interest in finding the relationships of this small fishes, circumglobally distributed in tropical to temperate near-shore benthic marine waters (Springer, 1993; Stepien *et al.* 1993). Stepien *et al.* (1993) sequenced the 281 base long rDNA internal transcribed spacer, located between the 18S and 5.8S rDNA, from seven representative blennioids. Analysis of these sequences suggest the monophyly of Chaenopsidae, Labrisomiade and Clinidae of blennioids in congruence of their relationships based on allozyme data from 40 presumptive gene loci (Stepien *et al.*, 1993).

Summary:

In the studies cited above, it appears that 18S rRNA sequences in part or full could not resolve the phylogeny of gnathostomes vis-à-vis fishes (Joss *et al.*, 1991; Stock *et al.*, 1991a). It is possible that in this partial sequence analysis, phylogenetically important information were left out, and in the case of full sequence analysis, phylogenetically non-informative sequences were included. In the case of 28S rDNA or rRNA sequence analysis, Le *et al.* (1989; 1993) selected significantly large number of taxa (12; 31-38); however, only 300 base long sequences in 1989 and 500 base long sequences in 1993 were used. Although, their (Le *et al.*, 1989) earlier phylogenetic tree was promising, the latter tree (Le *et al.*, 1993) has a number of robust nodes. However, it is possible that inclusion of additional 28S rDNA sequences might improve the present resolution. Hillis and Dixon (1989) and Hillis *et al.* (1991) on the other hand using a longer segment of 28S rDNA (2,000 bases) appeared to have reconstructed a fish-tetrapod phylogeny that agrees well with previously accepted phylogeny. However, the number of taxa included in these analysis was small. Further, inclusion of both 18S and 28S rDNA and rRNA sequences in the analysis need to be done in order to examine the ability of these sequenced regions to resolve gnathostome/fish phylogeny.

It has been reported that in DNA sequence alignment, stretches of multiple insertion sequences are often excised before determining phylogeny (Gatsey *et al.* 1993). On the other hand, Gatsey *et al.* (1993) demonstrated that removal of alignment ambiguous sites from mtrDNA decreased taxonomic congruence and resolution of both crocodilian and insect phylogeny. This supports that the inclusion of a larger rDNA segment increases the possibility of phylogeny resolution, while reduced rDNA segment reduces phylogeny resolution.

Since sequencing the entire length of rDNA of several euteleosts is highly time consuming, expensive, and cumbersome, Popodi *et al.* (1985) and Phillips *et al.* (1989) constructed restriction endonuclease maps of rDNA from euteleosts belonging to salmonidae alone. Although, it appears that their results are congruent with morphologically known relationships of these salmonids, addition of more diverse taxa will be necessary to examine the reliability of rDNA restriction enzyme maps in reconstructing phylogeny of fishes.

F. Proposal

As already depicted above, recent application of molecular systematics has not only illuminated *terra incognita,* the phylogenetic relationships between organisms, but also in most cases, has substantiated previous morphological resolutions on phylogeny (Hillis, 1987). Since the phylogenetic relationships among the Euteleostei are yet to be resolved, application of molecular techniques will significantly contribute in resolving their phylogeny.

Since the rDNA is (1) universal in occurrence, (2) similar in function, (3) organized as tandemly repeated units, (4) characterized by rapidly evolving and highly conserved domains, and (5) evolving in a concerted manner, it is an attractive molecule to study the phylogeny of both closely as well as distantly related euteleost fishes. As already detailed above, in addition to rDNA sequencing, the rDNA restriction endonuclease characteristics were found to be highly informative for phylogeny studies among various organisms (Wilson *et al.*, 1984; Hillis and Davis, 1986; Hillis and Davis, 1988; Hillis and Dixon, 1989; Wheeler, 1989). Further, to include character information from a larger segment of rDNA restriction endonuclease characterization of the entire length of rDNA transcription region (TR) may be more informative for euteleost phylogeny, instead of sequencing only a single or limited specific rDNA segment(s). Selected representative euteleost fishes will be characterized with their rDNA restriction endonuclease sites. The homology and diversity of their rDNA will be mapped with several selected restriction endonuclease recognition sites followed by generating a rDNA molecular character matrix. The character matrix will be analyzed using computer assisted parsimony analysis to obtain phylogeny. This will be compared with the morphological phylogeny of these representative taxa and the most parsimonious and analytically acceptable tree will be selected. Hence, it is being proposed to study the phylogeny of euteleost fishes based on the genomic diversity of ribosomal DNA as a function of evolution.

Representative samples from each of the following euteleostean clades, Ostariophysi, Protacanthopterygii and Acanthopterygii will form a cognate group with members of sufficient evolutionary distance to study their relationships using relatively conserved segments of their rDNAs.

Rationale behind the selection of specific outgroup and ingroup taxa, and techniques to be applied will be detailed in materials and methods.

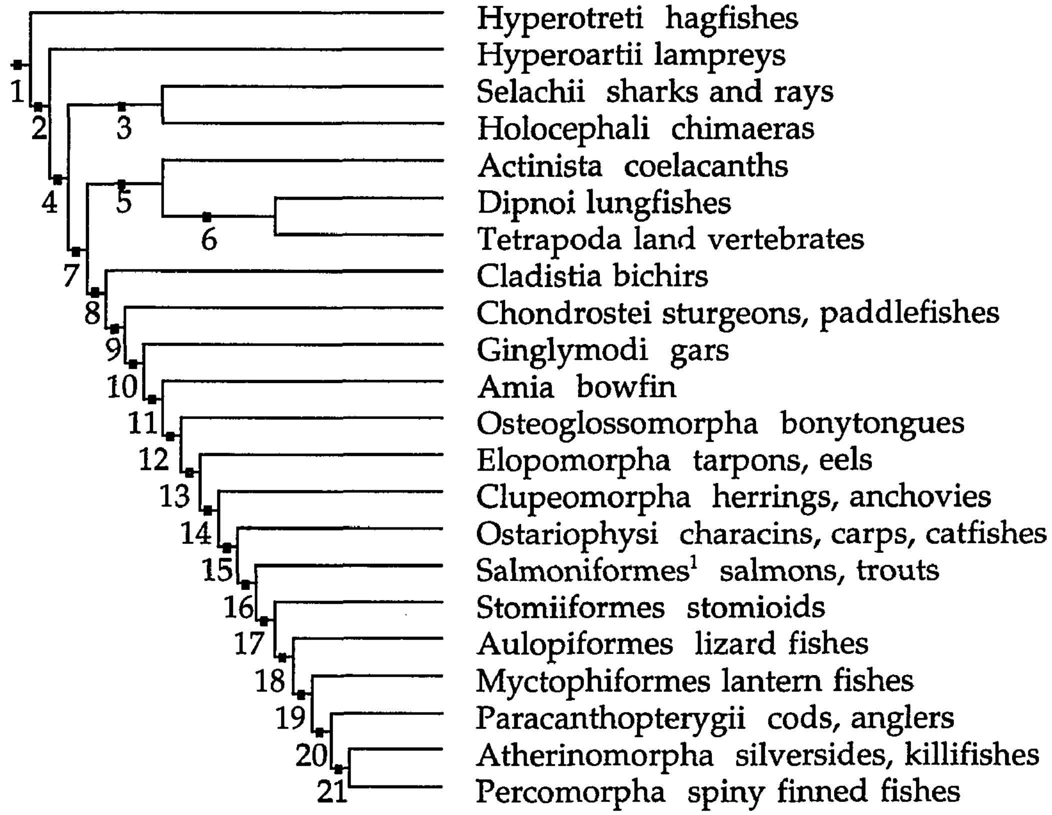

Figure 1. Interrelationships of the major groups of fishes (Nelson, 1989). Inclusive taxa: 1 Craniata, 2 Vertebrata, 3 Chondrichthyes, 4 Gnathostomata, 5 Sarcopterygii, 6 Choanata, 7 Osteichthyes, 8 Actinopterygii, 9 Actinopteri, 10 Neopterygii, 11 Halecostomi, 12 Teleostei, 13 Elopocephala, 14 Clupeocephala, 15 Euteleostei, 16 Neognathi, 17 Neoteleostei, 18 Eurypterygii, 19 Ctenosquamata, 20 Acanthomorpha, 21 Acanthopterygii. [1]Protacanthopterygii will be used in this study to include the all the Salmoniformes including Esocoidei and Salmonidae (Lauder and Liem , 1983) and Osmeroid (Begle, 1991).

Figure 2. A map of a repeating unit of ribosomal DNA of *Xenopus laevis*. ETS, external transcribed spacer; NTS, nontranscribed spacer. Spaces between 18S and 5.8S as well as between 5.8S and 28S rRNA genes are internal transcribed spacers. The length of NTS varies. (Sources: Long and Dawid, 1980 and Hillis and Davis, 1986. This map is not drawn to the scale, only an approximation, to show the relationship of the fragments.)

Table 1. Number of rRNA genes per haploid genome in teleost fishes.

Superorder*	Order*	No. of rRNA genes**
Protacanthopterygii	Salmoniformes	330 - 1190
Ostariophysi	Cypriniformes	120 - 290
Acanthopterygii	Pleuronectiformes	280***

*, Nelson, 1984; **, Long and Dawid, 1980; ***, based on one fish only.

MATERIALS AND METHODS

G. Selection of Representative Fish Species:

The aim of this study is to determine the diversity of rDNA in euteleost fishes belonging to the Superorders Ostariophysi, Protacanthopterygii and Acanthopterygii, as well as to determine their phylogenetic relationships based on the apomorphic characters of their ribosomal RNA genes (Figures 1 and 3, and Table 2). In order to achieve that aim, three out-groups were selected (one distant, Chondrostei, and two close, Elopomorpha and Clupeiomorpha; Table 2 and Figure 3). Thus this study encompasses a wide spectrum of actinopterygian fishes. Justification for the selection of representative samples are provided below:

G.1. The Chondrostei:

A representative chondrostean fish, lake sturgeon (*Acipenser fulvescens*) was selected in this study as a distant out-group of euteleost fishes. Evidence from chondrostean fossil records support their origin from paleoniscoid fishes in the fresh-water basins of northern Asia during Early Triassic period (review, Bristein, 1993). These fishes contain unusual morphological features, i.e., cartilaginous endocranium, presence of notochord in adults and heterocercal tail (reviews, Lauder and Liem, 1984; Bristein, 1993). Since they represent primitive actinopterygian fishes that existed 190-200 million years ago, they have been called "living fossils" (Gardiner, 1984). Hence, inclusion of a representative chondrostean in studying molecular evolution of rDNA may be highly informative, and also it will represent a distant euteleostean outgroup.

G.2. The Teleostei:

G.2.1. Elopomorpha:

The representative anguilliform (*Anguilla rostrata*) selected in this study belongs to a Teleostei group, Elopomorpha. The elopomorph is considered to be a natural monophyletic assemblage, since 1966, based on the leptocephalous larval stage common to all major groups of elopomorph life cycle (Lauder and Liem, 1983; Nelson, 1984). However, they vary tremendously in their body forms as adults, starting from a herringlike body to an eellike body (Nelson, 1984). Many scholars of fish phylogeny do not support this unique larval form to be a valid indicator of their phylogenetic antiquity (Nelson, 1984). Further, based on anguilliform fossils that dates back from Upper Cretaceous Period to Lower Eocene Epoch, Blot (1975) proposed the anguilliforms to be related to amiid fishes and should not be considered to be teleosts. Hence, the molecular relationships of rDNA of a representative anguilliform with other actinopterygians may be important in order to gain more insight into their interrelationships, and it may also function as an outgroup of euteleost fishes.

G.2.2. Clupeomorpha:

Another outgroup selected for this study was American shad (*Alosa sapidissima*), a representative clupeomorph. The fossil records support the first appearance of clupeomorph fishes in Lower Cretaceous Period (Lauder and Liem, 1983). In clupeomorphs, the swim bladder extends anteriorly in two lateral diverticula up to the prootic, where each is connected to the internal ear (Lauder and Liem, 1983; Nelson, 1984). A similar connection between the swim bladder and the internal ear was reported to be present in Megalopidae, the only elopomorph known, (Greenwood, 1970). However, the details of this swim bladder-internal ear connection present in the elopomorph is different when compared with clupeomorph (Nelson, 1984). Hence, this connection between the ear and the swim bladder in clupeomorph is unique and does not occur in any other groups of fish (Lauder and Liem, 1983). In addition, two prominent foramina in the temporal and auditory regions, recessus lateralis and the urostyle are present in clupeomorphs (Greenwood *et al.*, 1966; Forey, 1975). Further, there are several synapomorphic characters of the feeding apparatus known to be present in clupeomorph and euteleost fishes (Nelson, 1969 and 1973). Hence, it was important to select a representative clupeomorph as a close outgroup of euteleost fishes in this study. This will allow reexamination of the relationship of the clupeomorphs with the euteleost fishes based on rDNA evolution.

G.3. The Euteleostei:

As mentioned before, the Euteleostei is an arbitrary composition of a diverse group of fishes containing about twenty thousand species that encompasses more than 94% of the teleost fishes (Lauder and Liem, 1983, Nelson, 1984). However, their monophyly is not yet resolved (Maisey, 1986). In this study, representative fishes from three major groups, Ostariophysi, Protacanthopterygii, and Acanthopterygii (Nelson, 1984) with a major emphasis on the Protacanthopterygii were selected to study their phylogeny based on their rDNA.

G.3.1. Ostariophysi:

There are more than 6,000 ostariophysan species that represent about 72% of the freshwater fishes and 28% of all of the known fishes of the world (Lauder and Liem, 1983; Nelson, 1984). They possess (i) swim bladders separated anteriorly and posteriorly into two chambers, (ii) the anterior swim bladder chamber is attached to two pleural ribs, (iii) a pneumatic duct connects the bladder at the apposition of anterior posterior chambers, and (iv) epidermal club cells eliciting alarm substance (Pfeiffer, 1977, Lauder and Liem, 1983; Nelson, 1984). Again, the ostariophysans are separated into two Series, Anotophysi and Otophysi based on the presence of epibranchial organ in the former and a Weberian apparatus in the latter. The Anotophysi consists of 27 species while the Otophysans contain more than 6,000 species. The otophysans are again separated into three Orders, Cypriniformes, Characiformes and Siluriformes representing 2,422, 1,335, and 2,211 species, respectively (Nelson, 1984). The cypriniforms are known to be monophyletic based on the presence of kinethmoid bone, the dorsomedial palatal process, the structure of premaxilla and pharyngeal jaw complex (Fink and Fink, 1981). However, the internal relationships of the various cypriniforms are currently debated (Howes, 1981; Cavender and Coburn, 1992). The caraciforms are highly diverse and derived forms of otophysi, and their internal relationships are unresolved (Fink and Fink, 1981; Lauder and Liem, 1983; Nelson, 1984). Similarly, the siluriforms represents a highly diverse groups of otophysans which are highly modified with distinctive morphology (Lauder and Liem, 1983, Nelson, 1984). Based on the above facts three species of ostariophysan fish, *Cyprinus carpio, Labeo rohita,* and *Puntius gonionotus,* belonging to two separate lineages within Cyprininae of Cyprinidae, Cypriniformes, were selected for this study. This will help to establish the phylogenetic relationships of the two Cyprininae lineages vis-à-vis their relationships with other euteleosts based on rDNA.

G.3.2. Protacanthopterygii

The protacanthopterygii are composed of 320 species of salmoniform fishes belonging 15 families (Nelson, 1984). Some phylogeneticists consider them to be "primitive euteleostean fishes" representing argentinoids, esocoids, osmeroids and salmonoids (Fink and Weitzman, 1982; Lauder and Liem, 1983). The argentinoids represent 50 genera including argentines and herring smelts, esocoids include pikes and pickerels (*Esox*), mudminnows (*Dalia, Novambra,* and *Umbra*), and "scaled galaxiid" (*Lepidogalaxias*), the osmeroids include smelts and their relatives (Northern Hemispheric Osmeroidea, and Southern Hemispheric Glaxioidea), and the salmonids include salmons, trouts and charrs (*Oncorhynchus, Salmo* and *Salvelinus*), whitefishes (*Coregonus*), and graylings (*Thymallus*) as listed by Fink and Weitzman (1982) and Lauder and Liem (1983). Their monophyly is based on (i) enlarged toothplates associated with the 4th pharyngobranchial without fusion and (ii) fusion of the 3rd toothplate with 3rd pharyngobranchial (Rosen, 1974).

As indicated before in the introduction, the phylogenetic relationships of the protacanthopterygian clades are highly controversial. Fink and Weitzman (1982) summarized the Protacanthopterygii to be an artificial assemblage. The esocoids lacks adipose fin and possess toothplates on the 4th basibranchial unlike other protacanthopterygians, and similar to clupeiforms (Fink and Weitzman, 1982). The relationship of the esocids and umbrids need to be further investigated (Wilson *et al.*, 1992). The interrelationships of the osmeroids with esocoids and salmonids and other euteleosts are debated (Nelson, 1984; 1994; Begle 1991a; 1991b; 1992). Fink and Weitzman (1982) suggested the salmonids to be the sister group of Neoteleostei based on the basioccipital/exoccipital morphology, and the presence of medial cartilage between the ethmoid and premaxillae. Begle (1991a) also suggested the salmonid protacanthopterygians are closer to Neoteleostei than others, based on the presence of occipital condyle.

However, later, Begle (1992) summarized that the salmonids are primitive protacanthopterygians and they are closer to the ostariophysans based on the absence of toothplate on the 4th basibranchial. Further, in that study the osmeroids are again hypothesized to be close to Neoteleostei based on the presence of alveolar process of the premaxilla and reduced laminar bone of hyomandibula (Begle, 1992). In order to get more insight into their relationships, eight representative fishes belonging to four Families were selected for this study. They are Esocidae (*Esox americanus americanus, Esox niger*), Umbridae (*Umbra pygmaea*), Osmeridae (*Osmerus* sp.) and Salmonidae (*Coregonus clupeaformis, Oncorhynchus mykis, Salmo trutta, Salvelinus fontinalis*) (Table 2).

G.4. *The Neoteleostei:*

As already indicated, these euteleosts developed an important synapomorphic character, the retractor dorsalis (RD) (Rosen, 1973 and Winterbottom, 1974, Lauder and Liem, 1983; Rosen, 1985). Although the RD also evolved independently in *Lepisosteus, Amia, Pantodon,* and *Lepidogalaxius,* the neoteleosts are considered to possess RD proper (Lauder and Wainwright, 1992). The RD in neoteleosts originates posteriorly from any of the first to sixteenth vertebrae and inserts anteriorly into the gill-arch elements called, pharyngobranchials (Rosen, 1973; Winterbottom, 1974). Liem (1970 and 1978) showed that the RD plays a key role in manipulation, trituration and swallowing of prey in neoteleost fishes.

Although, (i) the unique functional design and structure of the RD, (ii) a rostral cartilage, (iii) type 4 tooth attachment mode, and (iv) exoccipital and basibranchial bones articulating with the vertebral column support the monophyletic origin of Neopterygii fishes. However, the internal relationships of various neopterygian forms are far from resolution as indicated before (Fink and Weitzman, 1982; Lauder and Liem, 1983). Lauder and Liem (1983), Johnson (1992), and Stiassny and Moore (1992) grouped the neoteleost clades with Stomiiformes as the most primitive, and Acanthopterygii as the most derived form. In this preliminary study, representative forms of highly derived acanthopterygians were selected to determine the restriction endonuclease characters of their rDNA for comparison with other euteleosts and for future use.

G.4.1. *Acanthopterygii*

Of the acanthopterygians, the highly derived forms are represented by Percomorpha, containing 10,345 species belonging to 13 Orders (Lauder and Liem, 1983; Nelson, 1984). Although Percomorpha is known to be a polyphyletic group, recently they have been suggested to be monophyletic based on their pelvic girdle morphology, excluding beryciforms, as discussed before (Stiassny, 1990; Stiassny and Moore 1992). However, this finding is not beyond controversy as shown by Roberts (1993) and Johnson and Patterson (1993), as discussed before in the introduction.

A percomorph ordial group, Perciformes, represents 7,800 species and includes highly diverse percomorphs. Although Rosen and Greenwood (1976) proposed the interarcual cartilage to be a synapomorphy of perciforms, Travers (1981) found it to be present also outside the perciforms. Hence, it will be important to have more character information on the perciforms to reveal their ancestry. As an initial step, three well accepted perciform Families were selected for this study, and one representative from each of the three Families, Cichlidae (*Oreochromis aureus*), Percichthyidae (*Morone saxatilis*), and Percidae (*Perca flavescens*) were sampled. Since, the inter-relationships of these families are poorly understood based on morphology, the present study is expected to bring-in more information and through some light in their interrelationships as well as to other euteleosts.

G.5. Summary:

As discussed above, seventeen representative actinopterygian fishes were selected for this study (Table 2), based on their known phylogenetic affiliations. This will help to find the phylogeny of the chondrostean, elopomorphan, clupeomorphan, ostariophysans (cyprinids), protacanthopterygians (esocids, umbrid, osmerid, and salmonids), and acanthopterygians (percichthyid, percid, and cichlid), based on rDNA. This will also allow for a comparison of the rDNA molecular phylogeny with the morphology based phylogeny of these representative protacanthopterygians, which might act as a beckon for future studies.

H. Fish Collection for DNA Samples:

All fish samples used in this study were collected alive from various locations in the States of New York, New Jersey, and Michigan, Montreal (Canada), and Mymensingh (Bangladesh). A list of these fishes with brief information on their location, time, and status at collections, and sex are provided in Table 3, while additional details are provided below. A list of persons, with their affiliations, identified the fishes are shown in Table 4. All individuals are professionally familiar with the species concerned, and could identify most of them with external morphology alone. All representative fishes were identified alive or fresh, while the rainbow smelt (*Osmerus mordax*), after sampling blood, was preserved in formaldehyde, and identified later. In the case of both rainbow smelt and white tilapia (*Oreochromis aureus*), morphological as well as meristic characters were examined for identification.

Lake sturgeon (*Acipenser fulvescens*) was caught in May 10, 1993, from the north side of Hydro-Quebec Dam at St. Lawrence River, Montreal, Canada, a site known for yearly spawning migration of lake sturgeon. Hence, it may represent a wild population of lake sturgeon. In the case of the American eel (*Anguilla rostrata*), they were purchased in June, 1992, from the Sea Dragon Fish Store, Church Avenue, Brooklyn, NY. However, they also represent a wild population, since, they were collected as migrating elvers from near-seashore waters off the Atlantic Coast, USA, and then grown to marketable size eels in aquaculture. The American shad (*Alosa sapidissima*) and a sample of striped bass (*Morone saxatilis*) were collected by gill netting from the Hudson River, from the south side of the Tappanzee Bridge in May, 1992. Another sample of striped bass (*Morone saxatilis*) was collected by Normandale Associates from the Hudson River at the west side of Manhattan, at about 25 miles south from the Tappanzee Bridge. Thus, they also represent wild populations.

Similarly, the rainbow smelt (*Osmerus mordax*) caught from Lake Champlain, NY, in April 1992, mudminnows (*Umbra pygmaea*) caught from Clarks Mill Stream, near the junction of Mannh'eim, Atlantic County, NJ, in June 1993, redfin pickerel (*Esox americanus americanus*) and one sample of chain pickerel (*Esox niger*), caught from Clark Brunch, Mullica River, Camden County, NJ, in June 1993, represent wild populations. Another sample of chain pickerel (*Esox niger*) was also collected from Whaley Lake P353, Dutches County, NY, in January, 1992. This represents a small size wild population since the lake is small in size (256 acres) and has limited exchange with other water bodies. One sample of yellow perch (*Perca flavescens*) was also collected from Whaley Lake P353, Dutches County, NY, in April 1992, and another from Lake Champlain, NY, in April 1992. Although the former represents a small size wild population, the latter represents a larger size wild population. The lake whitefish (*Coregonus clupeaformis*) was collected by Dr. Barbara A. Shields from spawning grounds in Leech Lake, North-Central Minnesota, by gill netting and, hence, represents a wild population.

Hence, it can be seen that the samples of lake sturgeon, American eel, American shad, rainbow smelt, mudminnows, redfin pickerel, chain pickerel, lake whitefish, striped bass, and yellow perch used in this study represent wild populations.

On the other hand, due to limited access to wild populations, common carp (*Cyprinus carpio*) was purchased alive from the Shapla Grocery, McDonald Avenue, Brooklyn, NY, supplied by fish farmers grown in their aquaculture facilities. The rohu (*Labeo rohita*) and Thailandian sarpunti (*Puntius gonionotus*) were netted out from the aquaculture ponds of Bangladesh Fisheries Research Institute located at Bangladesh Agricultural University Campus, Mymensingh, in January, 1990. Hence, the carp, rohu and sarpunti represent aquacultural stocks. Similarly, rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*), and brook trout (*Salvelinus fontinalis*) were obtained from the Cold Spring Harbor Fish Hatchery, Cold Spring Harbor, NY. This rainbow trout represents a slow maturing strain which was originally obtained by the Cold Spring Harbor Fish Hatchery from the Rainbow Spring Trout Hatchery, Thamesford, Ontario, Canada. On the other hand, the brown trout and brook trout were obtained originally by the Cold Spring Harbor Fish Hatchery from Connetquat State Park Fish Hatchery, Southshore, Long Island, NY. Although these trouts represent strictly in-bred stocks, there were possibilities of crosses between strains of respective trouts collected separately by the Cold Spring Harbor Fish Hatchery.

The white tilapia (*Oreochromis aureus*), used in this study was purchased in January, 1992, from the Shapla Grocery, McDonald Avenue, Brooklyn, NY. This was also grown by fish farmers in aquaculture ponds, and hence, represents a stock from aquaculture. In summary, the samples for common carp, rohu, sarpunti, rainbow trout, brown trout, brook trout, and white tilapia represent specially maintained stocks under conditions of aquaculture, and as a result, may differ from their wild populations.

Although sexual dimorphism in rDNA has not been reported from any vertebrate studied, all attempts were made to record the sexes of the sample fishes (Table 3). In some cases, where the sex could not be reliably determined, the fishes were immature, and in some other cases, the fishes were required to be released back after sampling blood, and there was no significant external sexual dimorphism. As a result, the sex of all fish samples could not be recorded.

I. Isolation of Nuclear DNA from the Experimental Fishes:

I.1. Tissue Collection:

Blood tissue samples were collected from each fish by caudal vein puncture using 20 G hypodermic needles attached to 10 ml fresh sterile Plastipak syringes containing 2 ml filter sterilized fresh 1 X SSC (0.15 M NaCl; 0.015 M Na-Citrate; pH 7.0) as shown in Figure 4. In the case of a small size fish, a 5 ml syringe containing 1 ml 1 X SSC was used for drawing blood. During the process of blood collection from a fish, the blood was mixed, intermittently, with the SSC inside the syringe to stop clotting and then transferred to a fresh sterile 30 ml Corning plastic flasket containing 5 ml 1 X SSC. The blood inside the flasket was also mixed gently and kept cold in ice-water in a bucket. In addition to blood, 5 ml milt was collected from lake sturgeon, diluted to 5 X volume with 1 X SSC in a fresh sterile 30 ml Corning plastic flasket, and stored in ice-water in a bucket, as above.

I.2. Preparation of Nuclear DNA:

Nuclear DNAs were extracted from the blood and sperm cells and purified by isopycnic CsCl gradients following the procedures of Eckhardt and Gall (1971) with modifications as detailed below.

Blood cells were pelleted by centrifugation at 750 g, resuspended in 1 X SSC and repelleted by centrifugation as before. The supernatant was poured off and the pelleted cells were homogenized (cell lysis) briefly in a homogenizing buffer containing 0.05 M tris-HCl, 0.025 M KCl, 0.005 M Mg-acetate, 0.35 M sucrose and 0.5% Triton X-100 at 4^0 C and pH 7.6. Lysis of the cells was confirmed by periodic microscopic observations of the solution with wet mount preparations. The same procedure was also followed for the sperm cells.

On the completion of cell lysis, the homogenate was centrifuged at 5×10^3 g for 10 minutes at 4^0 C to pellet the nuclei. The nuclear pellet was saved and the supernatant was discarded to eliminate the mitochondria and most of the RNA. The nuclear pellets were resuspended in a solubilizing buffer containing 0.5% w/v sarkosyl (NL-97, Geigy), 0.1 M EDTA, 0.05 M tris-Hcl, and 50 µg/ml Proteinase K at pH 8.4. As soon as the solution became viscous and clear, equal volume of water saturated redistilled cold phenol was added and gently mixed for 5 minutes. The emulsion was centrifuged at 12 X 10^3 g and 4^0 C for 10 minutes to separate the aqueous layer containing DNA from the rest. The aqueous phase was gently collected using a siliconized Pasteur pipet in a Corex tube to which equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1) mixture were added. It was mixed gently well and centrifuged again at 12 X 10^3 g and 4^0 C for 10 minutes. The aqueous layer containing the DNA was isolated, and the DNA was precipitated by adding 2 X volumes of cold absolute ethanol and kept at -20^0 C.

The precipitated DNA was pelleted by centrifugation at 12 X 10^3 g and 4^0 C for 10 minutes in Sorval SS-34 rotor. The supernatant was poured off, 5 ml cold 70% ethanol was added to bring the salts into solution, keeping the DNA as pellets. It was recentrifuged at 12 X 10^3 g and 4^0 C for 10 minutes, the supernatant was poured off, and dried under vacuum for 10 minutes. The DNA was brought into solution in 1 ml sterile TE, pH 8.0. A 5 µl DNA sample was diluted to 100 µl and the absorptions at λ 260 and 280 nm were read using a Gilford spectrophotometer Model 240. The ratio of UV absorption at λ 260 and 280 was calculated. This ratio above 1.8 indicates purity of nucleic acid, a value below 1.8 indicates the presence of protein impurities in the DNA sample.

If the ratio was found to be lower than 1.6 the DNA sample was further purified following isopycnic centrifugation in CsCl gradients. If the ratio was found to be above 1.6 and below 1.9, the DNA sample was redigested with Proteinase K (Sigma) followed by DNA extraction with phenol : chloroform :

isoamylalcohol as before.

I.3. Isopycnic Centrifugation of Nuclear DNA in CsCl:

Nuclear DNA was pelleted from the ethanol precipitate by high-speed centrifugation at 12 X 10^3 g in a Sorval SS-34 rotor for 10 minutes. Pelleted DNA was aspirated and air blown to dryness. The DNA was resuspended in 0.1 X SSC and mixed with an appropriate amount of saturated CsCl solution to a final ρ 1.79/cm^3 in a polyallomer tube, Beckman. The sample was ultracentrifuged using a Ti 50 rotor at 42 X 10^3 rpm and 20^0 C for 24 hours. After the run, the entire sample was collected in multiple ten-drop fractions from the bottom of the tube. The optical density of each fraction was read at λ 260 nm in a Gilford spectrophotometer Model 240 as before. The absorption profile of the fractions was plotted, and following the plot, all fractions representing the beginning to the end of the 2nd peak absorption were pooled together. Any shoulder that forms on either side of the second peak was considered to be the part of the peak.

From the pooled sample, the DNA was precipitated out by adding 3 X volume 70% ethanol, and subsequently storing at 4^0 C for 15 minutes. The precipitated DNA was pelleted at 12 X 10^3 g in SS-34 rotor, brought back into solution in 0.1 X SSC followed by reprecipitation with the addition of 2 X volume 100% ethanol and stored at 4^0 C for 15 minutes. The DNA was pelleted again, dried under vacuum and resolubilized in TE (10 mM Tris.HCl; 1 mM EDTA; pH 8.0). A small sample of this DNA was diluted, and read for absorptions at λ 230 -

300 nm through a cuvette of 1 cm internal path. Those values were plotted on an arithmetic graph for observing the UV absorption profile of the DNA sample, and the ratio of absorptions at λ 260 nm/280 nm was used as an indication of purity of the DNA: a value of this ratio below 1.8 indicates continued presence of protein impurities in the DNA sample, while a higher value indicates purity. However, the above procedure always resulted in a ratio of 1.9 or above for the DNA used in this study. The DNA concentration in µg/ml present in the stock solution was calculated by absorption at λ 260 nm X 50 X dilution factor. A constant 50 is used in this calculation, since, 50 µg/ml double stranded DNA has absorbance of 1 at λ 260 nm in a cuvette with one cm internal path (Berger, 1987).

In the case of whitefish (*Coregonus clupeaformis*), a sample of total DNA was kindly donated by Dr. Barbara A. Shields, Fish Division, Museum of Zoology, University of Michigan, Ann Arbor, Michigan, for this study. The sample was received as lyophilized DNA in three separate microcentrifuge tubes in a special container by air mail. From one of these microcentrifuge tubes, a sample DNA was brought into solution in TE pH 8.0 in cold and used in this study.

J. High Molecular Weight Fish DNA Digestion with Restriction Endonuclease, and Electrophoretic Separation:

J.1. Selection of Restriction Endonucleases for this Study:

Samples of 2 µg of DNA from several fishes were digested with eight different restriction endonucleases, *Bam*H I, *Eco*R I, *Hin*d III, *Kpn* I, *Pst* I, *Pvu* II, *Xba* I, and *Xho* I, supplied by Bioexcellence, Pharmacia, Promega, and Boehringer

Mannheim. These endonucleases recognize six base-pair digestion sites in a DNA molecule, and thus, cleave DNA infrequently. The set-up for DNA digestions was strictly followed as suggested by the supplier for specific endonuclease. Only five of these endonucleases, *Bam*H I, *Eco*R I, *Hin*d III, *Pst* I, and *Pvu* II, at a concentration of 1 unit/10 μl reaction volume, were found to digest 2 μg of nuclear fish DNA, significantly. Hence, these five restriction endonuleases were selected for this study.

J.2. Restriction Endonuclease Digestion Set-up:

Single and multiple restriction endonuclease digestions were set-up following the work of Ausubel *et al.* (1988) with modification as follows:

J.2.1. Digestion of DNA Samples with Single Restriction Endonuclease:

A two μg DNA sample from each representative fish was digested with one endonuclease in the specific buffer in a reaction volume of ≥50 μl overnight at 37^0 C to completion. One restriction endonuclease at a concentration of 1 unit/10 μl digestion mixture was added to each sample (Table 5). The reaction was stopped by adding 0.5 M EDTA, pH 7.5, to a final concentration of 10 mM EDTA. Hence, five such samples of DNA from every fish were digested by five different endonucleases, *Bam*H I, *Eco*R I, *Hin*d III, *Pst* I, and *Pvu* II.

J.2.2. Digestion of a DNA Samples with two Restriction Endonucleases:

A two μg DNA sample from each representative fish was initially digested to completion using a single endonuclease of choice, as above. The sample was heated 10 minutes at 60^0 C to inactivate the endonuclease added originally, chilled for 15 minutes in ice-water, and a specified buffer/deionized water was added and mixed well (Table 5). A sample of 1 unit/10 µl, of another endonuclease of choice was added (Table 5), digested overnight at 37^0 C to completion, and the reaction was stopped by adding 0.5 M EDTA, pH 7.5, as above. Hence, ten such samples of DNA from every fish were digested with ten different combinations of two endonucleases (*Bam*H I + *Eco*R I, *Bam*H I + *Hin*d III, *Bam*H I + *Pst* I, *Bam*H I + *Pvu* II, *Eco*R I + *Hin*d III, *Eco*R I + *Pst* I, *Eco*R I + *Pvu* II, *Hin*d III + *Pst* I, *Hin*d III + *Pvu* II, and *Pst* I + *Pvu* II; Table 5).

I.2.3. Digestion of a DNA Sample with Three Restriction Endonucleases:

In a few cases, samples of two µg fish DNA were digested with two combinations of three selected endonucleases (*Bam*H I + *Pst* I + *Pvu* II, and *Eco*R I + *Pst* I + *Pvu* II), if needed, for confirmation of digestion sites. A sample of a fish DNA digested with a single endonuclease was heated 10 minutes at 60^0 C to inactivate the endonuclease added originally, chilled 15 minutes in ice-water, and specified buffer/deionized water was added, and mixed well (Table 5). Additional samples of 1 unit/10 µl of each of the two endonuclease of choice were added (Table 5), digested overnight at 37^0 C to completion and stopped, by adding 0.5 M EDTA, pH 7.5, as above.

I.3. Agarose Gel Electrophoresis:

I.3.1 Processing Digested Fish DNA:

Each digested DNA sample was precipitated by adding 0.1 X volume 3 M Na-acetate followed by 2 X volume cold 100% ethanol and refrigerated 30 minutes at 4^0 C. The DNA was pelleted at 12 X 10^3 g for 10 minutes at 4^0 C. The supernatant was poured off and the pellet was washed by adding 100 µl cold 70% ethanol, and refrigerated for 20 minutes to bring most of the salts into solution. The DNA was repelleted as before at 12 X 10^3 g for 10 minutes at 4^0 C. The supernatant was poured off and the pelleted DNA was dried under vacuum for 10 minutes. The dried DNA was brought into solution by adding 27 µl TE pH 8.0, and kept cold in an ice-water bucket.

I.3.2. Staining Digested Fish DNA:

A volume of 3 µl 10 X loading buffer, containing 20% Ficoll 400, 0.1 M Na_2EDTA, pH 8.0, 1% sodium dodicyl sulphate, 0.25% Bromophenol Blue, and 5 µg/ml ethidium bromide was added to a digested DNA sample. The Ficoll 400 in the loading buffer increases the viscosity of the DNA sample, and thus minimizes the leakage of DNA during loading of the sample into the gel wells. Ethidium bromide is a DNA staining fluorescent dye which has a purple-red color at high concentration. However, the amount of stain used with the loading buffer, as above, in staining DNA do not show any color under visible light. In the DNA staining mechanism, one molecule of ethidium bromide binds to approximately every two base pairs of DNA by intercalating between the bases (Sambrook *et al.*, 1989).

Although, both the DNA and the ethidium bromide molecules absorb UV lights, the DNA transmits the absorbed light to the intercalated ethidium bromide molecule, and ultimately the ethidium bromide emits an orange-red light at λ 590 nm (Sambrook *et al.*, 1989). As a result, the fluorescence yield of ethidium bromide-DNA complex is much greater than the dye alone, and hence, a band containing as little as 1 ng DNA in a 6 mm lane can be detected with this stain (Sharp *et al.*, 1973). Another dye, Bromophenol Blue, used in the loading buffer is a tracking dye, which comigrates with DNA molecules of 0.5 Kb in length towards the positive pole in an electric field. This dye can be observed under visible light, and thus, provides an index of the mobility of the fastest DNA fragments in the gel (Ausubel *et al.*, 1988; Sambrook *et al.*, 1989).

J.3.3. Marker DNA:

Three types of marker DNAs (High Molecular Weight DNA Markers, 5618SA, BRL; λ DNA/*Hin*d III Fragments, 5612SA, BRL; 1 Kb DNA Ladder, 5615SA, BRL), each belonging to a separate molecular size gradients of known molecular sizes were used in this study. High Molecular Weight DNA Markers and λ DNA/*Hin*d III Fragments were used to determine the high molecular, and the 1 Kb DNA Ladder was used to determine low molecular size fish DNA bands after hybridization. Hence, in every gel, one high molecular size and another low molecular size DNA markers were included in addition to fish DNA during electrophoresis. A 2 µg DNA sample from each marker DNA of choice was pipetted out into a microcentrifuge tube, followed by TE, pH 8.0, as needed to make a volume of 27 µl, and 3 µl loading buffer.

I.3.4. Preparation of Agarose Gel:

A 1.2% agarose gel was freshly prepared for each electrophoresis. A sample of 240 mg Molecular Biology Certified Ultra Pure DNA Grade Agarose, Bio-Rad, was weighed out and added to a 80 ml glass beaker containing 17 ml deionized water, mixed well, and heated for 1 minute in a microwave oven. It was taken out and 4 ml 5 X TBE (0.225 M Tris-Borate; 0.005 M EDTA) was added, mixed well and then poured on a previously closed 10.2 cm X 6.4 cm UV transparent Lucite gel tray. The two short sides of the tray were previously closed with Scotch tape. An aluminum comb (5.6 cm X 0.3 cm) containing eight teeth (1.0 X 0.6 X 0.3 cm) was kept hanging into the gel from a bar resting on the walls of the long side of the tray. The position of the comb was 1 cm inside along the long side of the tray, and 1 mm above the bottom. The gel was solidified for 30 minutes at room temperature, the comb was removed, and the wells in the gel were examined under a binocular dissecting microscope for breakage. Each gel well was determined to have space to receive a 50 µl solution. Later, the Scotch tape from the two short sides of the tray were removed.

I.3.5. Set-up Electrophoresis:

The gel with the tray was placed in a electrophoresis trough and 1 X TBE buffer (0.045 M Tris-Borate; 0.001 M EDTA) was added, enough to cover the gel with a layer of 1 mm deep TBE. The digested fish DNA samples, their undigested control, and the marker DNAs, all treated with loading buffer, were individually loaded into separate wells. The cathode and the anode electrodes were connected close to and away from the well side of the gel, respectively, on the trough. The power supply unit was turned on and the voltage was adjusted to 40 volts, and it was run for about 3 hours or until the Bromophenol Blue moved close to the anode side of the gel. The power was turned off, the tray containing the gel from the trough was removed and placed on a transilluminator with a UV transparent ruler, containing scales marked in black on the side. The UV light at λ 254 nm was turned on, the mobility of the DNA fragments in the gel was observed, and photographed using DS-34 Polaroid camera with Plus-X Pan film.

K. Transfer of Fish DNA from Agarose Gels to Nylon Membranes by Southern Blotting:

K.1. Processing of the Gel:

Lanes of the marker DNAs were trimmed off from the agarose gel slab, and the fish DNAs were transferred from the gel on to a PhotoGene nylon membrane, Gibco-BRL, following the Southern blotting techniques (Ausubel *et al.*, 1988; Protocol I). The gel was treated with mild acid (0.25 M HCl) for 20 minutes for partial depurination followed by 2 X 30 minutes alkaline denaturation using

0.5 M NaOH. During the latter step, the depurinated DNA sites are cleaved making smaller DNA fragments which facilitate efficient transfer of DNA during Southern blotting. The gel was neutralized until the pH was found to be ≈7.8, measured by ColorpHast Indicator Strips, E. Merck, Germany, pH paper.

K.2. Preparation of Nylon Membrane, Support and Absorbent Papers:

A piece of PhotoGene Nylon Membrane, 3 mm smaller in both length and width of the gel size, was cut. In addition, 3 pieces of Whatman 3MM papers were cut, each of which is 7 mm smaller in both length and width of the gel. A wick paper was cut about 2 cm wider than the gel in both length and width. Twenty cm long two 10.2 X 6.4 cm Whatman Filter paper No. 1 was cut as wrapping paper for a 10.2 X 6.4 X 3 cm lucite box. Finally, paper towels were cut 7 mm smaller than the size of the gel in both length and width to make a 10 -15 cm thick stack.

K.3. Southern Blotting Set-up:

The 10.2 X 6.4 X 3 cm lucite box was wrapped with several folds of previously cut Whatman filter paper No. 1, and then put centrally as an island in a 1 L Pyrex glass baking dish. About 500 ml of 10 X SSC was added to the dish so that the SSC reaches about a half of the height of the box. The wrapping paper was made thoroughly wet by adding 10 X SSC on the wrapper. Any air bubbles trapped were removed by rolling a glass rod over the paper. The wick paper was put on top of the Whatman No. 1 paper over the box so that both of its ends hang into the 10 X SSC solution in the dish. The gel was inverted and laid on top of the wick paper, thus keeping the bottom side of the gel exposed. Any air bubble trapped by the gel was removed. The nylon membrane was hydrated by soaking in 10 X SSC, and then laid on top of the gel making sure that it does not overhang the gel, and also not to trap any air bubble.

Previously cut Whatman 3MM papers were put on top of the nylon membrane such that it did not overhang and also did not trap any air bubble. It was also made sufficiently wet by adding 10 X SSC. Finally, the 10-15 cm pile of previously cut paper towel was stacked on top of the 3MM paper. Care was taken not to create a short circuit by allowing any contact of the paper towel directly with the gel. The entire set-up including the dish was covered by Saran Wrap to minimize evaporation. A piece of glass (30 X 15 cm) was put above the Saran Wrap on top of the paper towel layer and a 0.4 kg weight was put on the glass. The glass was supported at the other end from outside of the tray to ensure stability. This allowed blotting 10 X SSC from the dish to the paper towel as follows, which was maintained overnight:

Dish ⇒ Wick paper ⇒ Gel ⇒ Nylon ═╗
Paper towel ⇐ 3MM paper⇐ ═╝

As a result, the DNA from the gel became transferred to the nylon membrane and stayed since it could not pass any further. Also, the DNA molecules being negatively charged, became electrically bound to the nylon membrane which contains positive charges.

At the end of blotting, the paper towels and the 3MM papers were removed. Before the nylon membrane was taken out, the position of the origin of each lane was marked on the nylon membrane with a No. 2 pencil. Then the nylon membrane was taken out, washed with 5 X SSC to get rid of any broken gel fragments, air dried under tungsten light, baked at 80^0 C for 2 hours, and stored under vacuum for Southern hybridization. In order to determine the amount of DNA remaining in the gel after the transfer, the gel was stained 15 minutes with 0.5 µg/ml ethidium bromide, transferred to a transilluminator and examined using UV light, and the percent DNA transfer was visually estimated and recorded.

L. Probe Preparation and Labelling:

L.1. Choice of rDNA Probes:

Based on the published literature, selected probes, pXlr101A and pXl212 containing *Xenopus laevis* rDNA were obtained from well known laboratories in the US and Switzerland. The pXlr101A contains a full length repeat unit of *X. laevis* rDNA inserted at the *Hin*d III restriction site of pBR322 (Bakken *et al.*, 1982). The pXl212 contains a part of the 18S, 5.8S and most of the 28S with the transcribed internal spacers of *X. laevis* rDNA in pCR1 at the *Eco*R I restriction site (Boseley, *et al.*, 1978). The pXlr101A was obtained as a DNA precipitate in ethanol as well as in transformed *Escherichia coli* HB101 cells from Dr. Brian McStay, Basic Sciences Division, Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, WA 98104). The pXl212 was only obtained as precipitated DNA in ethanol, and a sample of *Escherichia coli* DH52α cells (kindly provided by Professor Collier, B.C.) were transformed with pXl212. The transformed cells were grown in large volumes and the plasmid containing the rDNA insert was isolated following CsCl-ethidium bromide equilibrium centrifugation method and stored (Ausubel *et al.*, 1988).

Based on initial hybridization, according to procedures described later, the pXLr101A was found to hybridize with more fish rDNA fragment bands. This was expected, since the pXlr101A contains a full length rDNA repeat, and hence, the pXlr101A was selected to be used as the rDNA probe in this study.

L.2. Confirmation and Production of pXlr101A:

Transformed *Escherichia coli* HB101 cells containing pXlr101A, obtained from McStay (1991) were grown on LB agar plates containing 50 µg/ml ampicillin. Samples of cells from five separate colonies were grown in individual culture tubes containing 5 ml LB media with 50 µg/ml ampicillin. The plasmid DNA was extracted from each culture tube separately using the Magic Minipreps DNA Purification Systems (Promega A7100) with modifications as follows.

Cells were pelleted by centrifugation using swinging bucket rotor Sorval HB-4 at 3 X 10^3 rpm at 4^0 C for 5 minutes. Cells were resuspended in a wash solution containing 50 mM Tris-HCl, pH 7.5 and 10 mM EDTA and pelleted as above. This was repeated until the supernatant became clear. The cell pellet was well resuspended gently in 200 µl Resuspension Solution containing 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, and 100 µg/ml RNase A, and transferred to a microcentrifuge tube. The cells were lysed by adding 200 µl Cell Lysis Solution containing 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA. As soon as the solution becomes clear, 200 µl Neutralization Solution containing 2.55 M potassium acetate, pH 4.8, was added and mixed well.

The resultant solution was centrifuged at 14 X 10^3 g for 5 minutes and the supernatant was collected into a fresh microcentrifuge tube, 1 ml Magic Minipreps DNA Purification Resin, Promega, was added and mixed well. This slurry was passed through a Magic Minipreps mini-column attached to the Luer-lok of a 5 ml Plastipak B-D disposable syringe barrel using a vacuum manifold. The column was washed under vacuum by adding 2 ml Column Wash Solution containing 100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 2.5 mM EDTA and 50% ethanol. The column was dried under vacuum for 2 minutes, removed and transferred to a fresh microcentrifuge tube. The plasmid DNA was eluted by adding 60 µl pre-heated sterilized distilled water to the column followed by centrifugation at 14 X 10^3 g for 20 seconds.

The plasmid pXlr101A DNA, thus obtained from each column, was digested with *Bam*H I, *Eco*R I, *Hin*d III, *Sal* I, separated by agarose gel electrophoresis, mapped and compared with the original map (Figure 5) supplied by McStay (1991). The colony, whose DNA map exactly matched the original map was selected, grown in sterile LB media containing 50 µg/ml ampicillin, and then stored in microcentrifuge tubes in small aliquots of sterile LB media containing 50 µg/ml ampicillin and 15% glycerol at -80^0 C for future use.

The same procedure was followed using Magic Minipreps DNA Purification Systems to prepare fresh pXlr101A DNA from the selected colony, as needed, for the preparation of labelled probe.

L.3. Labelling pXlr101A rDNA Probe with Non-radioactive Marker, Digoxigenin:

Traditionally in most of the DNA hybridization techniques, the probe DNA is labelled with radioactive nucleotides containing ^{32}P. The instability and quick decay diminishes the usefulness of a ^{32}P labelled probe with time and becomes unusable after 14 days. Further, radioactivity of the probe has the potential to cause nucleotide mutations, and thus, affect hybridization. In addition, ^{32}P labelled nucleotides are expensive, hazardous to health, and their disposal has special requirements.

To overcome these problems, in this study, known amount of pXlr101A DNA was labelled with digoxigenin by random priming following the Genius System of Boehringer Mannheim (Protocol II) to be used as an rDNA probe. The digoxigenin is a plant steroid, found in *Digitalis purpurea* and *Digitalis lanata*. The DNA probes were synthesized using deoxyuridine 5′-triphosphate as one of the nucleotides to which digoxigenin is covalently bound. The digoxigenin is currently in wide use to label DNA probes since these probes can be immunologically detected after hybridization, which increases the specificity of detection (Zhang *et al.*, 1990).

The pXlr101A DNA template molecules were linearized by digesting with restriction endonuclease, *Sal* I, and then denatured by boiling into single stranded DNA molecules. A known amount of hexanucleotide primers and deoxynucleotide 5′-triphosphates containing digoxigenin labelled deoxyuridine 5′-triphosphate (DIG-dUTP) was added followed by Klenow polymerase I for DNA labelling. The reaction mixture contained 100 µg/ml DNA template, 0.21 µg/µl hexanucleotide, 0.1 mM dATP, 0.1 mM dCTP, 0.1 mmol/dGTP, 0.065 mM dTTP, and 0.035 mM dUTP, 50 mM Tris-HCl, 10 mM $MgCl_2$, 0.1 mM dithioerythritol, 1 mg/ml BSA and 1 U Klenow/10 µl at a pH of approximately 7.0.

The solution was incubated overnight at 37^0 C for 20 hours in an oven for the synthesis of labelled pXlr101A probe. The synthesis was stopped by adding 0.5 M EDTA, pH 8.0, to a final concentration of 20 mM EDTA. The probe DNA was precipitated by adding 0.125 µl 4.0 M LiCl, and 1 µg tRNA/µl reaction volume, followed by 3 X volume cold absolute ethanol, and stored for 15 minutes at -80^0 C. The DNA was pelleted by centrifugation for 10 minutes at 14 X 10^3 g, and 4^0 C. Cold 70% ethanol was added to the DNA pellet to bring all the salts into solution while keeping the DNA as precipitates. It was recentrifuged for 10 minutes at 14 X 10^3 g and 4^0 C. The supernatant was poured off and the pellet was dried under vacuum. The dried labelled probe DNA was brought into solution by adding 60.0 µl 0.1% SDS, and stored in a refrigerator. The concentration of digoxigenin labelled pXlr101A probe was quantitated in comparison to standard labelled DNA using colorimetric methods, as detailed below.

L.4. Quantitation of Digoxigenin Labelled pXlr101A rDNA Probe:

L.4.1 Preparation of Dot Blots on Nylon Membranes:

A digoxigenin labelled standard pBR328 DNA, Boehringer Mannheim, was diluted to 1 ng/µl, 100 pg/µl, 10 pg/µl, 1 pg/µl, and 0.1 pg/µl, shown as A, B, C, D, and E, respectively, in Table 6. Similarly, the digoxigenin labelled pXlr101A DNA was serially diluted to 10, 100, 1,000, 10,000, and 100,000 times as shown as V, W, X, Y, and Z, respectively, in Table 7.

A fresh sample of 10 cm^2 PhotoGene Nylon Membrane was cut and marked with a No. 2 pencil into five columns and two rows, thus making 10 squares, each of 1 cm^2 in size (Figure 6a). A circle of about 2 mm diameter was drawn at the center of each square using a No. 2 pencil. The squares on the top row were sequentially marked with a No. 2 pencil as A, B, C, D and E, and the lower row was marked as V, W, X, Y and Z, in such a way that A and V, B and W, C and X, D and Y, and E and Z fall in the same column (Figure 6a).

Every time, using a fresh micropipet tip, 1 µl of each of the solutions A, B, C, D, E, V, W, X, Y and Z were spotted on the nylon membrane within the circles of respective squares. The membrane was dried under tungsten light for 10 minutes, and baked under vacuum at 80^0 C for 2 hours to immobilize the DNAs.

L.4.2. Immunological Detection of DNA Labelled with Digoxigenin:

The labelled DNAs in the spots on the membrane were immunologically detected and visualized by colorimetric reactions following Protocol III, as detailed below. The baked membrane was washed with Buffer 1 (100 mM Tris-HCl, 150 mM NaCl, pH 7.5), incubated in Buffer 2 (1% blocking reagent, a denatured casein, Boehringer Mannheim, in Buffer 1) for 30 minutes, reincubated in Buffer 2 containing «DIG»AP-Conjugate (15 U/100 ml) for 30 minutes, washed twice with Buffer 1, and equilibrated two minutes with Buffer 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$ pH 9.5). The «DIG»AP-Conjugate was a polyclonal sheep anti-digoxigenin Fab-fragment antibody conjugated with alkaline phosphatase. Under the above condition, the «DIG»AP-Conjugate has been certified to immunologically bind only to digoxigenin labelled DNA.

The membrane was placed inside a plastic bag followed by adding 0.1 ml/cm^2 membrane freshly prepared color-substrate solution (3.4 mg nitroblue tetrazoluim and 1.75 mg 5-bromo-4-chloro-3-indolylphosphate/10 ml Buffer 3), and placed in the dark in a stainless steel tray with cover. During this time, the alkaline phosphatase conjugate present with the «DIG»AP-Conjugate dephosphorelates the colorless 5-bromo-4-chloro-3-indolylphosphate, and produces indoxyl which immediately gives away a H^+ ion and an e^- to form an indigo precipitate. The H^+ ion and e^- thus generated reduces nitroblue tetrazolium into a purple colored diformazan. The color generated by indigo and diformazan in combination in this reaction, along with the nylon membrane, was of different shades of brown, directly related to the concentration of the labelled DNA (Figure 6b). This method of detection is highly specific and can detect 0.1 pg homologous DNA through Southern Hybridization (Boehringer Mannheim, 1989). After 2 hours, the membrane was washed with Buffer 4 (10 mM Tris-HCl; 1 mM EDTA; pH 8.0), photocopied, and stored inside a fresh plastic bag with Buffer 1 in a refrigerator.

The intensity of the color developed in the spots of digoxigenin labelled pXlr101A (Dig-pXlr101A) and pBR328 were visually compared. By visual matching between the color intensity of the pXlr101A spots with the standard pBR328 DNA spots, the concentration of labelled pXlr101A was quantitated.

M. Southern Hybridization of Restriction Endonuclease Digested Fish DNA with Digoxigenin Labelled pXlr101A rDNA Probe and Immunological Detection:

M.1. Southern Hybridization:

Each nylon membrane with Southern transferred fish DNA were taken out of the vacuum and hybridized with Dig-pXlr101A rDNA probe according to Protocol IV. Each membrane was put in a plastic bag and 0.2 ml prehybrid buffer X cm^2 membrane was added to the bag and sealed using Pak'n Save Model 838, Decosonic Inc. The prehybrid buffer contained 5 X SSC, 1% denatured casein blocking agent, 0.1% N-lauroylsarcosine, 0.02% SDS. The bag was put in a shaker water bath maintained at 68^0C for >3 hours with constant agitation for uniform distribution of the prehybrid solution. The bag was taken out, wiped with paper towel to remove excess water from the outside. It was cut open through a corner and the prehybrid buffer was drained.

The bag was refilled with 0.025 ml hybrid buffer X cm^2 membrane. The hybrid buffer contained 50% formamide and 50% prehybrid buffer containing 5% blocking reagent and 10 ng/ml freshly heat denatured digoxigenin labelled pXlr101A probe rDNA. Extreme care was taken to eliminate all air bubbles trapped in the bag, and it was heat sealed as before. The bag was incubated again in a shaker water bath maintained at 42^0 C, and kept under agitation for overnight (>12 hours).

The bag was opened and the membrane was washed twice, 5 minutes each, at room temperature in 2 X SSC containing 0.1% SDS. This was rewashed twice, 15 minutes each, at room temperature with 0.1 X SSC containing 0.1% SDS. The rDNA hybrids on the membrane were detected using immunological detection techniques.

M.2. Immunological Detection of rDNA Hybrids:

Initially, the rDNA hybrids with digoxigenin labelled probes were detected immunologically using «DIG»AP-Conjugate followed by colorimetric method for visualization following Protocol IV for a couple of fishes in 1991. However, by 1992, the immunological detection of hybrids with digoxigenin labelled probes using «DIG»AP-Conjugate and Lumi-Phos 540 became well established in the field, and this was used for this entire study. However, it may be relevant to note here, no difference in terms of the number of rDNA bands was observed using either technique, in the fishes studied.

The digoxigenin labelled probe in the hybrid complex was detected using Lumi-Phos 350, Lumigen, Inc. Detroit, MI, at pH 9.5. Lumi-Phos 350 contains Lumigen PPD [0.33 mM 4-methoxy 4-(3-phosphatephenyl)-spiro(1,2-dioxetane-3,2′-adamantane) disodium salt], 750 mM 2-amino-2-methyl-1-propanol buffer (pH 9.5), 0.88 mM $MgCl_2$, 1.13 mM cetyltrimethylammonium bromide, and 0.035 mM fluorescent surfactant. The alkaline phosphatase dephosphorylates the Lumigen PPD into an unstable intermediate which decays and emits blue light at λ 477 nm. This light is further enhanced by the fluorescein surfactant present in the micelles formed by the cetyltrimethylammonium bromide emitting bright yellow light at λ 525 nm. Following this reaction, hybrids of digoxigenin labelled probes were recorded on an X-ray film. Sensitivity of this method is reported to be equivalent to ^{32}P labelled method, and it was found as reported to detect a hybrid much faster than the ^{32}P labelled probe (Boehringer Mannheim, 1992).

The membrane hybridized with pXlr101A rDNA probe was washed with Buffer 1, followed by Buffer 2 as in Protocol IV and as detailed before. The membrane was reincubated for 30 minutes at room temperature in a bag containing Buffer 2 with 15 U/100 ml «DIG»AP-Conjugate. After the immunological reaction, the solution was drained off and the unbound «DIG»AP-

Conjugate was removed by two washes with Buffer 1.

The membrane was equilibrated two minutes with Buffer 3 at pH 9.5, and transferred inside a 6" X 4" transparent PhotoGene Development Folder, Gibco-BRL, made of acetate film. The folder was opened, 10 µl X cm^2 membrane Lumi-Phos 530 was sterilely pipetted directly onto the membrane, and then the folder was reclosed. A glass rod was gently rolled over the folder in order to spread the Lumi-Phos 530 uniformly throughout the membrane and also to remove any entrapped air bubble.

The folder with the membrane was put inside a black opaque AQ X-RAY *Exposure Holder* immediately, and taken into a dark room. The exposure holder was opened, a previously cut, appropriate sized, Kodak XAR X-ray film was put on top of the acetate film of the folder, and the holder was closed.

After 2 hours the X-ray film was taken out and developed using GBX developer and fixed with GBX fixer following the supplier's protocol. If needed, exposure time was adjusted in order to record the hybrid bands adequately.

The membrane was washed 2 X 2 minutes with 1 ml Buffer 4 X cm^2 membrane, and stored in 1 ml Buffer 1 X cm^2 membrane in a sealed plastic bag, in a refrigerator to be rehybridized, if needed, later.

N. Mapping Fish Nuclear rDNA with Restriction Endonuclease Sites:

N.1. Determination of Sizes of rDNA Fragments:

N.1.1. Making Standard Plots:

For each gel a standard plot was constructed based on the mobility of the marker DNA fragments of known molecular sizes on the gel. In order to do so, the photographic negative of a gel was printed to actual size by enlargement. This was achieved by adjusting the enlarger so that the scale for length in the photographed negative becomes equal to the original scale placed on the printing pad at the level of printing paper. Multiple prints were made as needed. The mobility of each size DNA fragment was measured using the metric scale and plotted on a semi-logarithmic paper with mobility and molecular size in kilobase/s (Kb) on the X (arithmetic) and Y (logarithmic) axis, respectively.

N.1.2. Determining the Sizes of Fish rDNA Fragments:

The distance travelled by each rDNA fragment represented by a hybrid band from its origin, was measured from the X-ray negative for each gel using the metric scale, and recorded. The X-ray negative represents the actual size of the gel. Based on the standard plot related to this gel, the size of each hybrid rDNA band was determined in Kb. The fragment sizes were adjusted up to 300 bases in length during mapping, following (Phillips *et a.*, 1992).

N.2. Mapping Fish rDNA:

N.2.1. Construction of a Common Model for rDNA Mapping:

The length of the rDNA coding regions among vertebrates are reported to be very similar (Gerbi, 1985; Phillips *et al.*, 1989; Table 8). The total length of the rDNA coding region was reported to be 5.8 Kb in salmonids (*Oncorhynchus mykiss, Salmo salar, Salmo trutta, Salvelinus alpinus, Salvelinus fontinalis, Salvelinus namaycush*; Phillips *et al.*, 1989), that includes 18S, 5.8S, and 28S rDNA. Further, the total length of the coding region and the internal transcribed spacers (ITS) was shown to be 6.8 Kb in 17 salmonid species, including six *Salvelinus*, two *Salmo*, eight *Oncorhynchus*, and one *Hucho* (Popodi *et al.*, 1985; Phillips *et al.*, 1989 and 1992).

In addition, the total length of an rDNA repeat in the salmonids studied was found to vary between 22-26 Kb (Phillips *et al.*, 1989). Based on this information, an rDNA subunit was constructed as a model with a 6.8 Kb region containing 18S, 5.8S, and 28S plus the ITS with flanking non-transcribed regions (NTS) on either sides (Figure 7). Their relative positions in an rDNA repeat are known for vertebrates including salmonids to be NTS, 18S, ITS, 5.8S, ITS, and 28S in 5′ to 3′ sequence (Long and Dawid, 1980; Phillips *et al.*, 1992). The NTS, flanking the 5′ end of 18S rDNA is extended, since, it varies in length (Phillips *et al.*, 1989). The NTS flanking the 3′ end of the 28S rDNA region is a part of the next rDNA repeat unit. However, for the purpose of mapping, in addition to a complete rDNA repeat unit, the NTS of the adjacent rDNA repeat, bound to the

3′ end of a 28S rDNA of the complete repeat, was included in the map. This composition of rDNA region in mapping is needed since a restriction endonuclease may generate an rDNA fragment containing a part of the 28S of one repeat continuing with the NTS of the adjacent repeat.

N.2.2. rDNA Mapping:

The probe, pXlr101A, containing *Xenopus laevis* rDNA, is only expected to hybridize with the conserved regions of fish rDNA in the transcribed region since the NTS is highly variable and does not hybridize across species (Phillips *et al.*, 1992). Hence, a restriction endonuclease fragment of fish DNA that hybridized with this probe is expected to contain a part of the transcribed region of the rDNA of that fish. Based on this criteria, all the rDNA fragments of a fish generated by the restriction endonucleases were matched like a jigsaw puzzle. In case of problems in matching, fresh DNA samples were redigested with fresh enzymes in order to confirm the bands, and thus, an rDNA map construction was completed based on more than a single set of data.

N.2.3. Additional Consideration Given in rDNA Mapping:

Since the probe rDNA used in this study is heterologous in origin, from *Xenopus laevis*, the intensity of hybrid bands will not necessarily relate to the size or the amount of rDNA represented by the bands. In other words, a small size fish rDNA fragment may show an intense hybrid band than a larger size fish rDNA fragment if it contains sequences more complementary to the probe. Further, in measuring the size of rDNA fragments, as a standard procedure, a length of up to 300 bases was adjusted to match the sizes depending on the size of the rDNA fragment. It is known and also observed in this study, that DNA samples of similar size in length may have some differences in mobility through a gel in electrophoresis due to the differences in the gel and unequal electric potential in the field, in addition to other factors.

Phenol extraction of the digested fish DNA generally results in a loss of small size rDNA fragments, and thus, the small size rDNA fragments may became less detectable by hybridization with heterologous probes. Hence, at the end of digestion, the digested rDNA fragments were separated by agarose gel electrophoresis without further phenol extraction. Due to the presence of RE protein impurities with the DNA, the mobility of the digested fish rDNA fragments were cross examined in order to find out if any one of them was affected by the bound RE with the DNA after digestion. In such cases, the fragment sizes were also adjusted.

Further, in some cases, complete digestion was not always obtained. As a result, some fragments remained only partially digested. However, by cross examination of the digested fish DNA by various RE and their combinations, the partially digested and fully digested fragments could be determined.

It is known that under altered conditions, for example, non-optimal pH, increased enzyme concentration, reduced salt concentration, and the presence of

Co^{2+}, Mn^{2+} and Zn^{2+}, among others, may cause star activity of restriction endonucleases (RE) (Titus, 1991). This means that an RE may recognize DNA sequences in addition to the specific sequence for digestion. The RE, *Bam*H I, *Eco*R I, *Hin*d III, *Pst* I, and *Pvu* II, used in this study are known to have star activity under non-optimal conditions, as summarized before (Hsu and Berg, 1978; George and Chirikjian, 1982; Nasri and Thomas, 1986; Malyguine *et al.*, 1980; Titus, 1991). Hence, it is possible, when multiple enzymes were used, the buffer suggested by the supplier may not provide 100% optimal conditions, and in some cases may show star activity. There was also evidence of occasional mild to significant star activity of some of the RE, that resulted in the presence of an unexpected number and size of rDNA fragments. Presence of such a fragment was also confirmed during mapping.

O. Phylogeny Reconstruction of Major Groups of Actinopterygii Including Euteleostei, Based on rDNA Restriction Endonuclease Characters:

O.1. Alignment of Restriction Endonuclease Sites and Deduction of Data Matrix:

Restriction endonuclease (RE) maps of all seventeen fishes were compared with each other by aligning the transcribed region for matching restriction sites. The area of rDNA selected for RE recognition site characters was segmented (Figure 8). The number of segments was determined by the number of specific RE recognition sites present in the rDNA area selected for this study. An RE specific site present in a selected rDNA segment in the rDNA map of one fish was identified and its presence or absence in the rDNA map of rest of the fishes was determined.

Since both the 5′ and 3′ ETS rDNA is known to contain regulatory sequences (Gerbi, 1985), RE recognition sites starting from about 500 bases 5′ ETS until 500 bases 3′ ETS were included in generating the character matrix. The RE recognition sites in both the ETSs are generally included in reconstructing phylogeny (for example in salmonids, Phillips and Pleyte, 1991; Phillips *et al.*, 1989, 1992; in bats Baker *et al.*, 1991; Van Den Bussche, 1991). Suzuki *et al.* (1994) used 0.7 Kb of the 3′ ETS of rDNA as a probe in field mouse population relationships. Further, the total length of rDNA from the 5′ 18S to the 3′ 28S varies with the length of the ITS. The length of the ITS is known to vary from 1.5 Kb in 17 salmonids, 1.9 Kb in a Paracanthopterygii (*Opsanus tau*) to 4.5 Kb in a Percoidea (*Cynoscion nebulosus*) (Cortadas and Pavon, 1982; Phillips *et al.*, 1989, 1992; Tanhauser *et al.*, 1986). Further, the length of the 18S and 28S rDNA may also vary between species and populations depending on the expansion segments present in either of them (Gerbi, 1985). However, since a standard length of 7.8 Kb rDNA conserved region (6.8 Kb coding region and ITS, as stated before, plus 500 bases for either ETS) was used for mapping (Fig 8), some adjustments were made in aligning the homologous sites to accommodate for variations in the ITS lengths. When a consensus was reached on the alignment, presence or absence of restriction endonuclease sites through the conserved regions were recorded in the data matrix (Table 26).

O.2. Phylogeny Reconstruction Using Hennig86:

The phylogeny of the experimental fishes was reconstructed based on the rDNA restriction endonuclease data matrices, using the computer assisted parsimony analysis program, *Hennig86*. This program codes characters for calculating the most parsimonious trees, either exactly or by effective approximation methods. The speed and effectiveness of *Hennig86* in determining phylogeny has been found to be superior to PAUP and PHYLIP (Platnick, 1989; Sanderson, 1990). It is a fast and effective program for parsimony analysis (Swofford and Olsen, 1990).

The RE data matrix was analyzed using *Hennig86* options h, h*, mh, mh*, ie, ie* and ie- and the maximum parsimonious trees generated were recorded in a log file. The lake sturgeon, *Acipenser fulvescens*, was chosen as the principal outgroup by default. The trees were examined for the relationships of the major clades, and were also compared with the hypothetical tree based on morphology (Figure 96). The most parsimonious tree based on rDNA RE recognition characters matching with the hypothetical tree was selected and will be discussed.

P. Construction of Phylogeny Based on Morphology Alone and in Combination with rDNA RE Characters:

P.1. Hypothetical Cladogram of the Experimental Fishes Based on Morphology:

For comparing the rDNA RE based euteleost phylogeny obtained in this study with the morphology based relationship of the same euteleosts, a hypothetical cladogram of the experimental fishes was reconstructed based on available morphological information. The literature that was compared extensively for collecting specific information on the phylogenetic relationships of the experimental fishes were Bart and Page (1992), Begle (1991a; 1991b; 1992), Cavender and Coburn (1992), Fink and Fink (1981), Fink and Weitzman (1982), Fink (1984), Howes, (1991), Johnson (1993), Johnson and Paterson (1993), Lauder and Liem (1983), Le *et al.*, (1993), Nelson (1984; 1989; 1994), Parenti (1986); Phillips and Pleyte, (1991), Rosen (1974; 1985), Stearley, (1992), Stiassny (1990; 1993), Stiassny and Moore (1992), Wiley (1992), Wilson and Williams, (1992). Following the above comparison, a consensus was reached based on subjective evaluation and thus, a hypothetical and tentative cladogram was produced manually matching with the classical view (Nelson, 1989; 1994). This cladogram was compared with the rDNA phylogeny obtained from this study.

P.2. Morphological Phylogeny Based on Parsimony:

All the apomorphic morphological characters reported by the specific authors supporting the above hypothetical cladogram (Figure 96) were initially listed and examined carefully to eliminate autoapomorphic characters. Thus, a list of 21 apomorphic morphological characters was generated (Appendix 1) based on which a morphological character matrix was produced (Table 27). The character matrix was analyzed using all options available in *Hennig86*, parsimony analysis, and thus, many trees were generated. The option *mhennig* generated three most equally parsimonious trees (Figure 97). The trees generated by option *mhennig* were combined using option *nelsen* from *Hennig86* to generate a Nelsen consensus tree as presented in Figure 98.

P.3. Phylogeny based on rDNA RE Characters and Morphology:

The RE and morphological character matrices were combined together and analyzed with all options available in *Hennig86*. The option *mhennig* generated four trees which were processed further using option *nelsen* of *Hennig86* to generate a Nelsen consensus tree as presented in Figures 99 and 100.

Table 2

List of Experimental Actinopterygian Fishes and Their Phylogenetic Groups[1].

Order	Family	Species	Common name
Infraclass: Chondrostei			
Acipenseriformes			
	Acipenseridae		
		Acipenser fulvescens (Rafinesque, 1817)	Lake sturgeon
Infraclass: Neopterygii; Division: Halecostomi; Subdivision: Teleostei			
Infradivision: Elopomorpha			
Anguilliformes			
	Anguillidae		
		Anguilla rostrata (Lesueur, 1817)	American eel
Infradivision: Clupeomorpha			
Clupeiformes			
	Clupeidae		
		Alosa sapidissima (Wilson, 1811)	American shad
Infradivision: Euteleostei			
Superorder: Ostariophysi			
Cypriniformes			
	Cyprinidae		
		Cyprinus carpio (Linnaeus, 1758)	Common carp
		Labeo rohita (Hamilton)	Rohu
		Puntius gonionotus (Bleeker)	Thailandian sarpunti
Superorder: Protacanthopterygii			
Salmoniformes			
	Esocidae		
		Esox americanus americanus (Gmelin, 1788)	Redfin pickerel
		Esox niger (Lesueur, 1817)	Chain pickerel
	Umbridae		
		Umbra pygmaea (DeKay, 1842)	Eastern mudminnow
	Osmeridae		
		Osmerus mordax (Mitchill, 1814)	Rainbow smelt
	Salmonidae		
		Coregonus clupeaformis (Mitchill, 1818)	Lake whitefish
		Oncorhynchus mykiss (Walbaum, 1792)	Rainbow trout
		Salmo trutta (Linnaeus, 1758)	Brown trout
		Salvelinus fontinalis (Mitchill, 1814)	Brook trout
Superorder:Acanthopterygii			
Perciformes			
	Percichthyidae		
		Morone saxatilis (Walbaum, 1792)	Striped bass
	Percidae		
		Perca flavescens (Mitchill, 1814)	Yellow perch
	Cichlidae		
		Oreochromis aureus (Steindachner)	White tilapia

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[1] Nelson, 1984

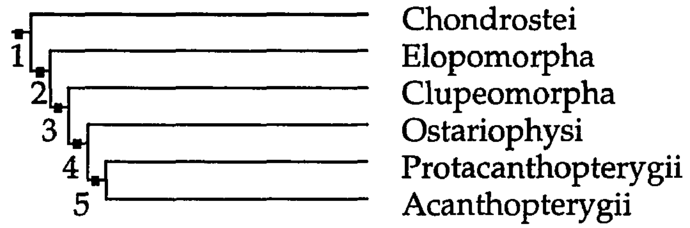

Figure 3. Compressed relationships of the major groups of fishes selected for this study (following Lauder and Liem, 1983; Nelson, 1989). 1 Actinopterygii, 2 Teleostei, 3 Clupeocephala, 4 Euteleostei, 5 Neognathi (Protacanthopterygii and Neoteleostei).

Table 3
List of Experimental Fishes with Location, Time, Status of Collection, and Sex.

Taxa	Place of Collection	Time	Status	Sex
Acipenser fulvescens[1]	St. Lawrence River, Montreal, Canada	1993	Live, Mature	♂
Anguilla rostrata	Sea Dragon, Fish Store, Brooklyn, NY	1992	Live, Mature	♀
Alosa sapidissima[2,8]	Hudson River, Tappanzee Bridge, NY	1992	Live, Mature	♂
Cyprinus carpio	Shapla Grocery, Brooklyn, NY	1992	Live, Mature	♀
Labeo rohita[3,11]	FRI, BAU, Mymensingh, Bangladesh	1990	Live, Immature	ND
Puntius gonionotus[3,11]	FRI, BAU, Mymensingh, Bangladesh	1990	Live, Mature	ND
Esox americanus americanus[4]	Camden, NJ	1993	Live, Mature	♀
Esox niger[4]	Camden, NJ; Whales Lake, NY	1992/3	Live, Immature	ND
Osmerus mordax [5,8]	Lake Champlain, NY	1992	Live, Mature	♀
Umbra pygmaea[4]	Atlantic, NJ	1993	Live, Mature	ND
Coregonus clupeaformis[7]	Ann Arbor, Michigan	1992	Live, Mature	♀§
Oncorhynchus mykiss[10]	Cold Spring Fish Hatchery, NY	1990/2	Live, Immature	ND
Salmo trutta[10]	Cold Spring Fish Hatchery, NY	1990/2	Live, Immature	ND
Salvelinus fontinalis[10]	Cold Spring Fish Hatchery, NY	1990/2	Live, Immature	ND
Morone saxatilis[2,6,8,9]	Hudson River, Manhattan/Tappanzee Br. NY	1992	Live, Im/Mature	ND
Perca flavescens[8]	Whales Lake and Lake Champlain, NY	1992	Live, Mature	♂/♀
Oreochromis aureus[8]	Shapla Grocery, Brooklyn, NY	1992	Live, Mature	♀

Help was obtained in each specific case from the following persons or the organizations they represent. [1]Mr. A. Schiavone, New York State Department of Environmental Conservation, [2]Mr. Robert Gabrielson, President, NYS Fishermen Association, [3]Dr. M.S. Shah, FRI, [4]Professor Rudolf G. Arndt, A.M. Teti and A.G. Dramstad, Stockton College, NJ; [5]Mr. Howard Swan, Day & Night Fishing, Moriah, NY, and [6]Mr. J. Miller, Delaware River Coordinator, US Department of Interior, Fish and Wildlife Service, through Normandale Associates captured the respective fishes; [7]Dr. Barbara A. Shields donated DNA, Ann Arbor, University of Michigan; [8]Earl Noyan, [9]A.K.M.N. Islam and [10]Mr. G. Ravert, Brooklyn College, helped in collecting fishes; [11]Bishan Chowdhury, BAU, helped in DNA extraction; FRI, Fisheries research Institute; BAU, Bangladesh Agricultural University; ND, could not be determined; ♂, male; ♀, female; §to be confirmed.

Table 4
List of Persons, with Affiliations, Identified the Experimental Fishes

Species	Identified by
Acipenser fulvescens[1,2]	Mr. A. Schiavone & Mr. A. Branchaud
Anguilla rostrata[3]	Dr. D. Thoney
Alosa sapidissima[4]	Mr. R. Gabrielson
Cyprinus carpio[5]	Z.M.G.S. Jahangir
Labeo rohita[5,6]	Dr. M.S. Shah; Z.M.G.S. Jahangir
Puntius gonionotus[6]	Dr. M.S. Shah
Esox americanus americanus[7]	Prof. R.G. Arndt
Esox niger[7]	Prof. R.G. Arndt
Osmerus mordax[3]	Dr. D. Thoney
Umbra pygmaea[7]	Prof. R.G. Arndt
Coregonus clupeaformis[8]	Dr. B. A. Shields
Oncorhynchus mykiss[9]	Mr. N. Soule
Salmo trutta[9]	Mr. N. Soule
Salvelinus fontinalis[9]	Mr. N. Soule
Morone saxatilis[4,10,11]	Mr. J. Miller; Dr. I.I. Wirgin
Perca flavescens[5,12]	Mr. H. Swan; Confirmed with NYSDEC
Oreochromis aureus[13]	Dr. M.L.J. Stiassny

Affiliations: [1]Mr. A. Schiavone, New York State Department of Environmental Conservation; [2]Alain Branchaud, Department des Sciences Biologiques, Université du Quebec à Montréal, Montréal, Québec, Canada; [3]Dr. Dennis Thoney, New York Aquarium, Brooklyn, NY; [4]Mr. Robert Gabrielson, President, NYS Fishermen Association; [5]Z.M.G. Sarwar Jahangir, Ph.D. Candidate, The Graduate School and University Center, New York, NY; [6]Dr. M.S. Shah, Fisheries research Institute, Bangladesh Agricultural University Campus, Mymensingh, Bangladesh; [7]Professor Rudolf G. Arndt, Stockton College, NJ; [8]Dr. Barbara A. Shields, Ann Arbor, University of Michigan; [9]Mr. Norman Soule, Cold Spring Harbor Fish Hatchery, Cold Spring Harbor, NY; [10]Mr. J. Miller, Delaware River Coordinator, US Department of Interior, Fish and Wildlife Service, through Normandale Associates; [11]Dr. Isaac I. Wirgin, New York University Medical Center, Tuxedo, NY; [12]Mr. Howard Swan, Day & Night Fishing, Moriah, NY; [13]Dr. Melanie L.J. Stiassny, Ichthyology, American Museum of Natural History, New York, NY.

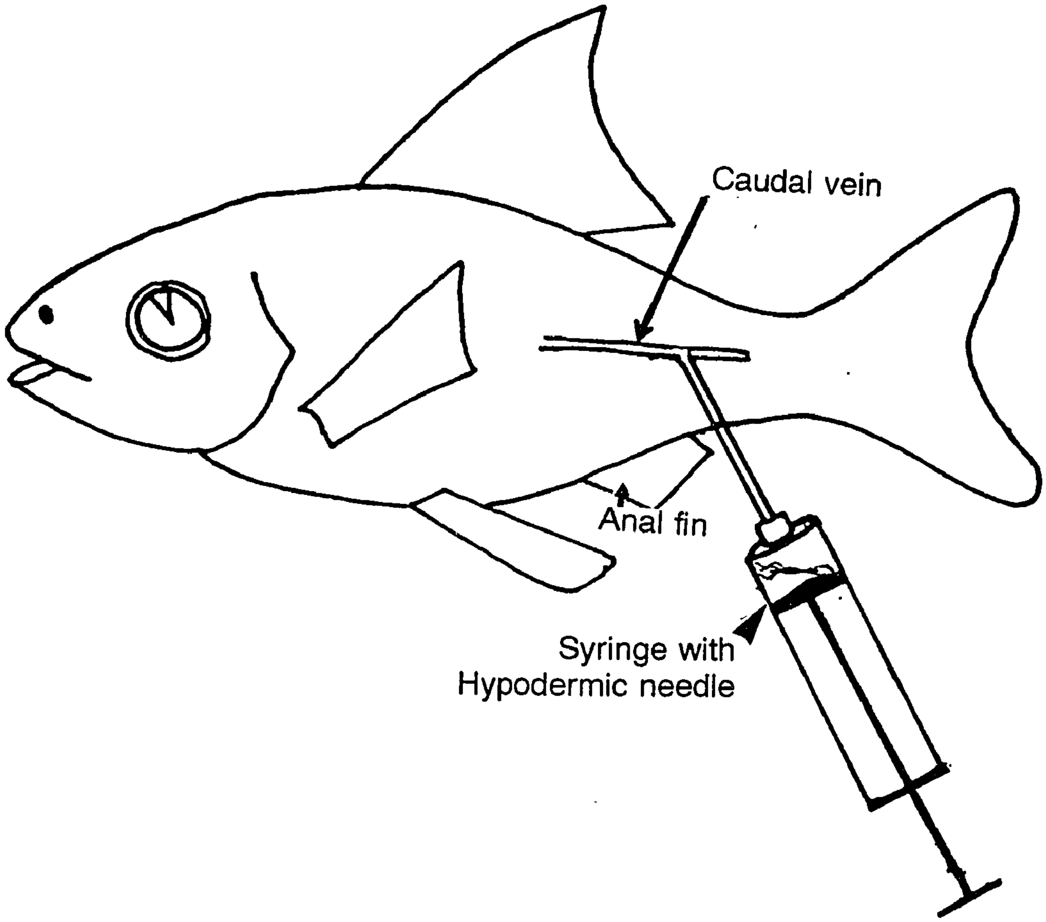

Figure 4. A typical representation of blood collection from a representative fish. The caudal vein passing through the hemal canal was reached by passing the hypodermic needle between the hemal spines. Blood was drawn into the syringe by pulling out the plunger. In order to prevent clotting of blood, 1-2 ml of 1 X SSC was drawn into the syringe before the blood collection.

Table 5

A typical set-up of fish nuclear DNA restriction endonuclease digestion.

1st digestion set-up:

Tube No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
µl DNA🐟 (2µg)	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
µl DW	62	41	46	41	78	62	62	62	62	41	41	41	41	46	46	41	26
µl 10X buffer	8^b	5^h	6^e	5^b	10^h	8^b	8^b	8^b	8^b	5^h	5^h	5^h	5^h	6^e	6^e	5^b	6^e
µl R. Enzyme	8H	2E	6B	2V	10S	8H	8H	8H	8H	2E	2E	2E	2E	6B	6B	2V	6B
µl Total	80	50	60	50	100	80	80	80	80	50	50	50	50	60	60	50	60

2nd digestion set-up: After the 1st digestion, each sample was heated at 60⁰ C for 10 minutes to denature the endonuclease used in the 1st digestion, chilled 15 minutes in ice-water and set-up for the 2nd digestion.

Tube No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
µl DNA🐟 (2µg)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
µl DW	-	-	-	-	-	16	44	16	88	18.5	-	61	77	18	94	61	110
µl 10X buffer	-	-	-	-	-	2^h	10^e	2^b	10^h	5.5^e	-	7^h	9^h	-	4^h	7^h	6^h
µl R. Enzyme	-	-	-	-	-	2E	6B	2V	$2S^*$	6B	2V	$2S^*$	2V	2V	$2S^*$	$2S^*$	2V
µl R. Enzyme	-	-	-	-	-	-	-	-	-	-	-	-	$2S^*$	-	-	-	$2S^*$
µl Changed Total						100	140	100	180	80	50	120	140	80	160	120	180

🐟Fish nuclear DNA; DW, deionized water; R. Enzyme, Restriction endonuclease; B, *Bam*H I; E, *Eco*R I; H, *Hind* III; S, *Pst* I; V, *Pvu* II; [b]Buffer B - 50 mM NaCl, 6 mM Tris-HCl, 6 mM $MgCl_2$, 1 mM DTT, and pH 7.5; [e]Buffer E, 100 mM NaCl, 6 mM Tris-HCl, 6 mM $MgCl_2$, 1 mM DTT, and pH 7.5; [h]Buffer H, 50 mM NaCl, 90 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, and pH 7.5; *new supply with a 5 X concentration of units/µl.

Protocol I

Transfer Fish DNA from Agarose Gel to Nylon Membrane by Southern Blotting

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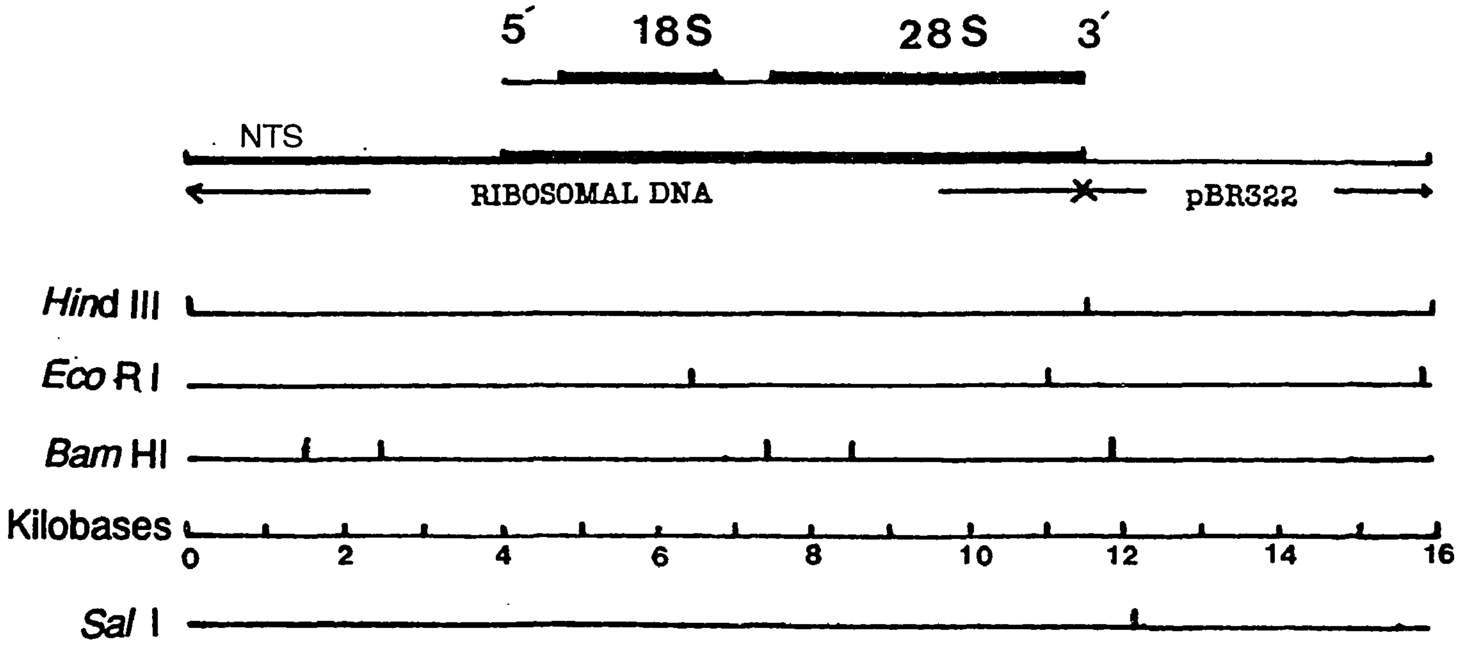
The gel was:

1. depurinated by soaking 20 minutes in 20 ml 0.25M HCl X gel volume at room temperature;
2. denatured by soaking 2 X 20 minutes in 20 ml denaturing solution X gel volume at room temperature;
3. neutralized by soaking 2 X 20 minutes in 20 ml neutralizing solution X gel volume at room temperature;
4. blotted in 10 X SSC with nylon membrane overnight to 36 hours;

The nylon membrane was:

5. washed 5 minutes in 5 X SSC;
6. air dried for 10 minutes;
7. baked under vacuum at 80^0 C for 2 hours, and stored under vacuum for hybridization at a later time.

Denaturing solution, 1.5 M NaCl and 0.5 M NaOH, pH >13.0;
Neutralizing solution, 1.5 M NaCl and 1.0 M Tris-HCL, pH 7.5.



pXlr101A Restriction Enzyme Map

Figure 5. Restriction endonuclease map of pXlr101A as supplied by Dr. Brian McStay, 1991. It contains ribosomal DNA inserted into a plasmid, pBR322, at the *Hind* III site. NTS, non-transcribed spacer region; 18S, 18S rRNA gene; 28S, 28S rRNA gene. The restriction endonuclease site map of the probe pXlr101A, used in this study, exactly matched this map.

Protocol II
Preparation of Digoxigenin Labelled *pXlr101A*

1. Took 9.8 μl (2 μg) pXlr101A DNA into a microcentrifuge tube;
2. Added 1.2 μl 10X Buffer D (150 mM NaCl, 6 mM Tris-HCl, 6 mM $MgCl_2$, 1 mM DTT, and pH 7.5; 1.0 μl (12 U) *Sal* I;
3. Mixed well, centrifuged, 3 X 10^3 rpm in HB-4 rotor, and incubated overnight at 37^0 C;
4. Added 78.0 μl distilled water, tightly sealed the tube, and mixed well;
5. Boiled 20 minutes; Chilled immediately in NaCl-ice for 2 minutes;
6. Added 12.0 μl 10X hexanucleotide; 12.0 μl 10X dNTP (dUTP with dig); 6.0 μl Klenow Polymerase I;
7. Incubated overnight for 20 hours at 37^0 C;
8. Added 4.8 μl 0.5 M EDTA pH 8.0; 15.0 μl 4.0 M LiCl; 2.0 μl 50 μg/μl tRNA; 450.0 μl cold absolute ethanol, stored 15 minutes at -80^0 C
9. Centrifuged 10 minutes, at 14 X 10^3 g, 4^0 C;
10. Poured off the supernatant; added 300.0 μl cold 70% ethanol, rolled well for uniform hydration;
11. Stored 15 minutes at -80^0 C; centrifuged 10 minutes at 14 X 10^3 g and 4^0 C;
12. Poured off supernatant; dried under vacuum;
13. Added 60.0 μl 0.1% SDS; brought the DNA into solution in refrigerator;
14. Digoxigenin labelling was quantitated by colorimetric method.

Table 6

Dilution of digoxigenin labelled standard pBR328 DNA (5 ng/µl) supplied with the labelling Kit.

Digoxigenin Labelled Control pBR328 DNA	Dilution Buffer Added	New Solution	Final Concentration
2 µl Original	8 µl TE buffer	A	1 ng/µl
1 µl Original	9 µl TE buffer	B	100 pg/µl
1 µl solution B	9 µl TE buffer	C	10 pg/µl
1 µl solution C	9 µl TE buffer	D	1 pg/µl
1 µl solution D	9 µl TE buffer	E	0.1 pg/µl

Table 7

Dilution of digoxigenin labelled pXlr101A using TE pH 8.0 as the dilution buffer.

Digoxigenin Labelled pXlr101A	Dilution Buffer Added	New Solution	Dilution
2 µl Stock Sol^n.	8 µl TE buffer	V	10 X
1 µl V	9 µl TE buffer	W	100 X
1 µl W	9 µl TE buffer	X	1,000 X
1 µl X	9 µl TE buffer	Y	10,000 X
1 µl Y	9 µl TE buffer	Z	100,000 X

Protocol III
Immunological Detection Using Colorimetric Technique

Immunological Reaction:

1. Washed the nylon membrane with 1 ml Buffer 1 X cm^2 membrane for 5 minutes
2. Incubated in 1 ml Buffer 2 X cm^2 membrane for 30 minutes.
3. Incubated 30 minutes with 0.2 ml Buffer 2 containing «DIG»AP-Conjugate 1 µl/5 ml buffer X cm^2 membrane.
4. Wash 2 X 15 minutes with 1 ml Buffer 1 X cm^2 membrane.

Colorimetric Detection:

5. Equilibrated the membrane 2 minutes with 0.2 ml Buffer 3 X cm^2 membrane.
6. Incubated the membrane with 0.1 ml color-substrate solution in Buffer 3 X cm^2 membrane. Observed periodically for the color development.
7. Wash the membrane after 2 hours with 1 ml Buffer 4 X cm^2 membrane.
8. Photocopied membrane to record the color developed; membrane stored in refrigerator.

Buffer 1, 100 mM Tris-HCl, 150 mM NaCl, pH 7.5; **Buffer 2**, 1% blocking reagent (denatured casein), Boehringer Mannheim, in Buffer 1; **Buffer 3**, 100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5; **Color-substrate solution**, 3.4 mg nitroblue tetrazoluim and 1.75 mg 5-bromo-4-chloro-3-indolylphosphate/10 ml Buffer 3); **Buffer 4**, 10 mM Tris-HCl, 1 mM EDTA, and pH 8.0.

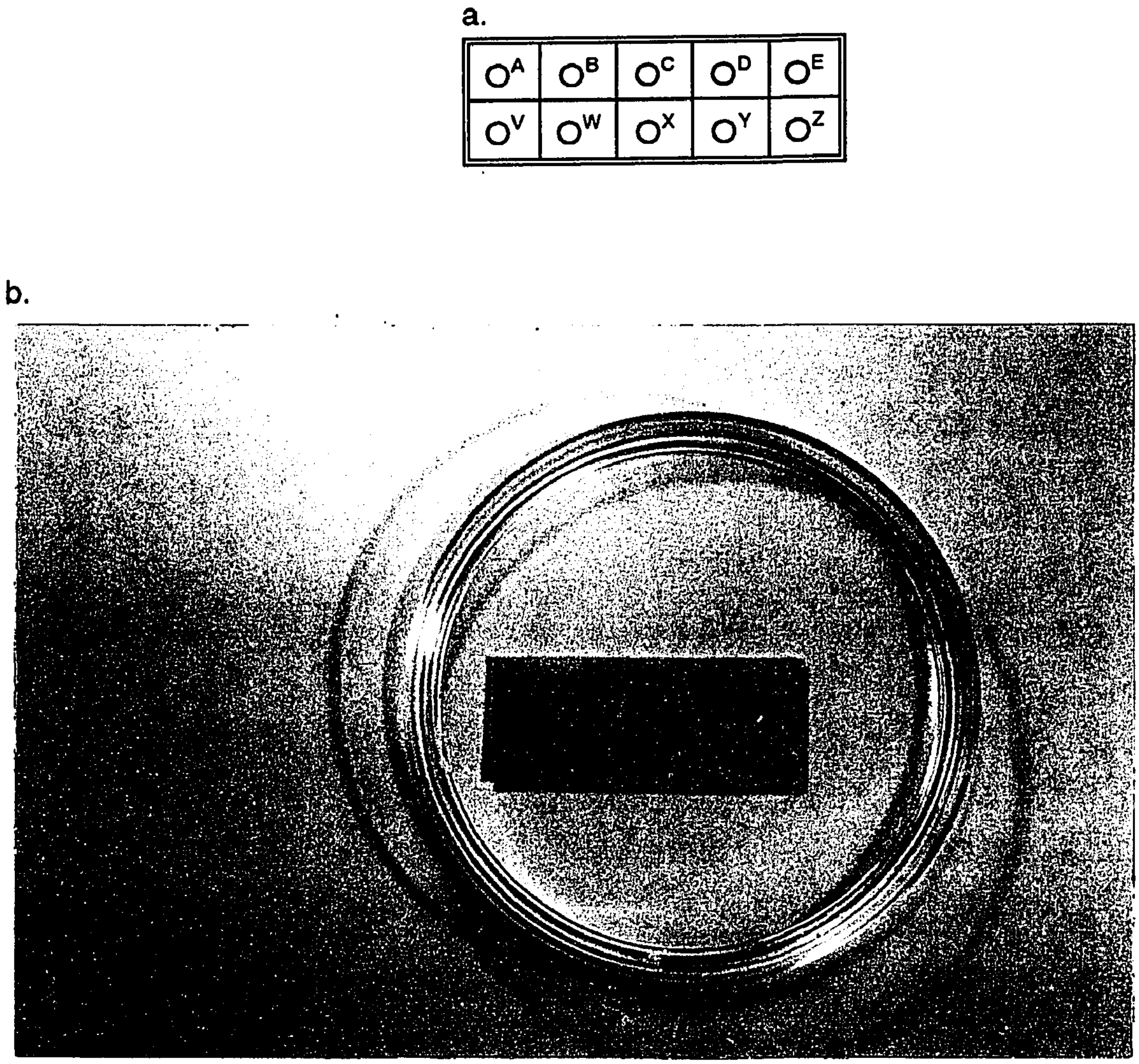


Figure 6. a. A 10 cm^2 PhotoGene Nylon Membrane showing the positions of digoxigenin labelled pBR328 and pXlr101A DNA spots for quantitating labelled pXlr101A. b. Relative amount of digoxigenin labelled pBR328 and pXlr101A present in respective spots as detected by anti-digoxigenin antibody followed by colorimetric reaction.

Protocol IV

Southern Hybridization of Fish rDNA with Digoxigenin Labelled pXlr101A *Xenopus laevis* rDNA Probe

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Southern Hybridization:

1. Prehybridize with 0.2 ml prehybrid buffer X cm^2 membrane at 68^0C for >3 hours.

2. Hybridize with .025 ml hybrid buffer X cm^2 membrane at 42^0C containing 10 ng/ml Dig-pXlr101A probe for >12 hours.

3. Wash 2 X 5 min at room temperature with 1 ml (2 X SSC + 0.1% SDS) X cm^2 membrane.

4. Wash 2 X 15 min at 68^0C with 1 ml (0.1 X SSC + 0.1% SDS) X cm^2 membrane.

Immunological Reaction:

1. Washed the nylon membrane with 1 ml Buffer 1 X cm^2 membrane for 5 minutes

2. Incubated in 1 ml Buffer 2 X cm^2 membrane for 30 minutes.

3. Incubated 30 minutes with 0.2 ml Buffer 2 containing «DIG»AP-Conjugate 1 µl/5 ml buffer X cm^2 membrane.

4. Wash 2 X 15 minutes with 1 ml Buffer 1 X cm^2 membrane.

Chemoluminescent Detection:

1. Equilibrated the membrane 2 minutes with 0.2 ml Buffer 3 X cm^2 membrane.

2. Put membrane in a acetate folder, added 10 µl/ cm^2 membrane Lumi-Phos 530; packed in a X-ray Exposure Holder.

3. Expose the folder to Kodak XAR X-ray film for about 2 hours.

4. Developed and fixed the film in GBX developer and fixer, respectively.

Composition of **Buffer 1**, **Buffer 2**, and **Buffer 3**, as described in Protocol III.

Table 8

Length of vertebrate rRNA or rDNA in terms of nucleotides

Organism	Length of rRNAs/rDNAs in bases				
	18S	28S	5.8S	Total	ITS
Toad (*Xenopus laevis*)[1]	1,925	4,471			
Chicken (*Gallus domesticus*)[1]	1,800	4,645			
Mouse (*Mus musculus*)[1]	1,950	4,712			
Mammals[1]			160	6,822[5]	
Salmonids (*Salmo, Salvelinus*)[2]				5,800[1,3]	1,500[1,4]

[1] rRNA, Lewin, 1994; [2] rDNA, Phillips *et al.*, 1989; [3] 5.8 Kb reported; [4] 1.5 Kb reported, probably including 5.8 S; [5] by adding mouse 18S and 28S rRNAs, and mammalian 5.8S rRNA; ITS, internal transcribed spacers; Kb, kilobases; Total includes 18S, 28S and 5.8S.

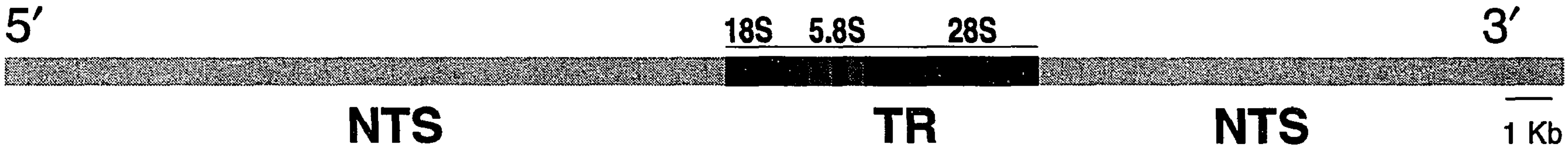


Figure 7. A model rDNA subunit with NTSs flanking at the 5′ and 3′ ends of the 18S and 28S rDNA regions, respectively, as used in fish rDNA mapping. The total length of the coding region (18S, 5.8S, and 28S rRNA genes; 5.8 Kb; shown in black) plus two internal transcribed spacers (ITS; on either side of 5.8S) is 6.8 Kb, as shown by a fine line above the wide line based on Popodi *et al.* (1985), and Phillips *et al.* (1989 and 1992). The positions of the ITSs and the coding regions are known to be 18S ITS 5.8S ITS 28S, in sequence, as shown in the model following Phillips *et al.* (1992). NTS, non-transcribed region; TR, transcribed region. The TR is known to extend beyond the 5′ and 3′ ends of the 18S and 28S rDNA regions, respectively (Phillips *et al.*, 1992).

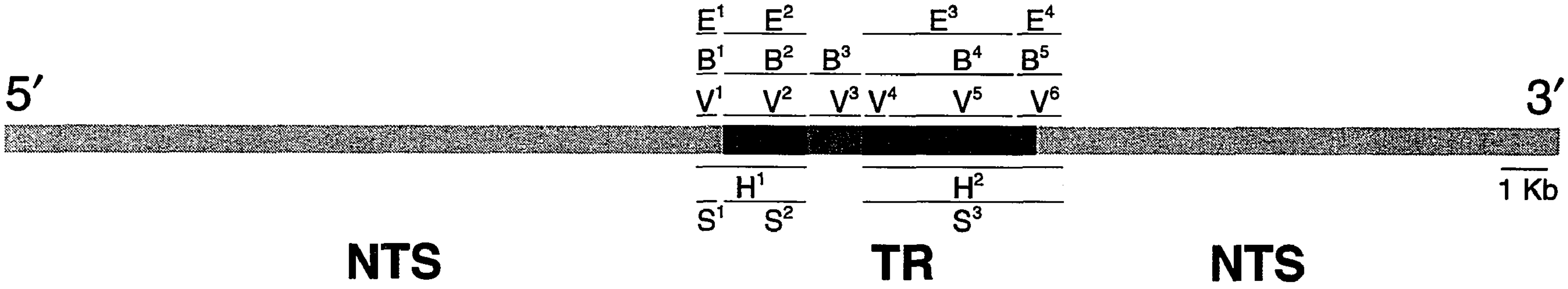


Figure 8. An rDNA, with flanking NTS at either sides, showing the specific regions of each restriction endonuclease recognition sites used in generating the character matrices. The relative positions of the 18S and 28S rRNA genes and ITS were shown before in Figure 7. ITS, Internal transcribed spacer; NTS, non-transcribed region; TR, transcribed region. The 5.8S rDNA is located within the ITS in this figure. The TR is known to extend beyond the 5′ and 3′ ends of the 18S and 28S rDNA regions, respectively (Phillips *et al.*, 1992). B, *Bam*H I; E, *Eco*R I; H, *Hin*d III; S, *Pst* I; V, *Pvu* II.

RESULTS

Q. General Information:

The results of the hybridization of RE fragmented *Acipenser fulvescens, Anguilla rostrata, Alosa sapidissima, Cyprinus carpio, Labeo rohita, Puntius gonionotus, Esox americanus americanus, Esox niger, Umbra pygmaea, Osmerus mordax, Coregonus clupeaformis, Salvelinus fontinalis, Salmo trutta, Oncorhynchus mykiss, Morone saxatilis, Oreochromis aureus,* and *Perca flavescens* rDNA with *Xenopus laevis* rDNA probe, the fragment sizes, and the rDNA maps are presented in Figures 9-94, and Tables 9-25. The fragment sizes were determined as detailed in the Materials and Methods, followed by mapping. After aligning the maps for homologous RE recognition sites within the conserved rDNA segments, a rDNA molecular character matrix was generated and presented in Table 26. The rDNA phylogenetic tree constructed by analyzing the rDNA character matrix using *Hennig86* is presented in Figure 95. Further, a hypothetical cladogram of the euteleosts based on published morphological information is provided in Figure 96. In addition, parsimonious trees based on morphology alone and in combination with rDNA RE characters are presented in Figures 97-100.

Under the Header, "Species Specific Information", additional information in map construction is provided, as needed. All the completely digested rDNA fragments generated are represented in the map. Most of the fragments became automatically aligned in the rDNA map constructed for each fish. However, in a few cases, one or a few rDNA fragments were needed to be cross-examined with the other rDNA fragments of the same sample in order to confirm their relative positions in the map.

The rDNA probe hybridized intensely with the fish rDNA fragments, as recorded by X-ray film. The detection procedure applied eliminated almost all non-specific noise. The intensity of hybridization appeared to be related to the presence of complementarity of the sequences rather than to the length of the rDNA fragments. There is some evidence of infrequent RE star activity and partial digestion which were easily detected and eliminated.

The rDNA phylogeny of the major groups of experimental fishes generated by *Hennig86* option *hennig* with length 43, consistency index 44, and retention index 55, is comparable to the morphological phylogeny. The most parsimonious trees based on morphology alone and in combination with the rDNA RE characters are presented and described later (Figure 97-100).

R. Species Specific Information:

R.1. *Acipenser fulvescens*, Lake sturgeon:

The results of lake sturgeon rDNA hybridization are presented in Figures 9-12, and Table 9. The respective sizes of all the rDNA fragments generated by the RE were measured in terms of nucleotide base pair lengths and aligned to construct a lake sturgeon rDNA map as presented in Figure 13. Most of the RE rDNA fragments hybridized strongly with the *Xenopus laevis* rDNA probe. Some rDNA fragments generated stronger bands than others as a reflection of sequence complementarity with the probe rDNA. The sizes of all the rDNA fragments thus generated were determined by comparison with the standards as described in the Materials and Methods. All these fragments were aligned according to their positions in the map. A few rDNA fragments were found to be partially digested and confirmed by cross-examination. The partially digested rDNA fragments were not directly included in the map, although they are represented by their respective counterpart fragments.

R.2. *Anguilla rostrata,* American eel:

The results of American eel rDNA hybridization are presented in Figure 14-17. The respective sizes of all the rDNA fragments generated by the RE were measured in terms of nucleotide base pair lengths following the procedures detailed in the Materials and Methods, and presented in Table 10. All the rDNA fragments were compared for matching and thus the American eel rDNA map was constructed as shown in Figure 18. Most of the rDNA fragments generated by the RE hybridized strongly with the *Xenopus laevis* rDNA probe. Some rDNA fragments generated stronger bands than others as a reflection of complementarity with the probe rDNA. All the completely digested fragments were aligned and placed according to their respective positions in the map. One rDNA fragment was found to be partially digested and confirmed by cross-examination. The partially digested rDNA fragment is not directly included in the map, although it is represented by its counterpart fragments.

All the rDNA fragments generated by digestion with the RE aligned well to generate the map with only two exceptions (Figure 18). (1) In comparison to the rDNA fragments generated by all the RE, alone or in combinations (Table 10; Figures 14-18), the eel rDNA digested in combination with *Bam*H I and *Pvu* II was expected to generate a 2.4 Kb fragment which was missing. On the contrary, an additional 1.1 Kb fragment was observed. It is possible that due to the star activity of either of the RE, the expected 2.4 Kb fragment became further fragmented into 1.1 and 1.3 Kb in length and comigrated. This was suggested by the spread of the 1.1 Kb hybrid band. (2) Similar to above, *Bam*H I and *Pst* I together was expected to generate a 3.5 Kb rDNA fragment which was also missing. It is possible that due to the star activity of these RE, this fragment was further fragmented into two fragments, and one fragment might have co-migrated with the 2.4 Kb fragment, and the other was not recognized by the probe since no additional small size fragment was observed.

R.3. *Alosa sapidissima*, American shad:

The results of hybridization of RE fragments of American shad rDNA with the probe rDNA are shown in Figures 19-22. Most of the shad rDNA fragments hybridized strongly with the *Xenopus laevis* rDNA probe as observed by the photographic recordings in the X-ray film (Figures 19-22). The sizes of these fragments were measured in comparison to standard DNA fragments of known molecular sizes according to the procedures mentioned in the Materials and Methods. The sizes of these rDNA fragments are listed in 11. While most of the fragments were completely digested, three of them were partial digests. The completely digested fragments were aligned to construct the American shad rDNA map and presented in Figure 23. All the completely digested fragments aligned steadily in generating the map.

A small rDNA fragment of about 0.5 Kb generated by *Bam*H I was not always detectable. An extremely light hybrid band of about 0.5 Kb DNA fragment was observed when the shad rDNA was digested with *Bam*H I in combination with either *Pst* I or *Pvu* II. Further, when the shad rDNA was digested with *Bam*H I and *Eco*R I in combination, the 4.6 Kb *Eco*R I rDNA fragment was split into two detectable fragments of approximately 1.7 and 2.5 Kb in length and in total they fall short of the original by about 0.4 Kb. This missing fragment of approximately 0.4 Kb in size was additionally generated by *Bam*H I which was not detected under the hybridization conditions. Inclusion of this fragment made a perfect alignment of the shad rDNA RE map presented in Figure 23.

R.4. *Cyprinus carpio*, Common carp:

Most of the carp rDNA RE fragments strongly hybridized with the *Xenopus laevis* rDNA probe as presented in Figure 24-27. The respective sizes of all the rDNA fragments generated were measured and are presented in Table 12. All the rDNA fragments were incorporated in the rDNA map and presented in Figure 28.

The RE, *Hin*d III, *Bam*H I, *Pvu* II and *Pst* I were found to have variable cut sites in the carp rDNA. However, the rDNA fragments generated by the RE combinations, *Eco*R I + *Hin*d III, *Eco*R I + *Bam*H I, *Eco*R I + *Pvu* II, *Eco*R I + *Pst* I, and *Eco*R I + *Pvu* II + *Pst* I were found to be precise. Based on these fragments, a basic map was constructed and the rest of the fragments were subsequently aligned. In some instances, the specificity of the RE probably became relaxed since star activity was observed. Fragments of rDNA thus generated due to star activity of an RE did not match with other corresponding fragments, and hence, were omitted. However, this did not affect the construction of the map since all RE fragments of the rDNA were obtained through the other combinations of RE digestions.

R.5. *Labeo rohita*, Rohu:

The results of hybridization of rohu rDNA RE fragments with the probe rDNA are shown in Figures 29-32. Most of the rohu rDNA fragments hybridized strongly with the *Xenopus laevis* rDNA probe. The sizes of these fragments were measured in comparison to standard DNA fragments of known molecular sizes according to the procedures mentioned in the Materials and Methods and shown in Table 13. These rDNA fragments were aligned to construct the rohu rDNA map and are presented in Figure 33.

The digestion of the rohu rDNA with the RE was complete and the alignment of the fragments in order to generate the map was reproducible (Figure 29-33; Table 13). The *Bam*H I 28S rDNA 0.5 Kb fragment was detectable as a very light band when the DNA was digested with *Bam*H I alone. At other times, this band was not detectable under the hybridization conditions. However, presence of this fragment was further confirmed when *Bam*H I and *Eco*R I together digested the rDNA refragmenting the 5.0 Kb *Eco*R I rDNA fragment into 1.7 and 2.7 Kb fragments with a shortage of a 0.6 Kb fragment, approximately. This missing fragment must be the 0.5 Kb *Bam*H I 28S rDNA fragment, considering a minor variation in measurements. Incorporation of this fragment in generating the rohu rDNA map made a perfect alignment of all the rDNA fragments.

R.6. *Puntius gonionotus*, Thailandian sarpunti:

The sarpunti rDNA fragments generated by the RE are shown in Figures 34-37. Most of the sarpunti rDNA fragments hybridized strongly with the *Xenopus laevis* probe rDNA. The sizes of all these fragments were determined in comparison to standard DNA fragments of known molecular sizes and presented in Table 14. A sarpunti rDNA map constructed by aligning these rDNA fragments are shown in Figure 38. All the rDNA fragments became completely aligned in the map.

An rDNA fragment containing a part of the ITS and the 5′ end of the 28S generated by *Bam*H I and *Pvu* II together, was not found to hybridize under the experimental conditions. However, presence of this rDNA fragment is supported by other rDNA fragments covering this area. This probably relates to the high degree of diversity between the probe and the rDNA of this region of the sarpunti. Inclusion of RE recognition sites of this fragment made a complete alignment of all the sarpunti rDNA fragments in the map (Figure 38).

R.7. *Esox americanus americanus*, Redfin pickerel:

Most of the redfin pickerel rDNA fragments hybridized strongly with the *Xenopus laevis* rDNA probe as shown in Figures 39-42. The sizes of all these fragments were determined based on standard DNA fragments of known molecular sizes and presented in Table 15. While most of the fragments were completely digested, four fragments remained as partial digests. All the completely digested rDNA fragments were aligned and incorporated to construct the redfin pickerel rDNA RE map (Figure 43).

Only a single rDNA fragment was generated by each of the three RE, *Hin*d III, *Pst* I, and *Pvu* II of about 26.0, 19.0 and 17.9 Kb in length, respectively, that hybridized with the probe under the experimental conditions. On the other hand, *Bam*H I and *Eco*R I generated three hybridizable rDNA fragments each. Further, simultaneous digestion of the DNA with *Hin*d III and *Pst* I generated one 17.9 Kb fragment that hybridized with the probe. Since the *Pst* I cut site was found to be within the TR, *Hin*d III and *Pst* I together are expected to generate two fragments hybridizable with the probe. It is possible that both the fragments are of equal sizes and as a result only one hybrid band was obtained.

R.8. *Esox niger*, Chain pickerel:

Most of the chain pickerel rDNA RE fragments hybridized strongly with the *Xenopus laevis* rDNA probe as shown in Figure 44-47. The respective sizes of each rDNA fragment is determined as described in the Materials and Methods and are presented in Table 16. While most of the rDNA fragments were completely digested, only five of them were partial digests. All the completely digested rDNA fragments were aligned and incorporated in constructing the chain pickerel rDNA map (Figure 48).

There was striking similarities between the numbers and sizes of rDNA fragments of the redfin and chain pickerels generated by five RE singly or in combinations as shown in the rDNA map (Figures 44-48; Table 16). However, in contrast to redfin pickerel rDNA, *Hin*d III and *Pvu* II individually generated chain pickerel rDNA fragments of varying lengths that was reflected by smeared rDNA hybrid bands. Again, a large *Eco*R I chain pickerel rDNA fragment including a part of the NTS and most of the 18S appeared to be 14 Kb in length. Hence, this piece was found to be approximately 3 Kb smaller than the corresponding rDNA fragment of redfin pickerel. This is possible if (i) there was a deletion of a part of the rDNA NTS in chain pickerel, (ii) there was an insertion of a DNA fragment in the rDNA NTS of the redfin pickerel, or (iii) the mobility of the fragment in the gel was affected by bound RE or other factors.

In mapping, rDNA fragment lengths were aligned by cross-examination. As an example, it was determined that the *Eco*R I generated a chain pickerel 3.5 Kb rDNA fragment composed of a part of the 18S which continued until the middle of 28S. The length matches with the same rDNA fragment of redfin pickerel. When the same fragment was digested with *Bam*H I, it generated two fragments determined to be 1.8 and 2.4 Kb in lengths. However, the length of the 2.4 Kb rDNA fragment as above was adjusted to 2.3 Kb, and thus, the total length of the two fragments became 3.5 Kb matching to the size of the original fragment (Table 16). Following similar procedures a few other fragments were also aligned during mapping.

R.9. *Umbra pygmaea*, Eastern mudminnow:

Results of hybridization of the Eastern mudminnow rDNA fragments with *Xenopus laevis* rDNA probe, their sizes, and the rDNA map are presented in Figures 49-53, and Table 17. The probe rDNA strongly hybridized with most of the Eastern mudminnow rDNA. The sizes of all the rDNA fragments were determined as described in the Materials and Methods. All these fragments were completely digested and were aligned and incorporated in constructing the map.

In generating the map, three fragments were found to be the product of star activity, one fragment was found to be variable, and the mobility of three fragments were found to be affected (Table 17). However, they were cross-examined with other corresponding rDNA fragments generated by separate RE combinations which confirmed their alignments.

R.10. *Osmerus mordax*, Rainbow smelt:

The rainbow smelt rDNA fragments generated by all the RE, singly or in combination, fragment sizes and the rDNA map are presented in Figures 54-58, and Table 18. Most of the smelt rDNA hybridized strongly with the *Xenopus laevis* rDNA probe. All of the rDNA fragments obtained were found to be full digests, with one exception. All the completely digested rDNA fragments were aligned and represented in the rDNA map.

The smelt rDNA digested with *Eco*R I generated one fragment of 4.6 Kb and another fragment of ≥ 9.4 Kb in length. In comparison to other rDNA fragments generated by other RE alone and in combination with *Eco*R I, it was determined that *Eco*R I alone generated three rDNA fragments of 4.6, 9.4 and 9.9 Kb in length, as shown in the map (Figure 58), while the 9.4 and 9.9 Kb fragments migrated very closely. Similarly, based on critical evaluation and comparison, all the rDNA fragments were aligned and included in the map.

R.11. *Coregonus clupeaformis*, Lake whitefish:

Most of the lake whitefish rDNA RE fragments hybridized strongly with the *Xenopus laevis* rDNA probe (Figure 59-62). Sizes of all the rDNA fragments were determined and are presented in Table 19. All the fragments are fully digested except four partially digested fragments. All the completely digested fragments were aligned and incorporated in the rDNA map, while the partial digests are represented by corresponding fragments (Figure 63).

One of the two *Hin*d III sites was found to be variable. As a result, this RE fragment did not generate a sharp rDNA band. However, when whitefish DNA was digested with *Hin*d III in combination with other RE, sharp bands were obtained. Had both the *Hin*d III sites been variable, there would be a possibility to have smeared bands when used in combination with other RE also. However, all the fragments were matched and incorporated in the map, except the partial digests.

R.12. *Oncorhynchus mykiss*, Rainbow trout:

Most of the rainbow trout rDNA hybridized strongly with the *Xenopus laevis* rDNA probe (Figures 64-67). The sizes of all the rDNA fragments generated were determined and presented in Table 20. Most of the fragments were found to be completely digested while three were partial digests. All the completely digested fragments were aligned to generate the rDNA map (Figure 68). Some fragments showed less intense hybrid bands as a reflection of their reduced sequence complementarity with the probe rDNA. In particular, fragments containing the ITS or 3′ end of 28S rDNA, hybridized less strongly with the rDNA probe probably as a reflection of less complementarity with the probe rDNA.

R.13. *Salmo trutta,* Brown trout:

Most of the brown trout rDNA RE fragments hybridized strongly with the *Xenopus laevis* rDNA probe (Figure 69-72). The sizes of all the fragments were determined and are presented in Table 21. While most of the fragments were completely digested, a few were partial digests. All the completely digested fragments were aligned to match their respective positions, and thus, a brown trout rDNA RE map was constructed (Figure 73).

The RE, *Bam*H I, has a alternate site at the 5′ end of 18S rDNA. On the other hand, *Eco*R I has an additional site at the 3′ flanking region of 28S rDNA. Another rDNA fragment generated by *Bam*H I and *Pst* I containing part of the 18S along with ITS, hybridized weakly with the *Xenopus laevis* rDNA probe.

R.14. *Salvelinus fontinalis,* Brook trout:

Most of the brook trout rDNA RE fragments hybridized strongly with the *Xenopus laevis* rDNA Figures 74-77. The sizes of these rDNA fragments were determined against standard DNA of known molecular sizes and are presented in Table 22. Most of the fragments were completely digested while a few were partial digests. All the completely digested fragments were aligned to match their respective positions for the construction of brook trout rDNA map (Figure 78).

R.15. *Morone saxatilis,* Striped bass:

Most of the striped bass rDNA RE fragments hybridized strongly with the

Xenopus laevis rDNA probe (Figures 79-82). The sizes of all the rDNA fragments were determined in comparison to standard DNAs of known molecular sizes and are presented in Table 23. All the rDNA fragments were completely digested and aligned to their respective positions to construct the striped bass rDNA map as shown in Figure 83.

A 0.6 Kb *Bam*H I striped bass rDNA fragment hybrid with the probe was observed as a faint band only in one lane but not in others where *Bam*H I was one of the RE in combination with others. Further, the other RE applied in addition to *Bam*H I was not expected to digest that fragment as confirmed from other sets of digestions. This may be due to less complementarity of this fragment with the probe rDNA. Similarly, in other fishes in this study, the corresponding fragment also hybridized poorly with the probe DNA. Further, incorporation of this fragment in the map perfectly aligned the rest of the fragments in their respective positions. Further, there was an 1.0 Kb rDNA fragment generated by the combination of *Eco*R I and *Pst* I in the digest that produced a light band upon hybridization. This fragment has no matching space within the TR. Hence, it is possible that it was generated by the star activity of both or either of these RE. It is also possible that some of the 0.6 Kb fragments had bound RE proteins that slowed their migration in the gel. The later seems to be more logical since no additional rDNA hybrid fragment was detected.

R.16. *Oreochromis aureus*, White tilapia:

Most of the tilapia rDNA RE fragments hybridized strongly with the *Xenopus laevis* rDNA probe as shown in Figures 84-87. As with other samples, the sizes of all the rDNA fragments were determined in comparison to standard DNA of known molecular sizes (Table 24). Although most of the fragments were completely digested, a few were still found to be partial digests. All the completely digested rDNA fragments were aligned in order to generate the tilapia rDNA map as shown in Figure 88.

The RE, *Hin*d III, has generated a single rDNA fragment of 9.6 Kb in length. Digestion with *Eco*R I has generated five fragments of rDNA: fully digested 2.3, 2.85 and 4.0 Kb fragments and partially digested 6.2 and 7.0 Kb fragments, in length. This was determined by comparing all the fragments generated by each RE and constructing a map (Figure 88). When *Hin*d III and *Bam*H I digested tilapia DNA in combination, a star activity was observed. Identification of the specific RE responsible for the star activity was beyond the scope of this research protocol. However, George and Chirikjian (1982) and Nasri and Thomas (1986) have demonstrated star activity of *Bam*H I and *Hin*d III, respectively, under suboptimal reaction conditions. Since some digestions were conducted using a common buffer, as suggested by the supplier, there was a potential for mild star activity for either of the RE.

R.17. *Perca flavescens*, Yellow perch:

Most of the yellow perch rDNA RE fragments hybridized strongly with the *Xenopus laevis* rDNA probe (Figure 89-93). Since, under certain hybridization conditions the hybrid resolution was less clear, the hybridization was repeated with different RE combinations in order to compare between experiments (Figure 93). Combination of the two hybridization results resolved the number of rDNA fragments generated by these RE alone and in combinations. All the fragments were determined for their sizes as presented in Table 25, using standard DNA fragments of known molecular size. All the fragments were completely digested and aligned to generate the yellow perch rDNA map as shown in Figure 94.

A 1.5 Kb rDNA fragment was generated by digesting the yellow perch DNA with *Hin*d III and *Pst* I together, while lengths of the corresponding other rDNA fragments support it to be 1.1 Kb. It is possible that the restriction endonuclease bound to it significantly delayed its mobility and thus represented it as a larger size DNA.

S. The Molecular Character Matrix of the rDNA:

The molecular character matrix of the rDNA of the 17 actinopterygii taxa are presented in Table 26. The specific regions of rDNA related to each of the RE character sites were presented before in Figure 8. Since, a universal rDNA model was used to map the RE recognition sites for all the fishes, they required some adjustments to match their homologous sites in other taxa. The *Bam*H I recognition sites (1) in the 5′ ETS rDNA in *Coregonus clupeaformis,* (2) and outside the 5′ ETS rDNA in *Morone saxatilis,* were adjusted to be homologous to the *Bam*H I sites (1) in the adjacent area of the 18S, and (2) 5′ ETS rDNA, respectively in the respective fish. Similarly, the *Hin*d III site at the (1) 3′ ETS rDNA of *Salvelinus fontinalis,* and (2) 5′ NTS rDNA of *Oreochromis aureus* were adjusted to be homologous to the *Hin*d III sites in the adjacent area of the (1) 3′ NTS and (2) 5′ ETS rDNA, respectively, in respective fish.

The rDNA RE characters B^2, E^2, and S^2, are synapomorphic to protacanthopterygians, actinopterygians, and clupeocephalids, respectively. Another rDNA RE character, V^5 is commonly occurring in umbrid-osmerid-salmonid-acanthopterygian lineage, while H^2 is partially synapomorphic for the osmerus-salmonids-acanthopterygian lineage. The acanthopterygians have common several rDNA RE sites, for example at B^2 and E^4, with homoplasy.

T. rDNA Phylogeny:

The molecular character matrix of the rDNA of the 17 actinopterygians studied were analyzed using *Hennig86* options *hennig* and *maximumhennig (mhennig).* Both the options generated a single tree but the tree generated by the option *hennig* was found to be more resolved and consistent with the already established relationships of the major groups of actinopterygians (Nelson, 1989). The length of the tree is 43, consistency index is 44 and retention index is 55 which is not significantly different from the trees generated by other options.

Since, a single tree was generated by option *hennig* no consensus tree was needed.

In this analysis, *Acipenser* has been selected as the outgroup by the analytical program *Hennig86*, by default, and therefore *Anguilla* and *Alosa* became two additional euteleost outgroups as determined by the analysis. In addition, the Clupeocephala, Euteleostei, Neognathi, *Salmo-Salvelinus*, and Acanthopterygii have been found to be monophyletic, while the Ostariophysi, Protacanthopterygii, Esocoidei, and Salmonidae have not been found to be monophyletic.

U. Phylogeny of the Actinopterygians in this Study Based on Morphology:

U.1. A Hypothetical Cladogram:

A hypothetical cladogram of the actinopterygians included in this study was reconstructed based on known morphological relationships and presented in Fig 102. These relationships are not based on any character matrix, and hence, are not analyzed with parsimony analysis. The relationships of the major groups are based on Lauder and Liem (1983) and Nelson (1989) in addition to others as listed in the Materials and Methods. The intra-relationships of the (1) cyprinids, and (2) salmonids are supported by (1) Howes (1991) and Cavender and Coburn (1992), and (2) Rosen (1974), Phillips and Pleyte (1991), Wilson and Williams (1992), Wilson *et al.*, 1992, and Nelson, (1994), respectively. The interrelationships of the perciform acanthopterygians are based on Lauder and Liem (1983) and Bart and Page (1992).

U.2. Morphological Phylogeny Based on Parsimony:

All the apomorphic morphological characters reported by the specific authors supporting the above hypothetical cladogram (Figure 96) were initially listed and examined carefully to eliminate autoapomorphic characters. Thus a list of 21 apomorphic morphological characters was generated as presented in Appendix 1, based on which a morphological character matrix was created (Table 27). The character matrix was analyzed using all options available in *Hennig86,* parsimony analysis generating many trees. The option *mhennig* generated three equally most parsimonious trees (Figure 97), one of which (tree 2) was more resolved and closer to the current view of the relationships of the representative actinopterygians used in this study (Figure 96; Lauder and Liem, 1983; Wilson, 1984; Nelson, 1989; Cavender and Coburn, 1992; Nelson, 1994). All the three trees generated above were combined by the command *nelsen* from *Hennig86* to generate a Nelsen consensus tree as presented in Figure 98.

In this analysis, the *Acipenser* has been selected as the outgroup by default, and the *Anguilla* and *Alosa* have been found to be two additional euteleost outgroups. The Nelsen consensus tree supported the Clupeocephala, Ostariophysi, Neognathi, Esocidae, Salmonidae, and Acanthopterygii to be monophyletic, while the Euteleostei, Protacanthopterygii, and Esocoidei were not found to be monophyletic. Opposite to the findings in Nelsen consensus tree, two of the three most parsimonious trees supported the Clupeocephala, Euteleostei, Ostariophysi, Neognathi, Esocidae, Salmonidae and Acanthopterygii to be monophyletic, while the Protacanthopterygii, and Esocoidei were not found to be monophyletic.

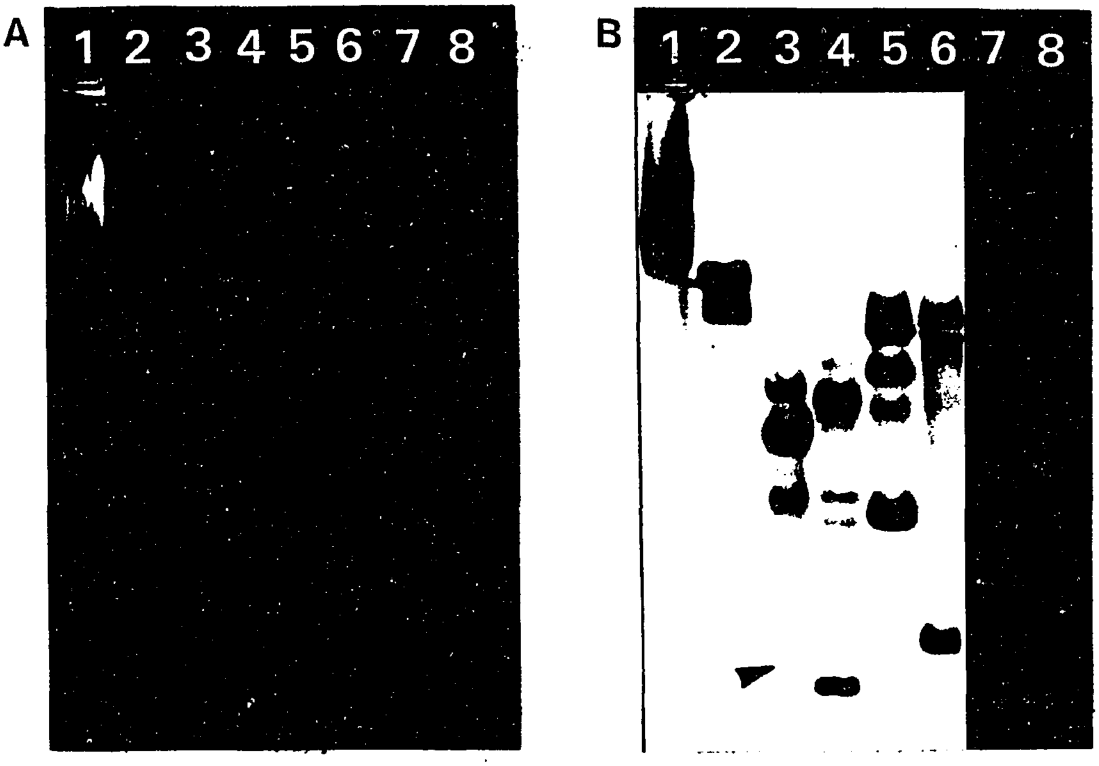
U.3. Phylogeny based on rDNA RE Characters and Morphology:

The RE and morphological character matrices were combined together and analyzed with all options available in *Hennig86*. The trees generated by option *mhennig* are more resolved and comparable to some of established relationships as shown by Nelson (1989). The option generated four trees which were further analyzed using option *nelsen* of *Hennig86* that generated a consensus trees with length 68 ci 60 ri 73, as presented in Figure 99, and 100.

In this analysis, the *Acipenser* has been selected as the outgroup by default, and the *Anguilla* and *Alosa* have been found to be two additional euteleost outgroups. The Nelsen consensus tree based on the rDNA RE and morphological characters supported the Clupeocephala, Euteleostei, Ostariophysi, Neognathi, Esocidae, *Salmo-salvelinus*, and Acanthopterygii to be monophyletic, while the Protacanthopterygii, Esocoidei, and Salmonidae not to be monophyletic. On the other hand, all four most parsimonious trees supported the monophyly of Clupeocephala, Euteleostei, Ostariophysi, Neognathi, Esocidae, and Acanthopterygii, while three of the four parsimonious trees supported the monophyly of Salmonidae, and none supported the Protacanthopterygii, and Esocoidei to be monophyletic.

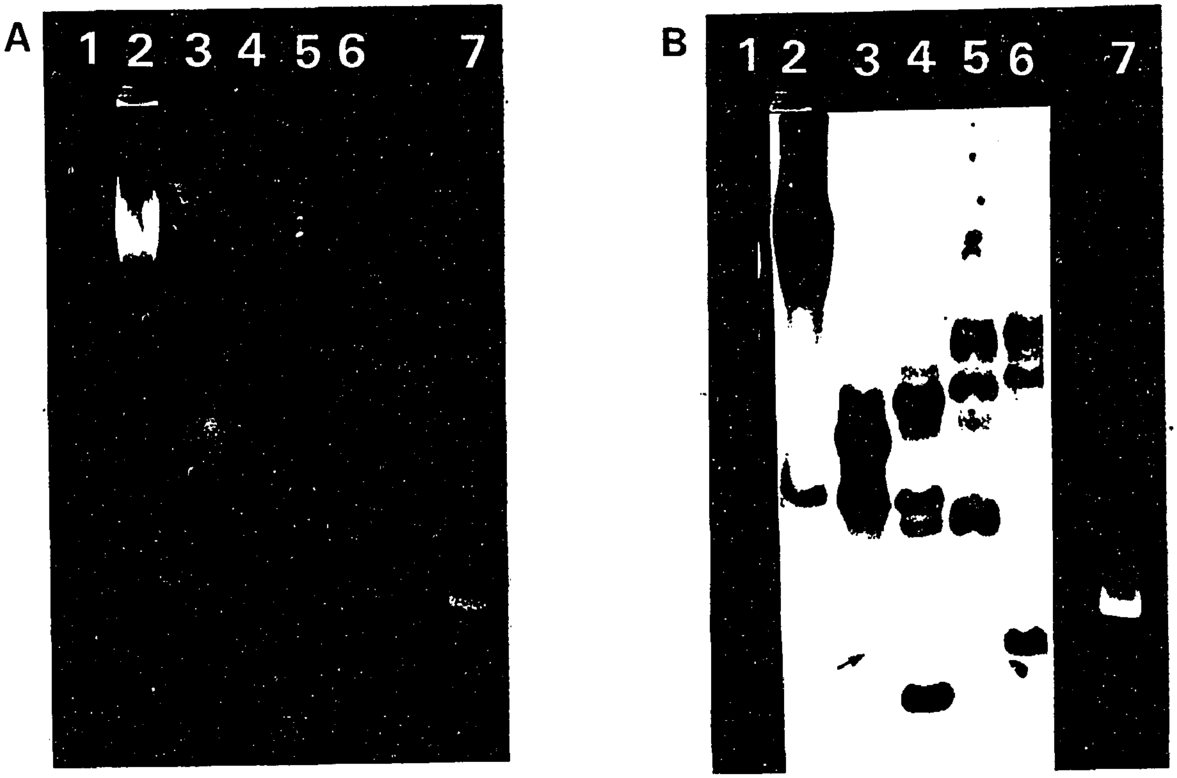
Acipenser fulvescens

Lake sturgeon



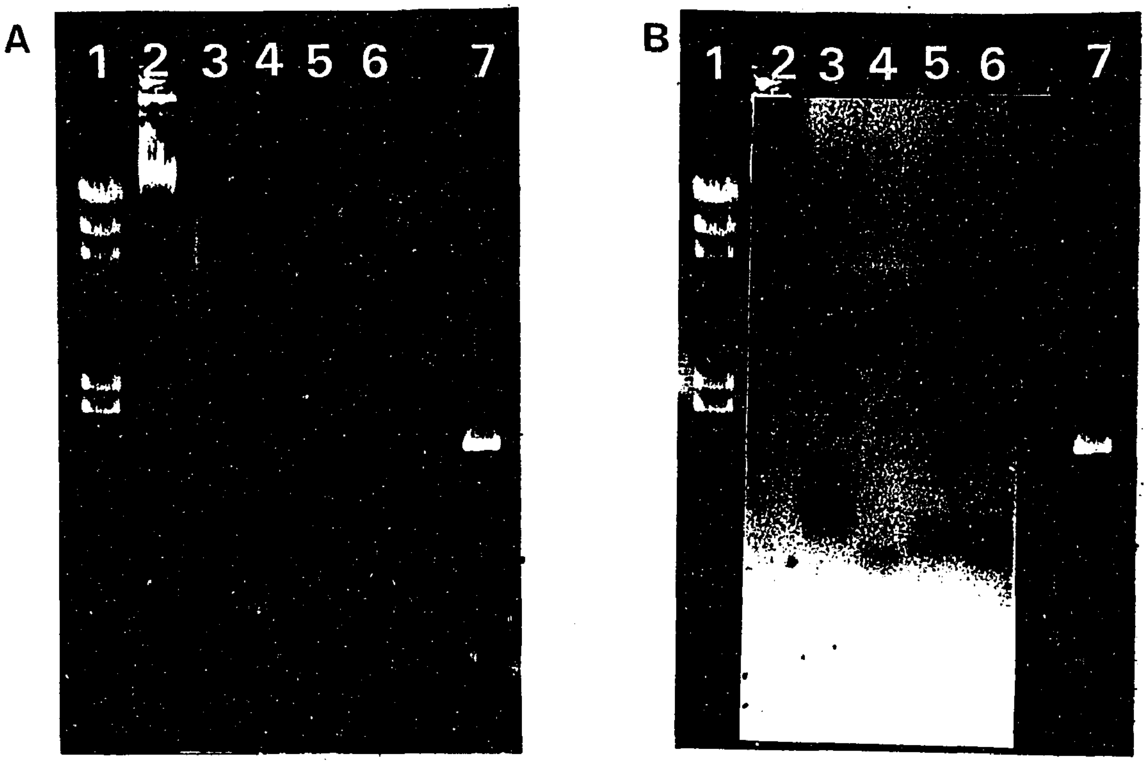
***Acipenser fulvescens*, lake sturgeon, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 9. *Acipenser fulvescens*, lake sturgeon, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 1-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, undigested; 2, *Hin*d III digest; 3, *Eco*R I digest; 4, *Bam*H I digest; 5, *Pvu* II digest; 6, *Pst* I digest; 7, λ DNA/*Hin*d III Fragments; 8, 1 Kb DNA Ladder. Thick arrow head points to a light hybrid band. Both A and B plates represent their actual sizes.



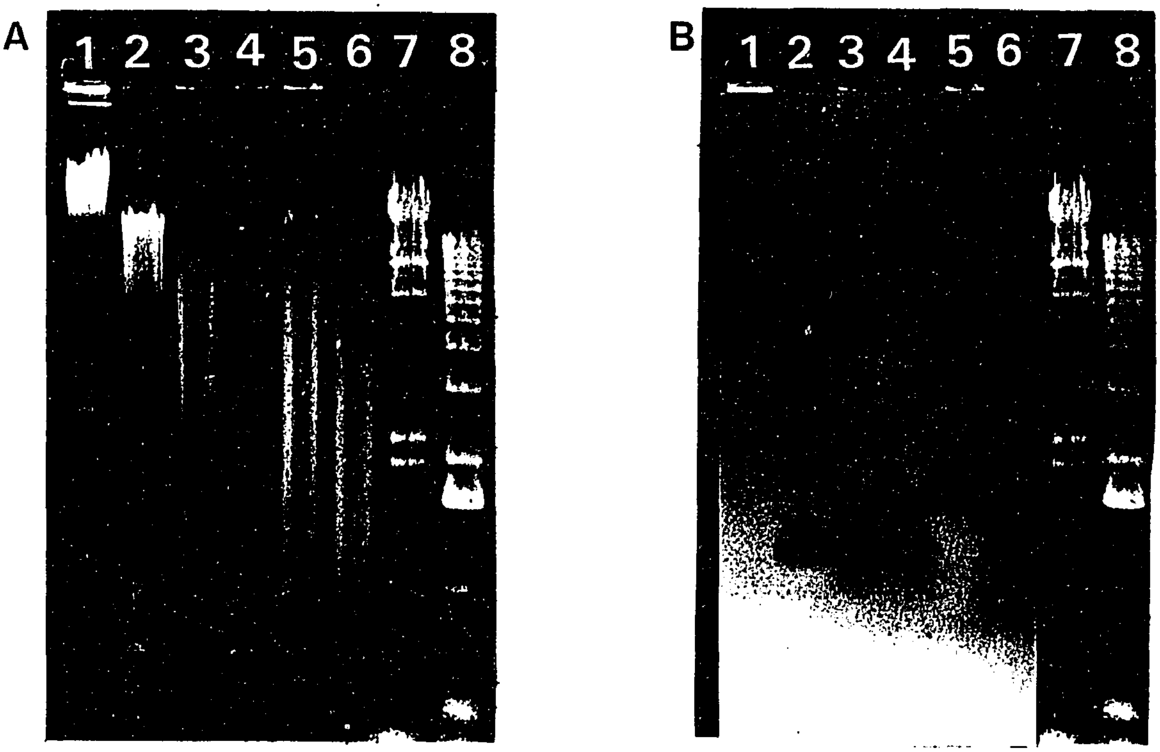
***Acipenser fulvescens*, lake sturgeon, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 10. *Acipenser fulvescens*, lake sturgeon, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Hin*d III + *Eco*R I digest; 4, *Hin*d III + *Bam*H I digest; 5, *Hin*d III + *Pvu* II digest; 6, *Hin*d III + *Pst* I digest; 7, 1 Kb DNA Ladder. Thin arrow points to a light hybrid band. Both A and B plates represent their actual sizes.



***Acipenser fulvescens*, lake sturgeon, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 11. *Acipenser fulvescens*, lake sturgeon, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Eco*R I + *Bam*H I digest; 4, *Eco*R I + *Pvu* II digest; 5, *Eco*R I + *Pst* I digest; 6, *Eco*R I + *Pvu* II + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes. Small arrow head points to a light hybrid band.



***Acipenser fulvescens*, lake sturgeon, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 12. *Acipenser fulvescens*, lake sturgeon, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, undigested; 2, *Bam*H I digest; 3, *Bam*H I + *Pvu* II digest; 4, *Bam*H I + *Pst* I digest; 5, *Pvu* II + *Pst* I digest; 6, *Bam*H I + *Pvu* II + *Pst* I digest; 7, λ DNA/*Hin*d III Fragments; 8, 1 Kb DNA Ladder. Both A and B plates represent their actual size. Small arrow heads point to light hybrid bands.

Table 9

Acipenser fulvescens, Lake sturgeon, rDNA fragments generated by restriction endonucleases

Restriction Endonuclease	Fragment sizes in kilobases
Hind III	9.8; (16)
*Eco*R I	1.2; 2.9; (3.6); 4.4; (5.6)
*Bam*H I	1.0; 2.6; 3.2; 5.0; (6.5)
Pvu II	2.7; (4.5); 6.0; (8.9)
Pst I	1.4; (6.5); 7.6
Hind III + *Eco*R I	1.2; 2.9; 4.4
Hind III + *Bam*H I	1.0; 2.6; 3.2; 5.0; (7.0)
Hind III + *Pvu* II	2.7; 6.0; (8.5)
Hind III + *Pst* I	1.3; (2.9); 7.6; (9.0)
*Eco*R I + *Bam*H I	1.1; 1.2; 2.4; (3.9)
*Eco*R I + *Pvu* II	0.9; 3.0
*Eco*R I + *Pst* I	1.0; 2.9; 4.4
*Eco*R I + *Pvu* II + *Pst* I	1.0; 3.0
[*]*Bam*H I + *Pvu* II	1.0; 1.2; 3.2; (5.0)
[*]*Bam*H I + *Pst* I	1.0; 1.2; (1.5); 2.5; 3.2
[*]*Pvu* II + *Pst* I	1.6; (3.0); (5.0); 6.2
[*]*Bam*H I + *Pvu* II + *Pst* I	1.2; 1.6; 3.0, (6.0)

In some cases minor adjustments of up to 200 bases in length were made in mapping. [*]The mobility of the digested rDNA fragments was slower than the marker DNA, since, restriction endonuclease protein remained bound to the DNA after digestion. Fragment lengths shown in parenthesis are partial digests.

RESTRICTION ENZYME MAP OF LAKE STURGEON, *ACIPENSER FULVESCENS*, RIBOSOMAL DNA

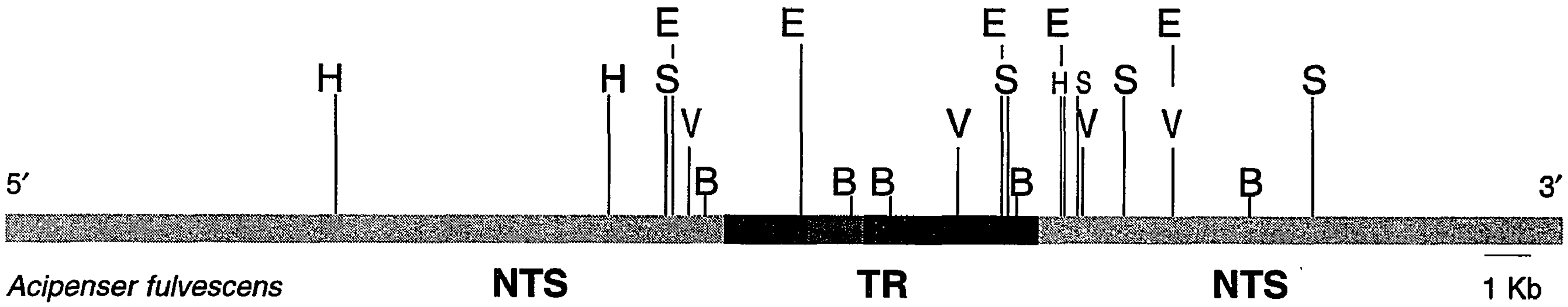
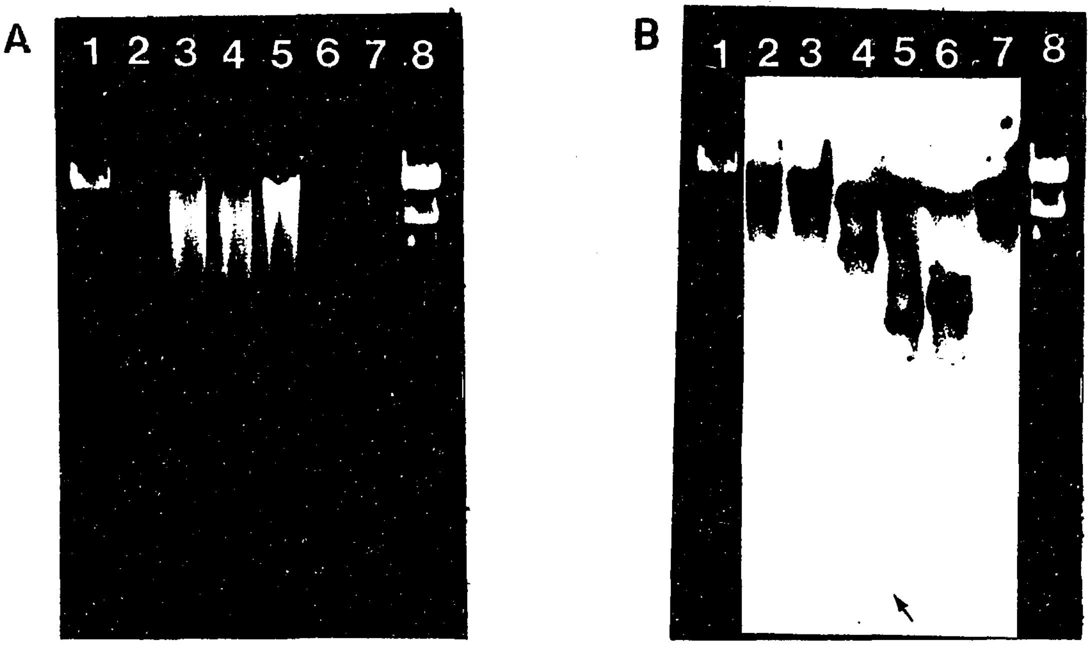


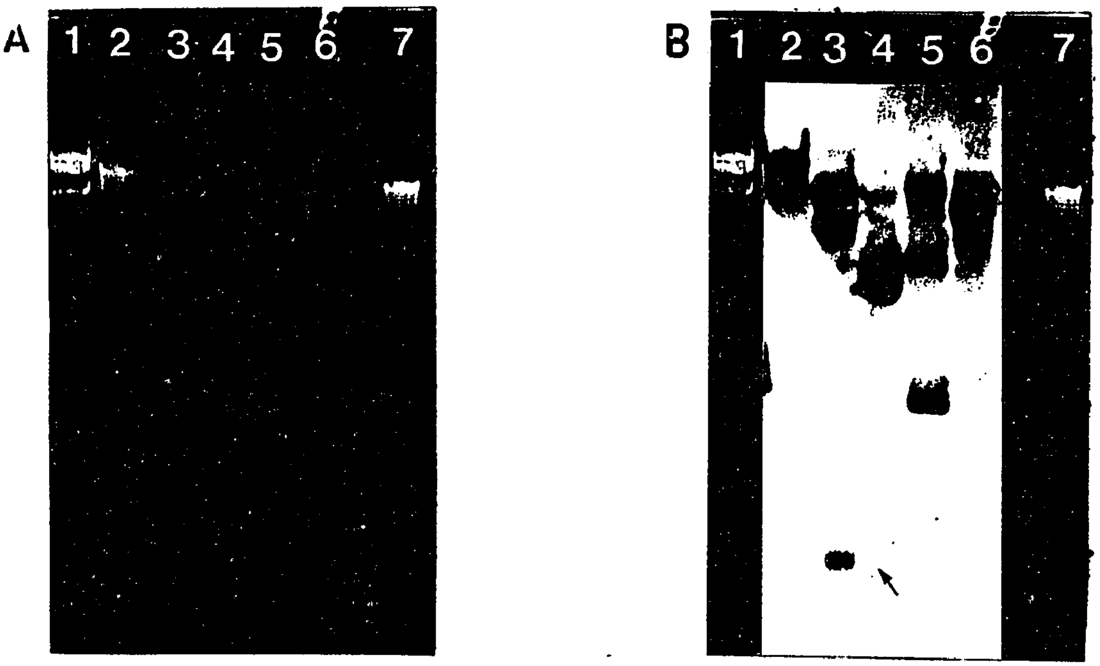
Figure 13. Proposed restriction enzyme map of lake sturgeon ribosomal DNA. B, *Bam*H I; E, *Eco*R I; H, *Hind* III; S, *Pst* I; V, *Pvu* II; NTS, nontranscribed spacer; TR, transcribed region. Relative positions of the respective restriction enzymes are shown in the map in approximate scale.

Anguilla rostrata
American eel



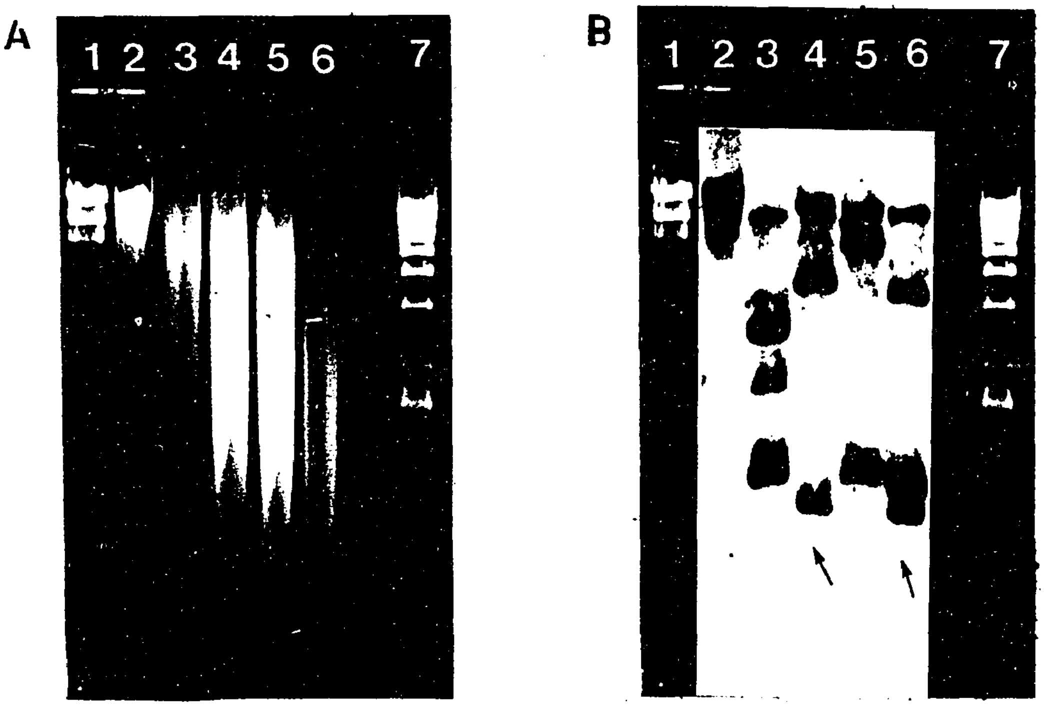
Anguilla rostrata, American eel, nrDNA hybridized with *Xenopus laevis* nrDNA

Figure 14. *Anguilla rostrata*, American eel, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-7) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, High Molecular Weight DNA Markers, BRL; 2, undigested; 3, *Hin*d III digest; 4, *Eco*R I digest; 5, *Bam*H I digest; 6, *Pvu* II digest; 7, *Pst* I digest; 8, λ DNA/*Hin*d III Fragments. Both A and B plates are reduced to 86% of their actual size. Thin arrow points to a light hybrid band.



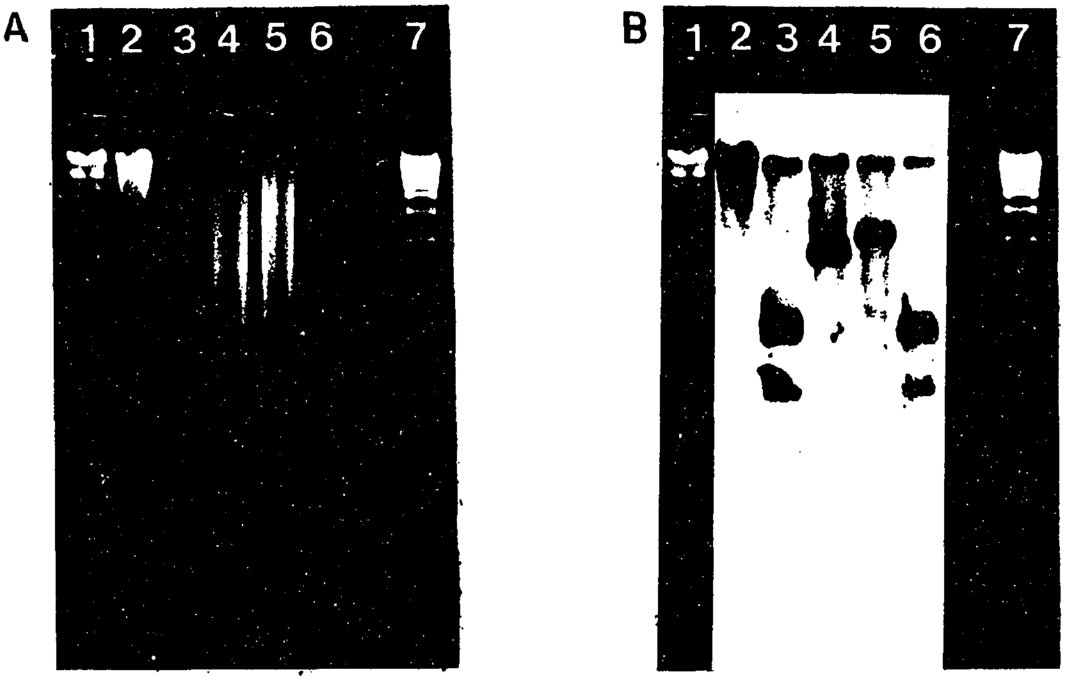
***Anguilla rostrata*, American eel, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 15. *Anguilla rostrata*, American eel, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Hin*d III + *Eco*R I digest; 4, *Hin*d III + *Bam*H I digest; 5, *Hin*d III + *Pvu* II digest; 6, *Hin*d III + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates are reduced to 86% of their actual size. Thin arrow points to a light hybrid band.



***Anguilla rostrata*, American eel, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 16. *Anguilla rostrata*, American eel, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Eco*R I + *Bam*H I digest; 4, *Eco*R I + *Pvu* II digest; 5, *Eco*R I + *Pst* I digest; 6, *Eco*R I + *Pvu* II + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates are reduced to 86% of their actual size. Thin arrows point to light hybrid bands.



***Anguilla rostrata*, American eel, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 17. *Anguilla rostrata*, American eel, nrDNA hybridized with *Xenopus laevis* rDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hind* III Fragments; 2, undigested; 3, *Bam*H I + *Pvu* II digest; 4, *Bam*H I + *Pst* I digest; 5, *Pvu* II + *Pst* I digest; 6, *Bam*H I + *Pvu* II + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates are reduced to 86% of their actual size.

Table 10

Anguilla rostrata, American eel, rDNA fragments generated by restriction endonucleases

Restriction Endonuclease	Fragment sizes in kilobases
*Hin*d III	20.0
*Eco*R I	4.7; 11.0
*Bam*H I	0.5; 3.5; 11.2
Pvu II	4.4; 11.0
Pst I	15[*]
*Hin*d III + *Eco*R I	0.6; 4.7; 11
*Hin*d III + *Bam*H I	0.6[**]; 3.0; 11.2
*Hin*d III + *Pvu* II	1.4; 4.5; 11
*Hin*d III + *Pst* I	6.4-8.0[***]
*Eco*R I + *Bam*H I	1.1; 1.7; 2.5; 9.4
*Eco*R I + *Pvu* II	0.6; 0.8; 3.7; 10.3
*Eco*R I + *Pst* I	1.1; 4.7; 9.5
*Eco*R I + *Pvu* II + *Pst* I	0.6; 0.8; (1.0); 4.7; 9.5
*Bam*H I + *Pvu* II	1.1[****]; 1.7; 8.9
*Bam*H I + *Pst* I	2.8; 3.5[?]; 8.3
Pvu II + *Pst* I	4.5; 10.5
*Bam*H I + *Pvu* II + *Pst* I	1.1[****]; 1.7

[*] Variable; [**] very light band; [***] bands merged; [****] possibly generated by RE star activity; [?] missing band probably due to star activity. Fragment lengths shown in parenthesis are partial digests.

RESTRICTION ENZYME MAP OF AMERICAN EEL, *ANGUILLA ROSTRATA*, RIBOSOMAL DNA

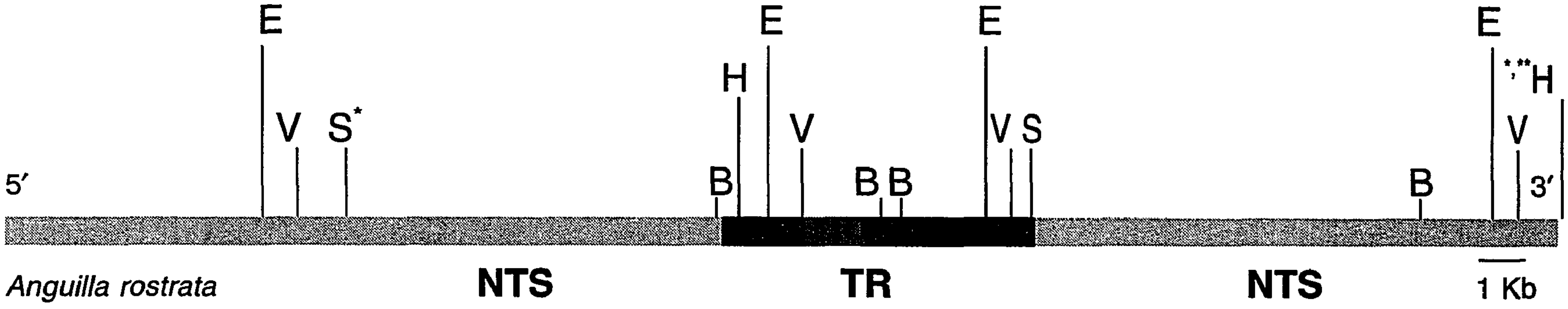
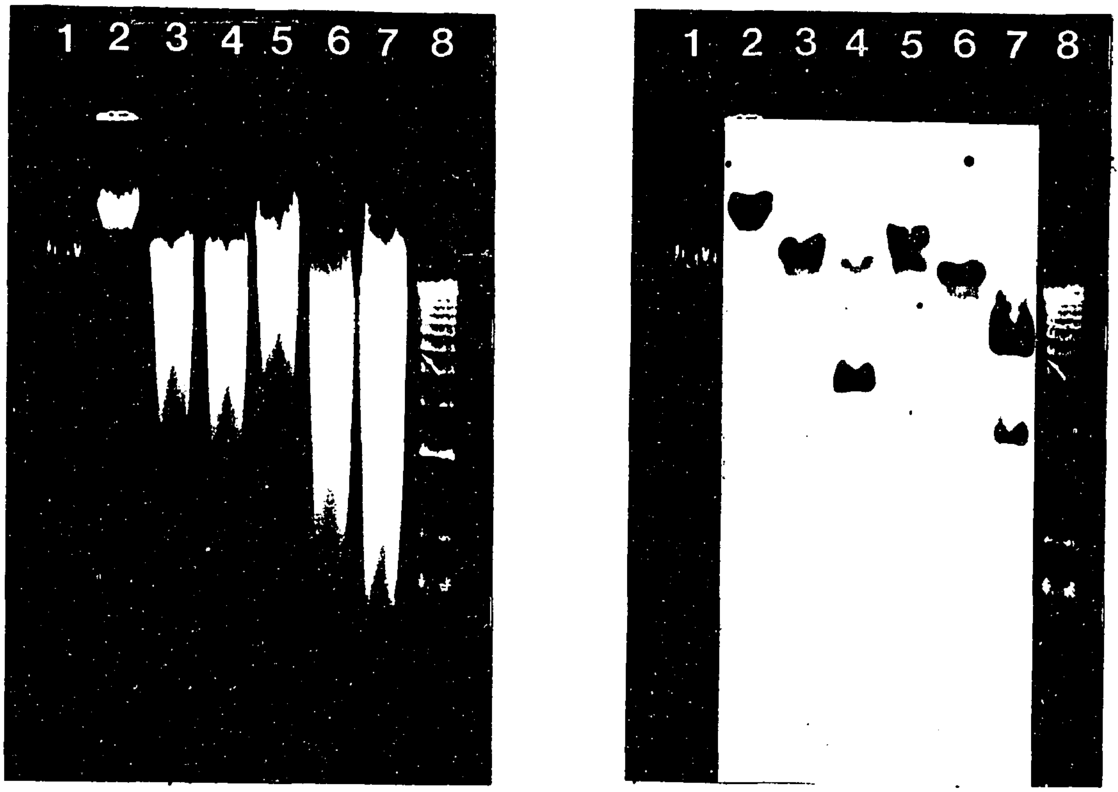


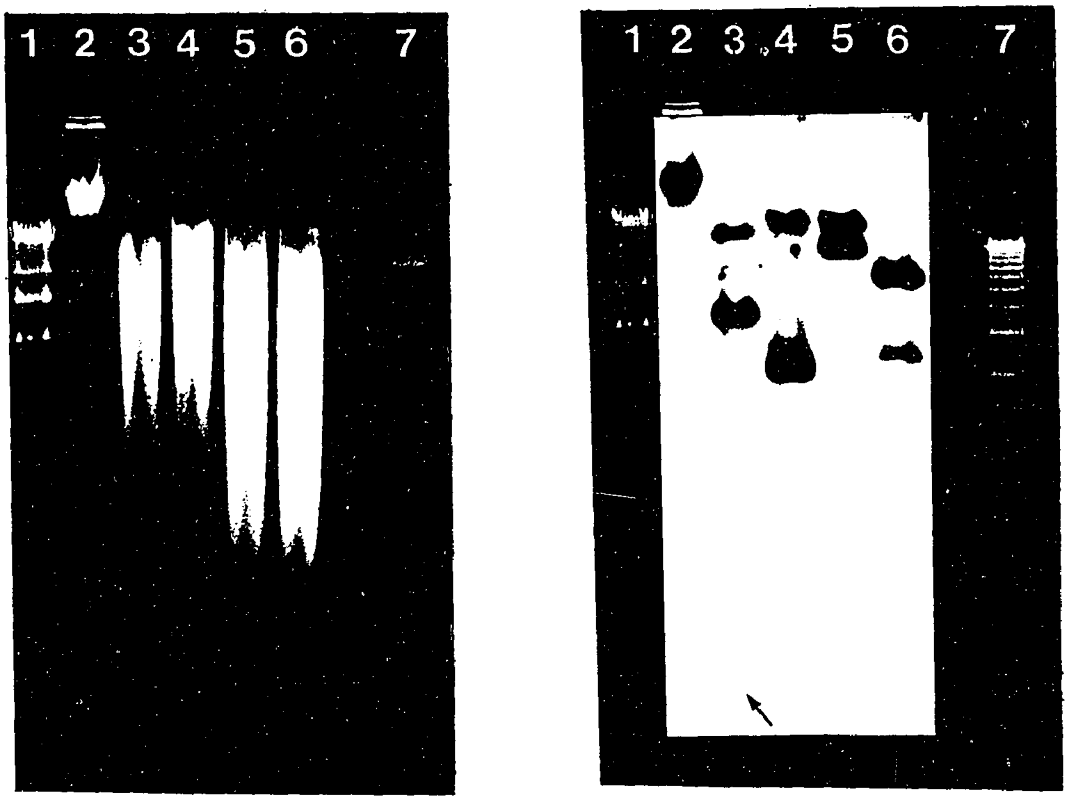
Figure 18. Proposed restriction enzyme map of American eel ribosomal DNA. B, *Bam*H I; E, *Eco*R I; H, *Hind* III; S, *Pst* I; V, *Pvu* II; NTS, nontranscribed spacer; TR, transcribed region. *variable; **should be 2 Kb out-side. Relative positions of the respective restriction enzymes are shown in the map in approximate scale.

Alosa sapidissima
American shad



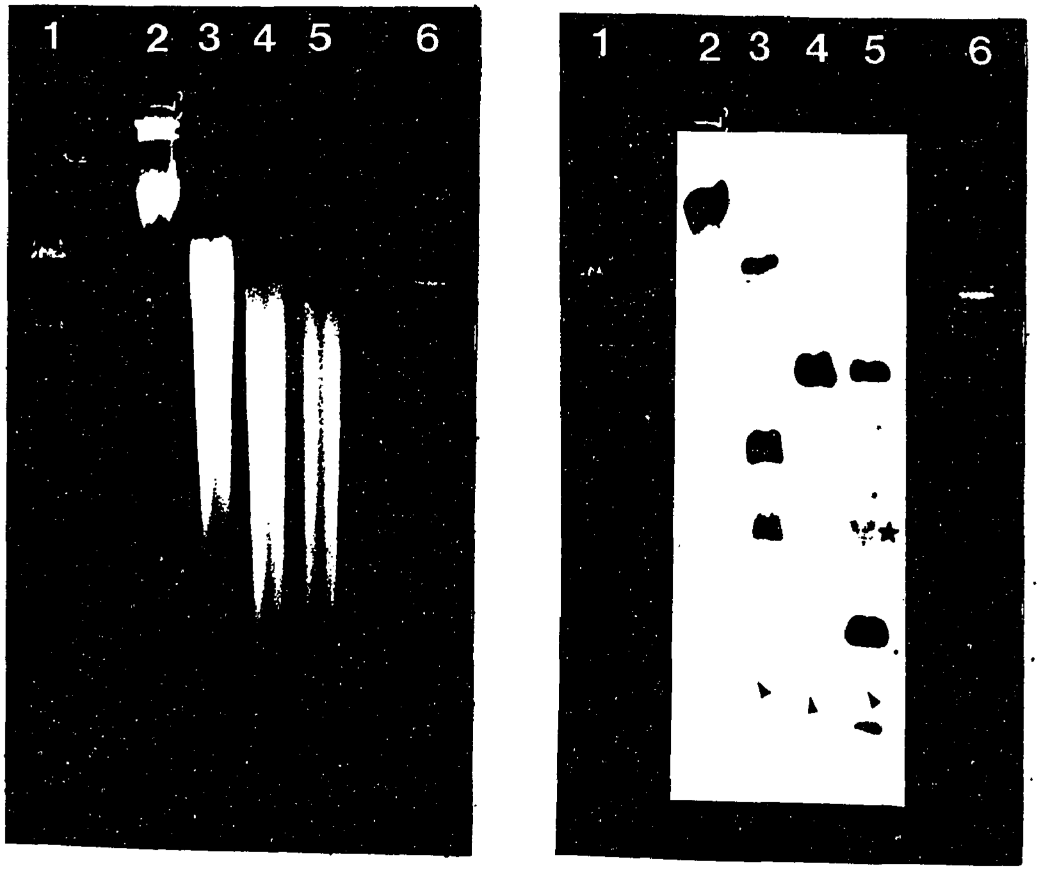
***Alosa sapidissima*, American shad, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 19. *Alosa sapidissima*, American shad, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-7) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Hin*d III digest; 4, *Eco*R I digest; 5, *Bam*H I digest; 6, *Pvu* II digest; 7, *Pst* I digest; 8, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes.



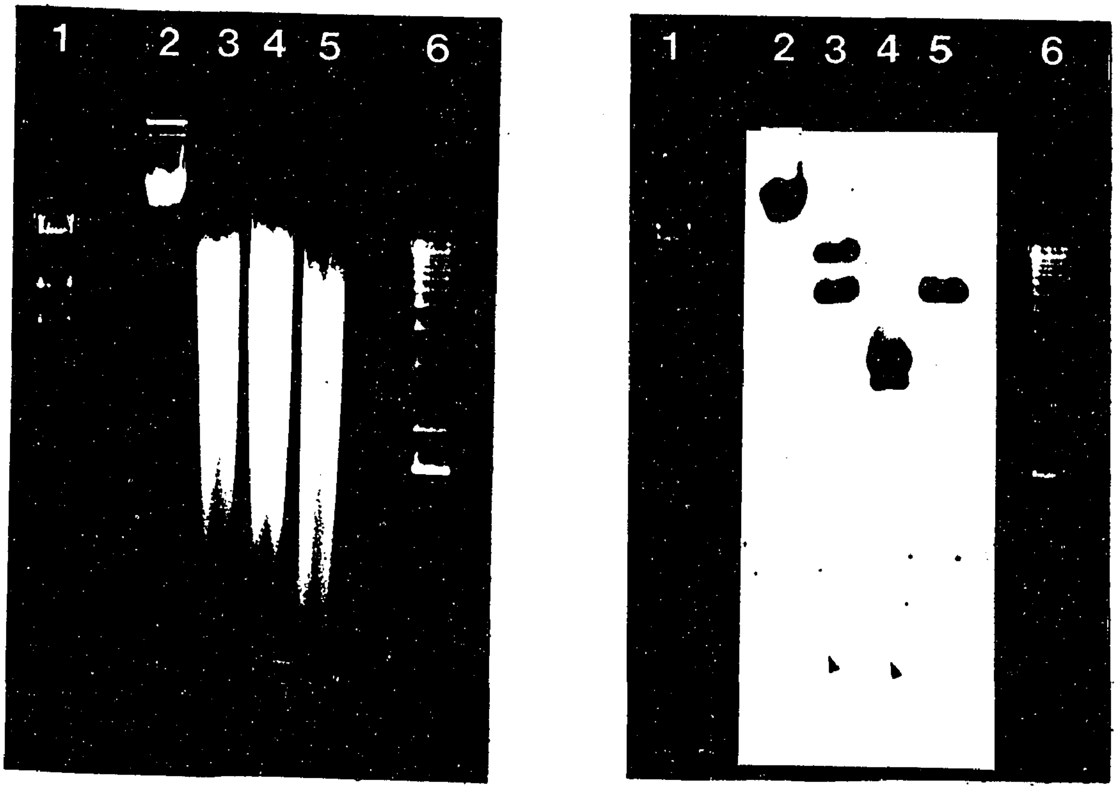
***Alosa sapidissima*, American shad, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 20. *Alosa sapidissima*, American shad, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Hin*d III + *Eco*R I digest; 4, *Hin*d III + *Bam*H I digest; 5, *Hin*d III + *Pvu* II digest; 6, *Hin*d III + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes. Thin arrow points to a light hybrid band.



***Alosa sapidissima*, American shad, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 21. *Alosa sapidissima*, American shad, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-5) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Eco*R I + *Bam*H I digest; 4, *Eco*R I + *Pvu* II digest; 5, *Eco*R I + *Pst* I digest; 6, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes. Small arrow heads point to light hybrid bands; ★, not a hybrid band.



***Alosa sapidissima*, American shad, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 22. *Alosa sapidissima*, American shad, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-5) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Bam*H I + *Pvu* II digest; 4, *Bam*H I + *Pst* I digest; 5, *Pvu* II + *Pst* I digest; 6, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes. Small arrow heads point to light hybrid bands.

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Alosa sapidissima, American shad, rDNA fragments generated by restriction endonucleases

Restriction Endonuclease	Fragment sizes in kilobases
*Hin*d III	26
*Eco*R I	4.6; 23.1
*Bam*H I	23.1; 27.0
Pvu II	19.3
Pst I	3.6; 6.1
*Hin*d III + *Eco*R I	0.7; 4.6; 19.4
*Hin*d III + *Bam*H I	3.1; 21.2
*Hin*d III + *Pvu* II	9.5; (19.3)
*Hin*d III + *Pst* I	3.6; 6.2
*Eco*R I + *Bam*H I	0.8; 1.7; 2.5; 23-24
*Eco*R I + *Pvu* II	0.8; 4.3-4.6*
*Eco*R I + *Pst* I	0.5; (0.8); 1.0; 3.5; (4.5)
*Bam*H I + *Pvu* II	0.5; 6.2; 12.8
*Bam*H I + *Pst* I	0.5; 2.8; 3.1-3.5*
Pvu II + *Pst* I	3.2; 6.1

*Comigrated; fragment lengths shown in parenthesis are partial digests.

RESTRICTION ENZYME MAP OF AMERICAN SHAD, *ALOSA SAPIDISSIMA*, RIBOSOMAL DNA

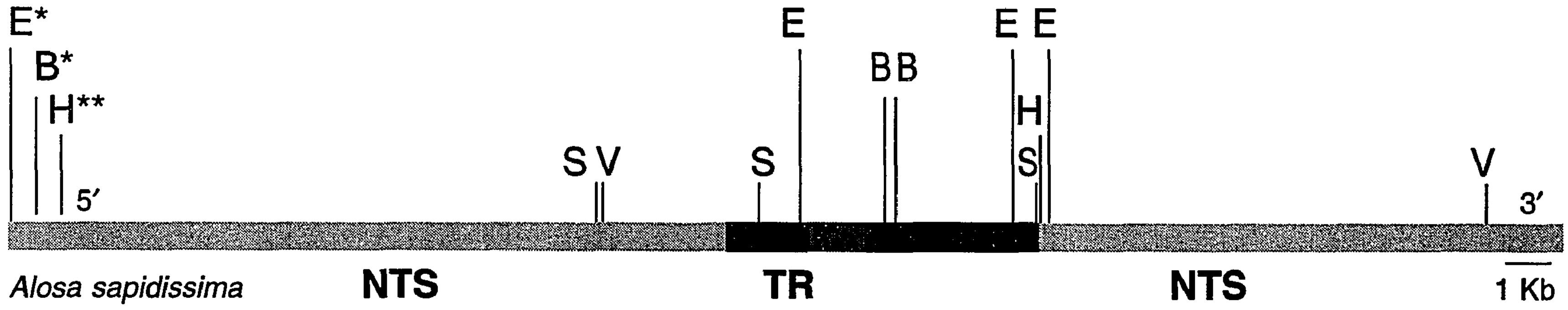
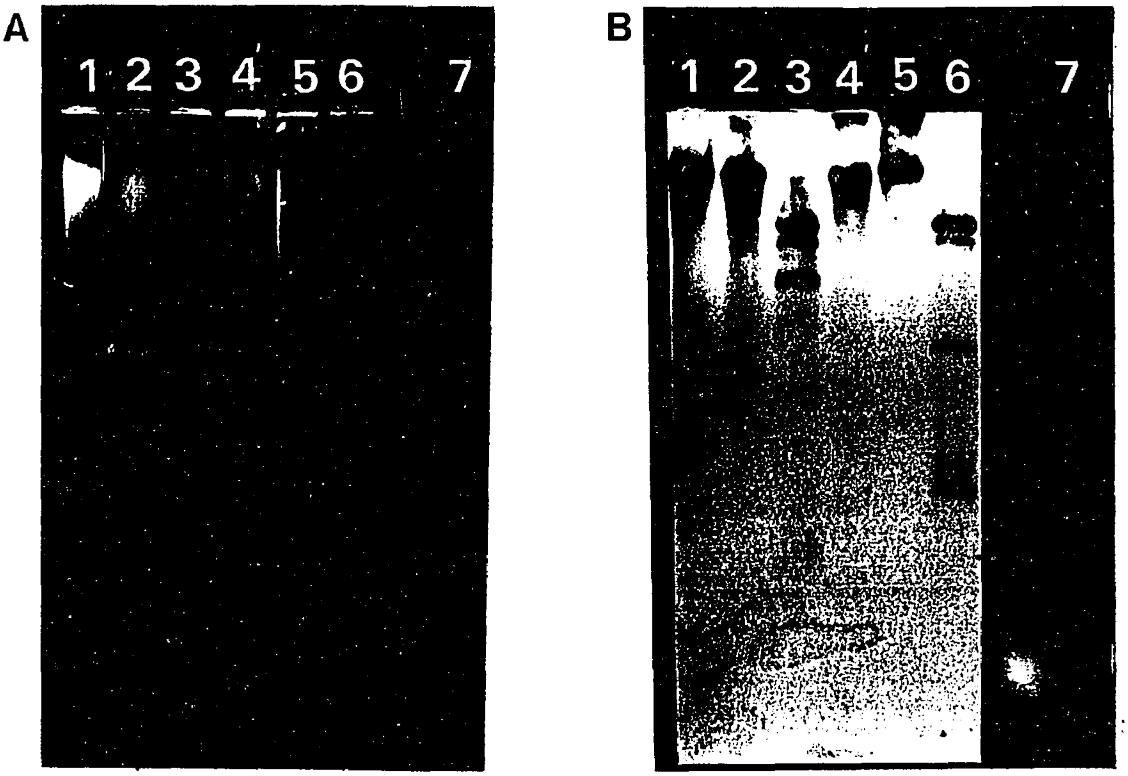


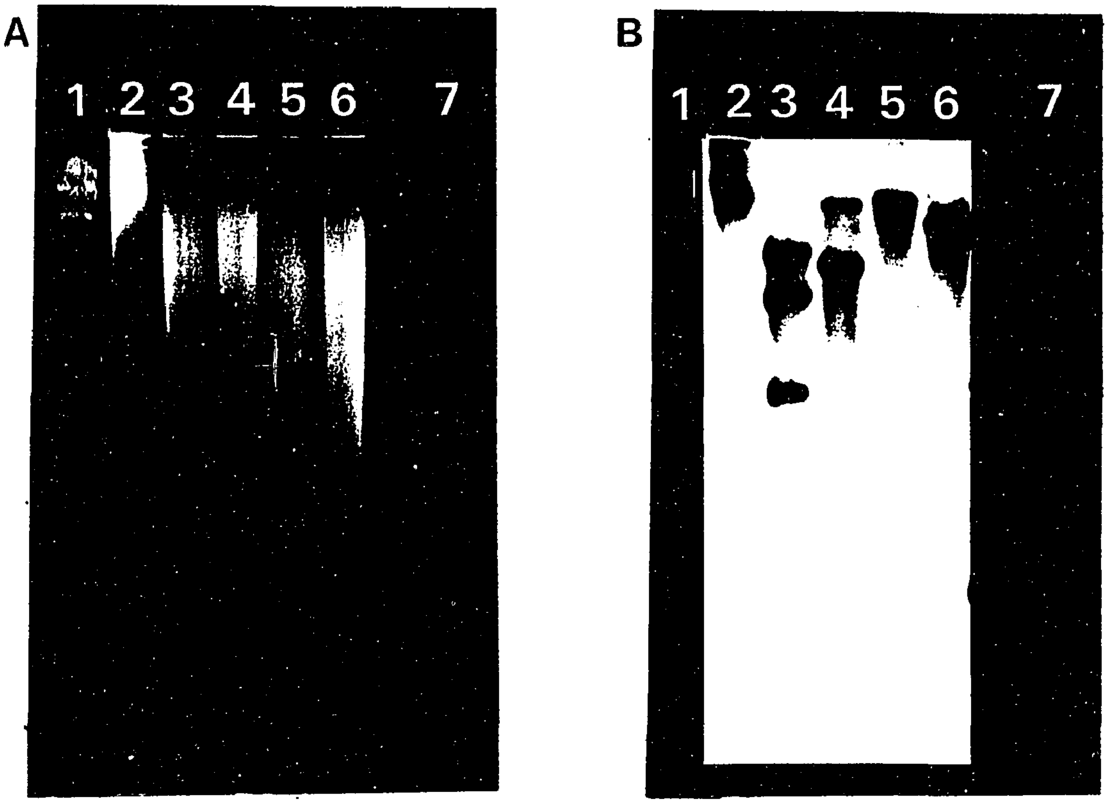
Figure 23. Proposed restriction enzyme map of American shad ribosomal DNA. B, *Bam*H I; E, *Eco*R I; H, *Hin*d III; S, *Pst* I; V, *Pvu* II; NTS, nontranscribed spacer; TR, transcribed region; *variable; **actual position 3.25 cm to the right. Relative positions of the respective restriction enzymes are shown in the map in approximate scale.

Cyprinus carpio
Common carp



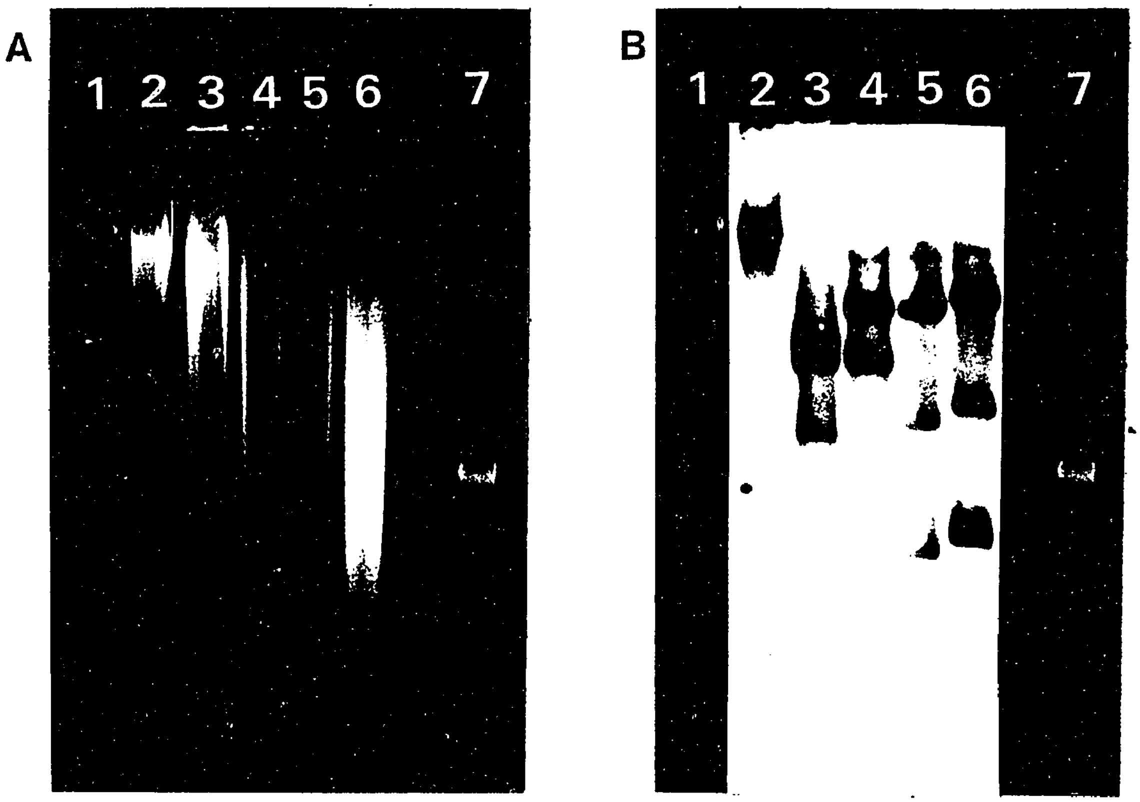
***Cyprinus carpio*, carp, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 24. *Cyprinus carpio*, carp, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 1-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, undigested; 2, *Hin*d III digest; 3, *Eco*R I digest; 4, *Bam*H I digest; 5, *Pvu* II digest; 6, *Pst* I digest; 7, λ DNA/*Hin*d III Fragments. Both A and B plates represent their actual sizes.



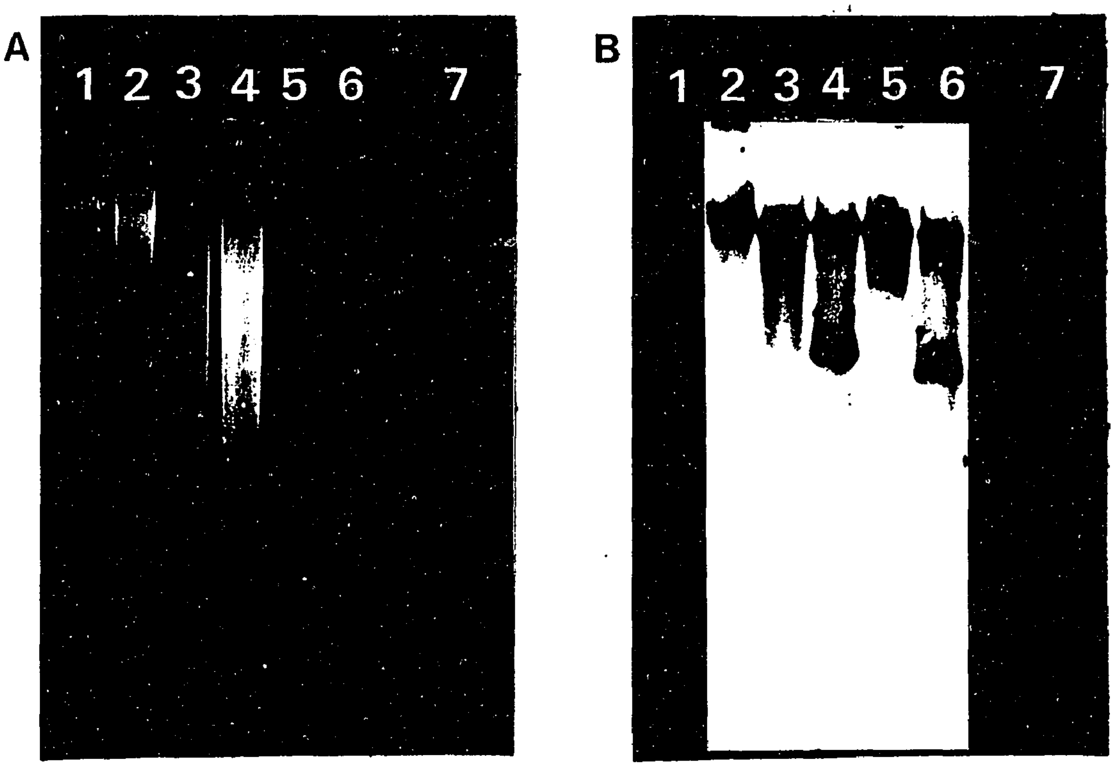
***Cyprinus carpio*, carp, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 25. *Cyprinus carpio*, carp, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Hin*d III + *Eco*R I digest; 4, *Hin*d III + *Bam*H I digest; 5, *Hin*d III + *Pvu* II digest; 6, *Hin*d III + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes.



***Cyprinus carpio*, carp, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 26. *Cyprinus carpio*, carp, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Eco*R I + *Bam*H I digest; 4, *Eco*R I + *Pvu* II digest; 5, *Eco*R I + *Pst* I digest; 6, *Eco*R I + *Pvu* II + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes.



***Cyprinus carpio*, carp, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 27. *Cyprinus carpio*, carp, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Bam*H I + *Pvu* II digest; 4, *Bam*H I + *Pst* I digest; 5, *Pvu* II + *Pst* I digest; 6, *Bam*H I + *Pvu* II + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes.

Table 12

***Cyprinus carpio*, Common carp, rDNA fragments generated by restriction endonucleases**

Restriction Endonuclease	Fragment sizes in kilobases
*Hin*d III	23.0[*],[§]
*Eco*R I	3.1; 5.6 + (0.8; 4.25; 4.5)[**,1]
*Bam*H I	15.0[*],[§]
Pvu II	≥23.0[*]
Pst I	none considered[**,1]
*Hin*d III + *Eco*R I	1.7; 3.2; 5.6
*Hin*d III + *Bam*H I	4.9; 18.2
*Hin*d III + *Pvu* II	22.1[*],[§]
*Hin*d III + *Pst* I	9.0[*],[§]
*Eco*R I + *Bam*H I	2.1; 3.1; 3.5
*Eco*R I + *Pvu* II	3.1; 5.6
*Eco*R I + *Pst* I	1.1; 2.1; 5.6
*Eco*R I + *Pvu* II + *Pst* I	1.1; 2.1; 5.6
[*]*Bam*H I + *Pvu* II	15.0[*],[§]
[*]*Bam*H I + *Pst* I	3.1; 15.0[*],[§]
[*]*Pvu* II + *Pst* I	15.0[*],[§]
[*]*Bam*H I + *Pvu* II + *Pst* I	2.6[**,2]; 15.0[*],[§]

[*]Variable; [**]star activity; [§]major fragment; [1]not considered for mapping; [2]possibly represent the 3.0 Kb rDNA fragment generated by *Bam* HI and *Pst* I digest.

RESTRICTION ENZYME MAP OF COMMON CARP, *CYPRINUS CARPIO*, RIBOSOMAL DNA

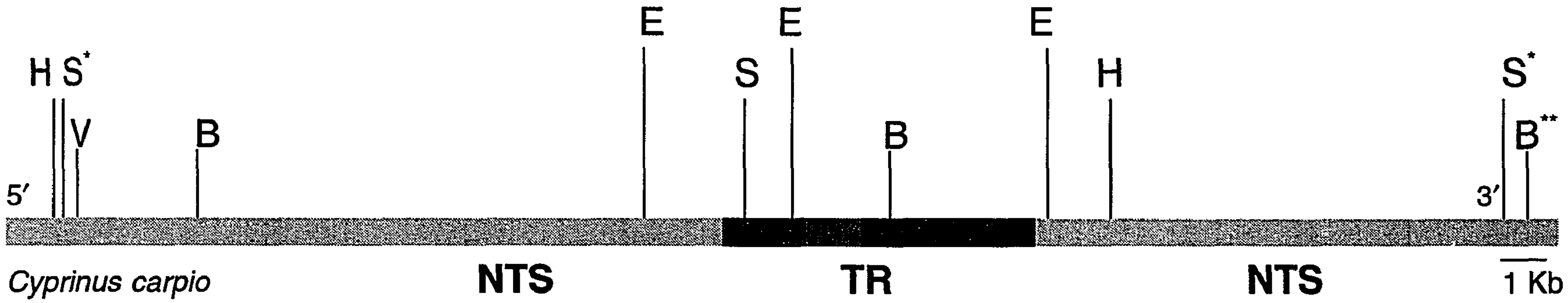
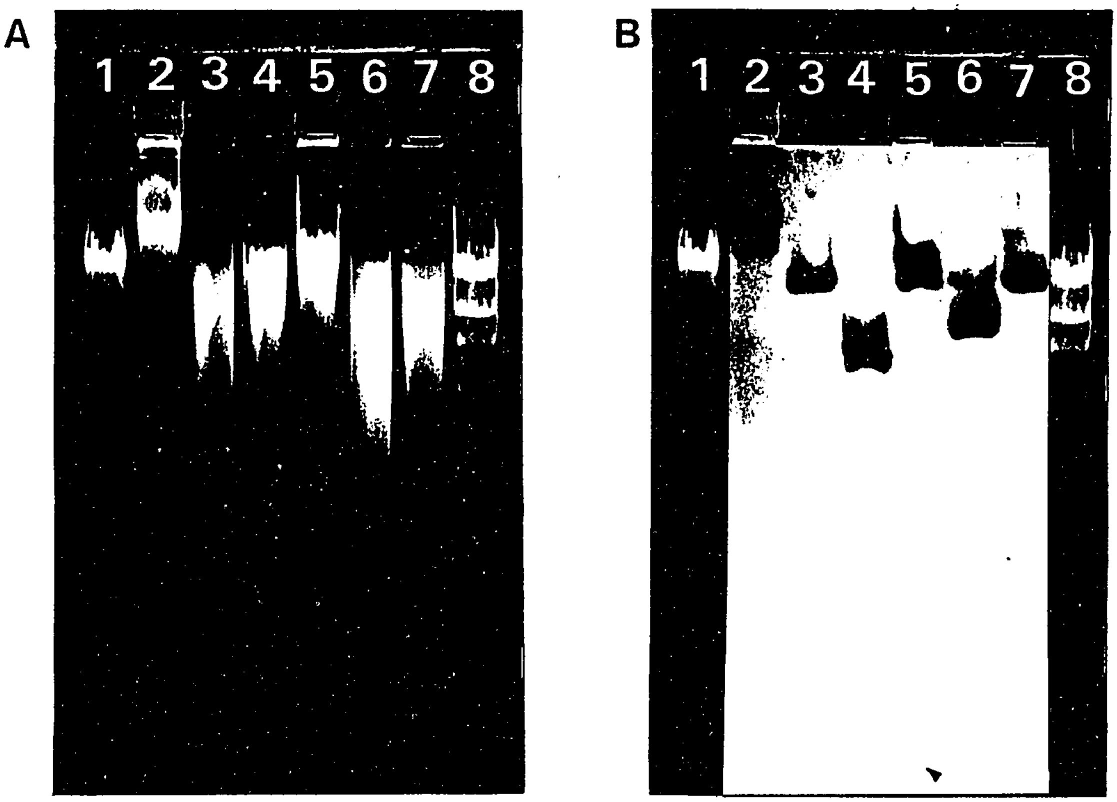


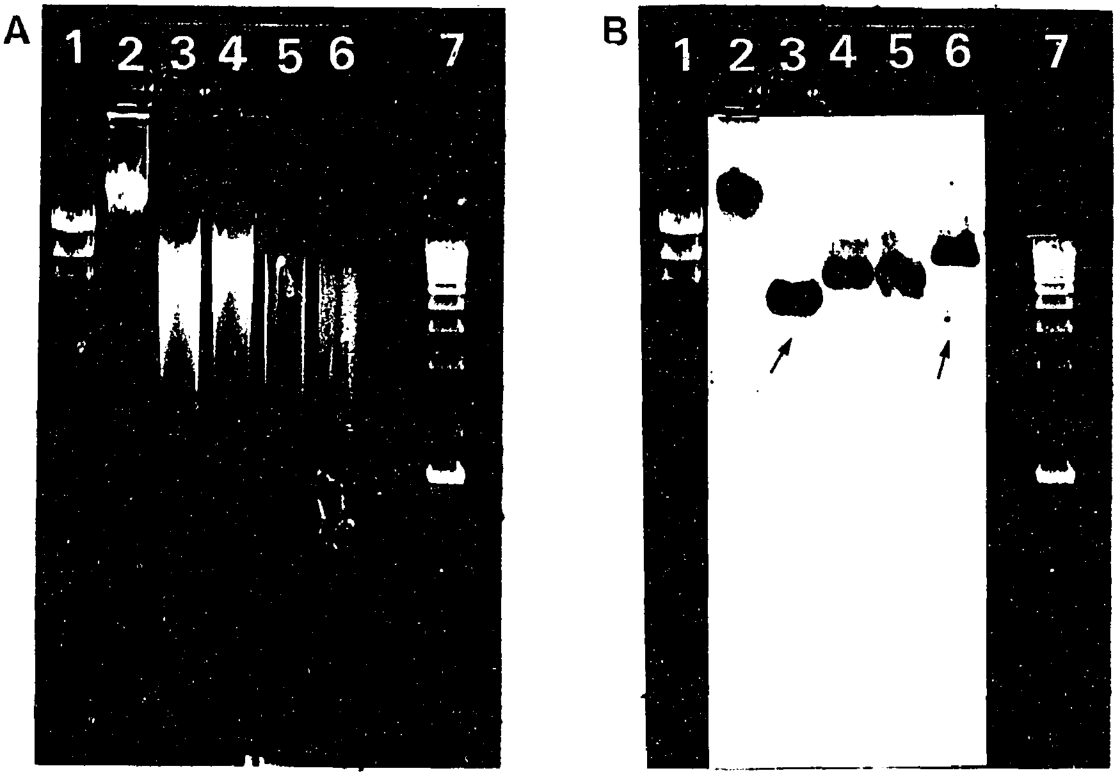
Figure 28. Proposed restriction enzyme map of carp ribosomal DNA. B, *Bam*H I; E, *Eco*R I; H, *Hin*d III; S, *Pst* I; V, *Pvu* II; NTS, nontranscribed spacer; TR, transcribed region; **actual position is at 1 cm to the right. Relative positions of the respective restriction enzymes are shown in the map in approximate scale.

Labeo rohita
Rohu



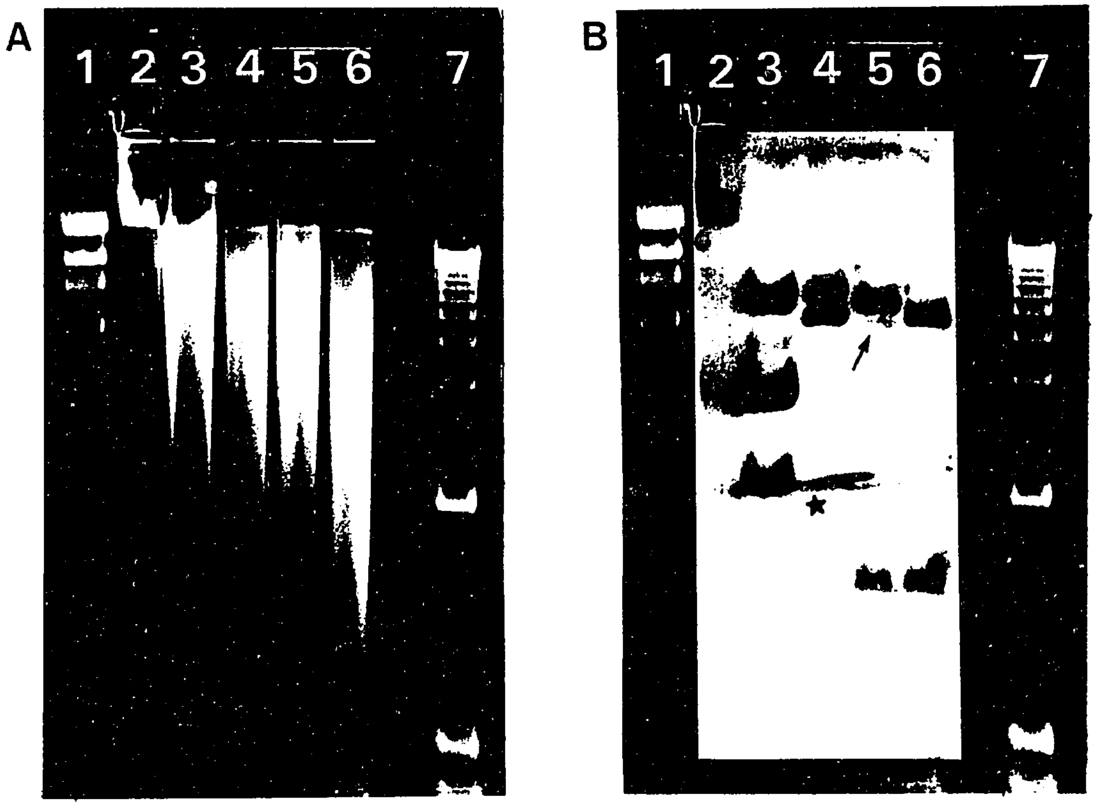
***Labeo rohita*, rohu, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 29. *Labeo rohita*, rohu, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-7) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, High Molecular DNA Marker, BRL, 2, undigested; 3, *Hin*d III digest; 4, *Eco*R I digest; 5, *Bam*H I digest; 6, *Pvu* II digest; 7, *Pst* I digest; 8, λ DNA/*Hin*d III Fragments. Both A and B plates represent their actual sizes. Arrow head, light hybrid band.



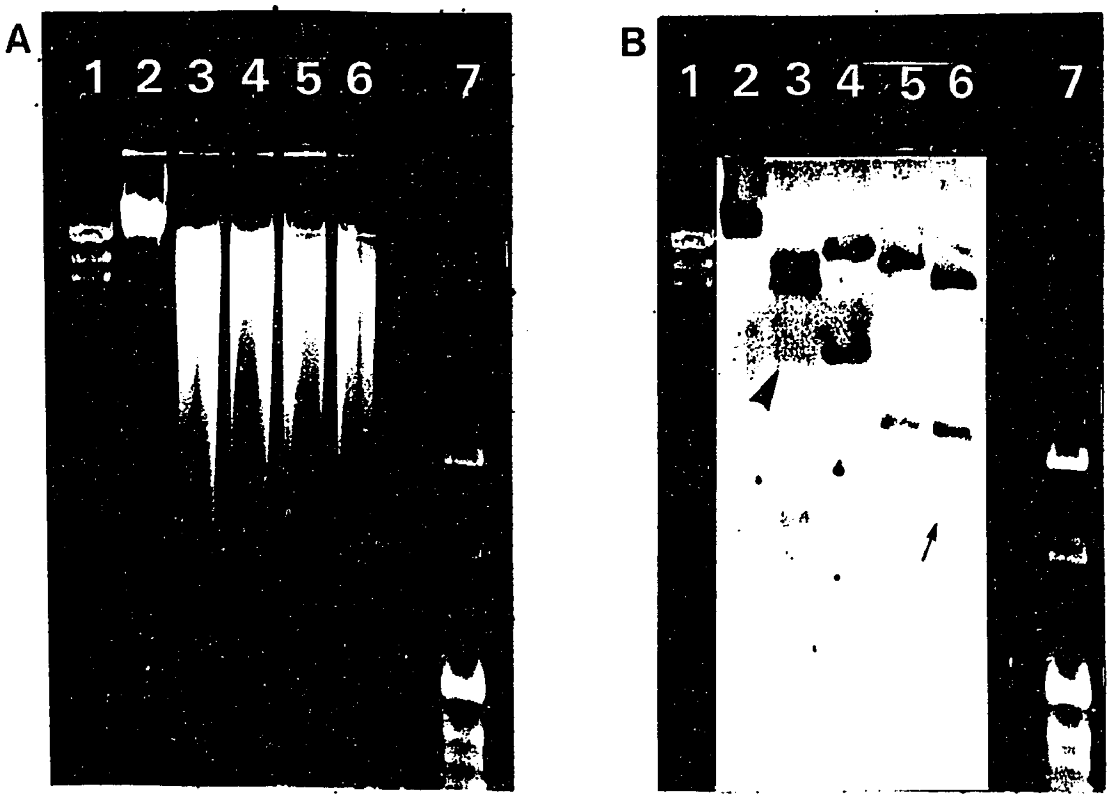
***Labeo rohita*, rohu, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 30. *Labeo rohita*, rohu, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Hin*d III + *Eco*R I digest; 4, *Hin*d III + *Bam*H I digest; 5, *Hin*d III + *Pvu* II digest; 6, *Hin*d III + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes. Thin arrows point to hybrid bands.



***Labeo rohita*, rohu, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 31. *Labeo rohita*, rohu, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hind* III Fragments; 2, undigested; 3, *Eco*R I + *Bam*H I digest; 4, *Eco*R I + *Pvu* II digest; 5, *Eco*R I + *Pst* I digest; 6, *Eco*R I + *Pvu* II + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes. Thin arrow points to a hybrid band; ★, not a hybrid band.



***Labeo rohita*, rohu, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 32. *Labeo rohita*, rohu, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hind* III Fragments; 2, undigested; 3, *Bam*H I + *Pvu* II digest; 4, *Bam*H I + *Pst* I digest; 5, *Pvu* II + *Pst* I digest; 6, *Bam*H I + *Pvu* II + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes. Large arrow head, not a hybrid band; thin arrow points to a hybrid band.

Table 13

Labeo rohita, Rohu¶, rDNA fragments generated by restriction endonucleases

Restriction Endonuclease	Fragment sizes in kilobases
Hind III	13.4
*Eco*R I	5.0
*Bam*H I	0.5; 14
Pvu II	7.5; 8
Pst I	15.3
Hind III + *Eco*R I	3.8*; 5.0
Hind III + *Bam*H I	6.5
Hind III + *Pvu* II	5.5; 8.0
Hind III + *Pst* I	3.5*; 9.9
*Eco*R I + *Bam*H I	1.7; 2.7; 5.0
*Eco*R I + *Pvu* II	4.35; 5.0
*Eco*R I + *Pst* I	1.1; 3.8*; 5.0
*Eco*R I + *Pvu* II + *Pst* I	1.1, 4.35
**Bam*H I + *Pvu* II	1.0; 6.5; 7.5
**Bam*H I + *Pst* I	2.9; 12
**Pvu* II + *Pst* I	1.8; 8.0
**Bam*H I + *Pvu* II + *Pst* I	1.8; 6.5

*Very light band; ¶Indian major carp.

RESTRICTION ENZYME MAP OF ROHU, *LABEO ROHITA*, RIBOSOMAL DNA

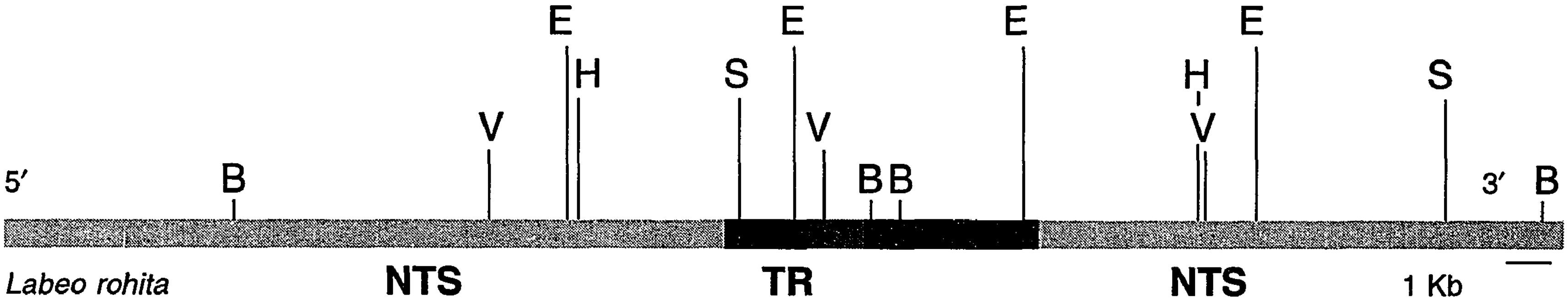
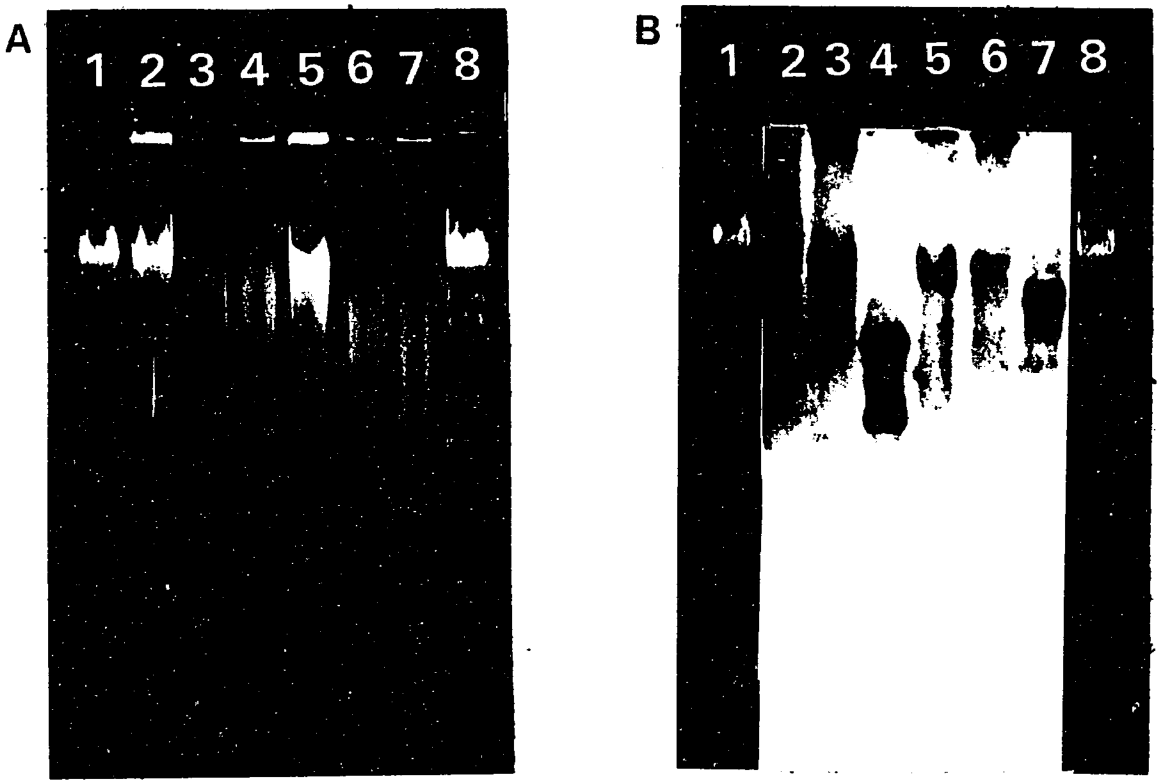


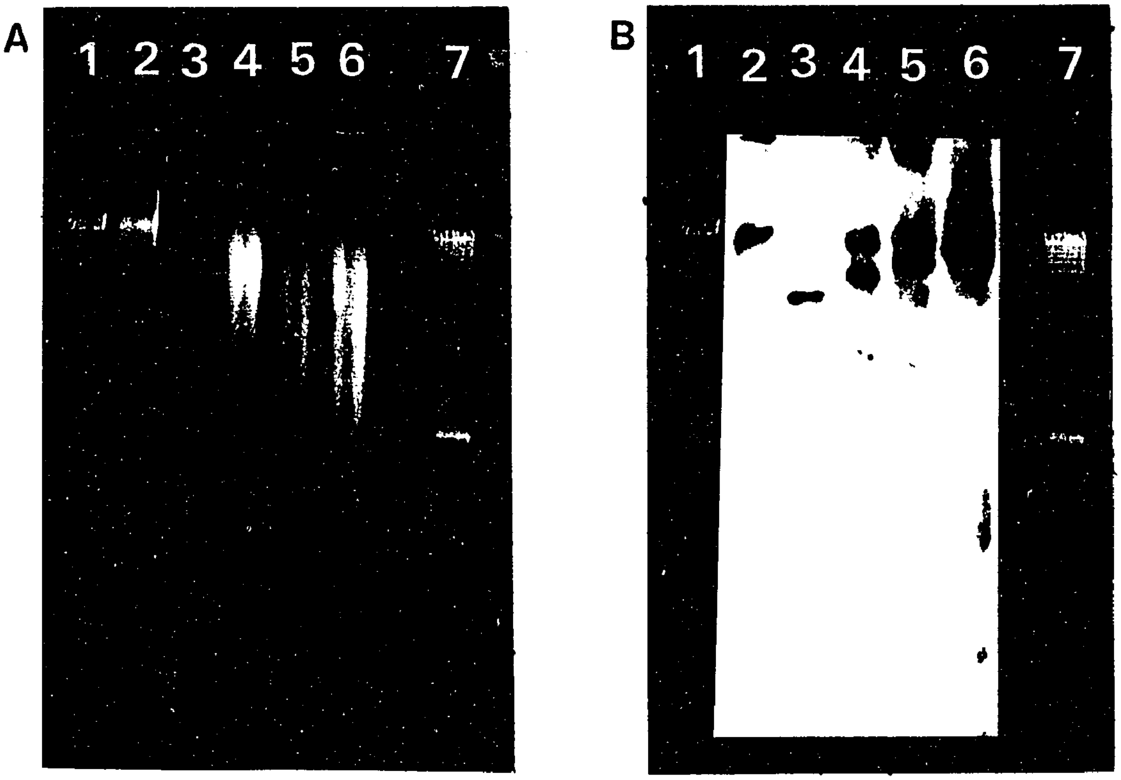
Figure 33. Proposed restriction enzyme map of rohu ribosomal DNA. B, *Bam*H I; E, *Eco*R I; H, *Hin*d III; S, *Pst* I; V, *Pvu* II; NTS, nontranscribed spacer; TR, transcribed region. Relative positions of the respective restriction enzymes are shown in the map in approximate scale.

Puntius gonionotus
Thailandian sarpunti



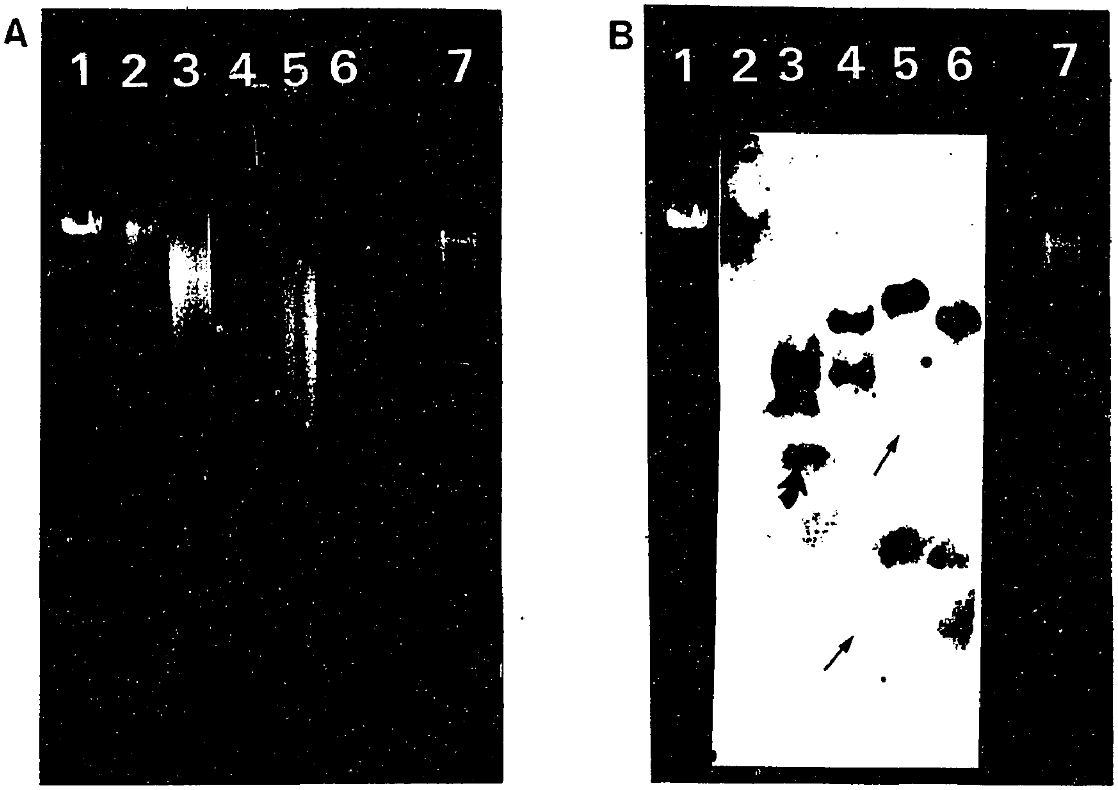
***Puntius gonionotus*, Thailandian sarpunti, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 34. *Puntius gonionotus*, Thailandian sarpunti, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-7) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, High Molecular DNA Marker, BRL, 2, undigested; 3, *Hin*d III digest; 4, *Eco*R I digest; 5, *Bam*H I digest; 6, *Pvu* II digest; 7, *Pst* I digest; 8, λ DNA/*Hin*d III Fragments. Both A and B plates represent their actual sizes.



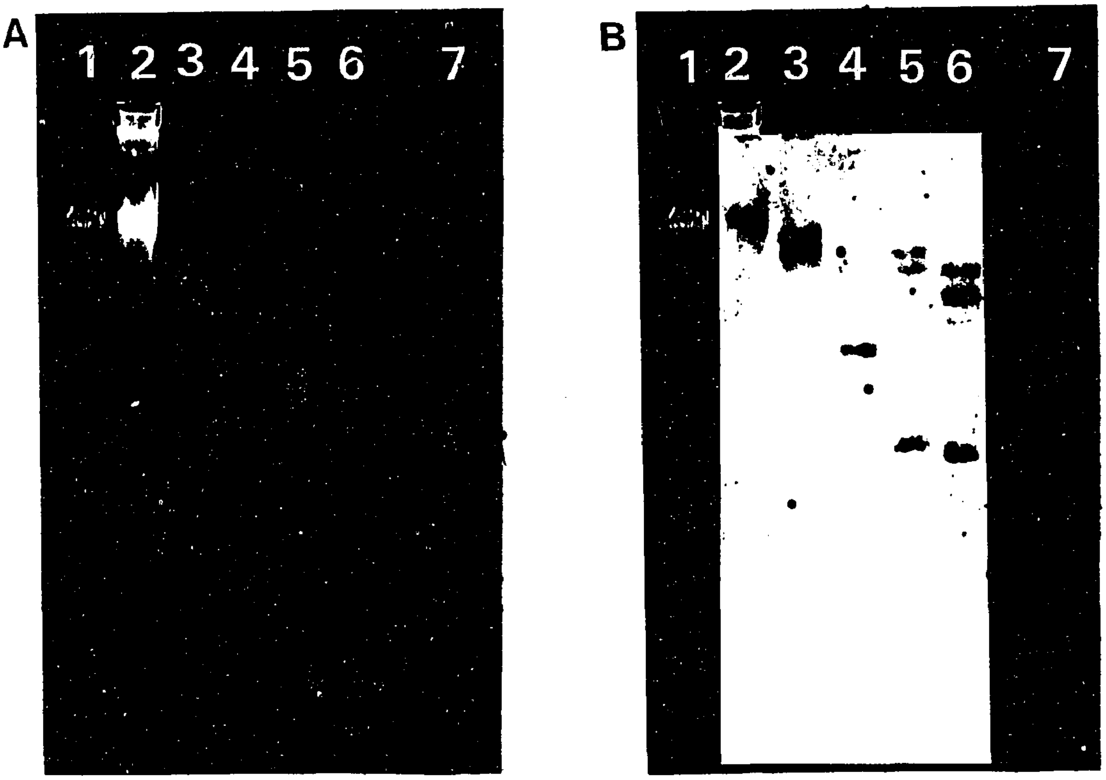
Puntius gonionotus, Thailandian sarpunti, nrDNA hybridized with _Xenopus laevis_ nrDNA

Figure 35. *Puntius gonionotus*, Thailandian sarpunti, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Hin*d III + *Eco*R I digest; 4, *Hin*d III + *Bam*H I digest; 5, *Hin*d III + *Pvu* II digest; 6, *Hin*d III + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes.



***Puntius gonionotus*, Thailandian sarpunti, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 36. *Puntius gonionotus*, Thailandian sarpunti, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Eco*R I + *Bam*H I digest; 4, *Eco*R I + *Pvu* II digest; 5, *Eco*R I + *Pst* I digest; 6, *Eco*R I + *Pvu* II + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates represent their actual size. Large arrow, not a hybrid band; thin arrows point to hybrid bands.



***Puntius gonionotus*, Thailandian sarpunti, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 37. *Puntius gonionotus*, Thailandian sarpunti, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Bam*H I + *Pvu* II digest; 4, *Bam*H I + *Pst* I digest; 5, *Pvu* II + *Pst* I digest; 6, *Bam*H I + *Pvu* II + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes.

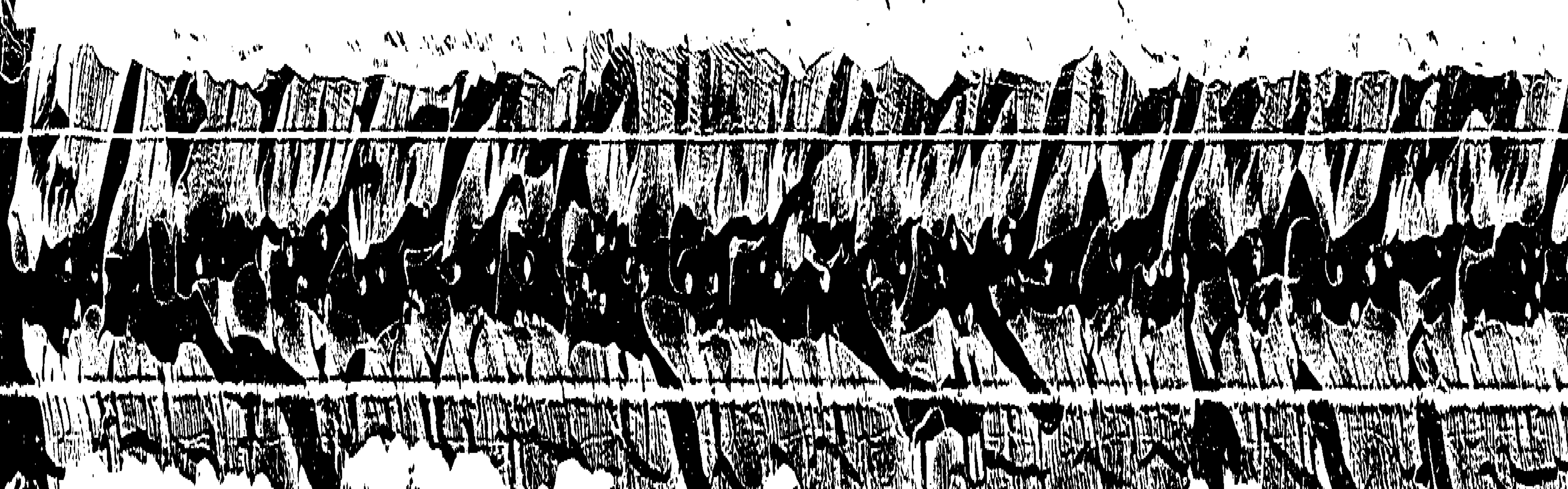
Table 14

Puntius gonionotus, Thailandian sarpunti[¶], rDNA fragments generated by restriction endonucleases

Restriction Endonuclease	Fragment sizes in kilobases
*Hin*d III	18.9
*Eco*R I	2.4; 5.0
*Bam*H I	11.5
Pvu II	17
Pst I	8.4; 11.4
*Hin*d III + *Eco*R I	2.4; 5.0
*Hin*d III + *Bam*H I	5.2; 11.5
*Hin*d III + *Pvu* II	6.9; 12.0
*Hin*d III + *Pst* I	8.4-10.2*
*Eco*R I + *Bam*H I	2.3; 2.6-2.8*
*Eco*R I + *Pvu* II	0.6; 2.6; 4.5
*Eco*R I + *Pst* I	1.1; 2.1; 5.0
*Eco*R I + *Pvu* II + *Pst* I	0.6; 1.1; 4.5
*Bam*H I + *Pvu* II	9.8; 11.5
*Bam*H I + *Pst* I	3.3; 4.9; 8.0
Pvu II + *Pst* I	1.8; 6.6; 11.5
*Bam*H I + *Pvu* II + *Pst* I	1.7; 4.9; 8.0

*Comigrated; [¶]known in Bangladesh.

RESTRICTION ENZYME MAP OF THAILANDIAN SARPUNTI.



RESTRICTION ENZYME MAP OF THAILANDIAN SARPUNTI, *PUNTIUS GONIONOTUS*, RIBOSOMAL DNA

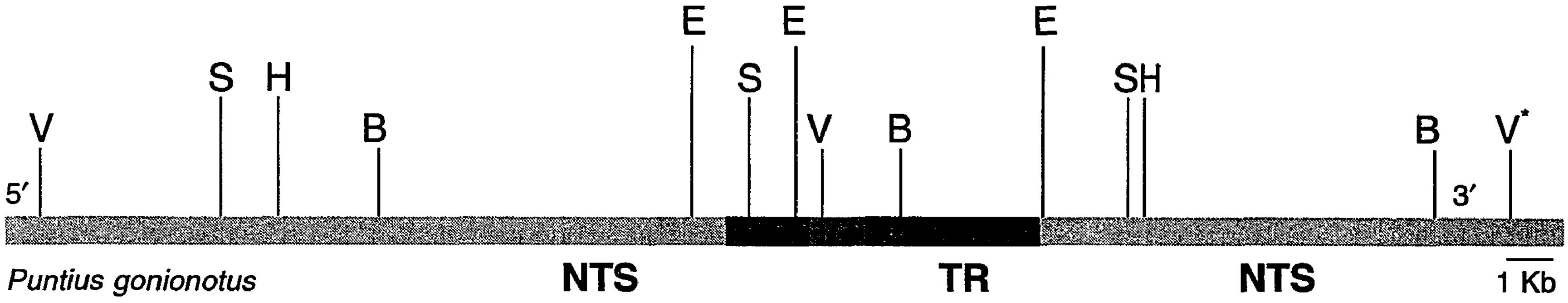
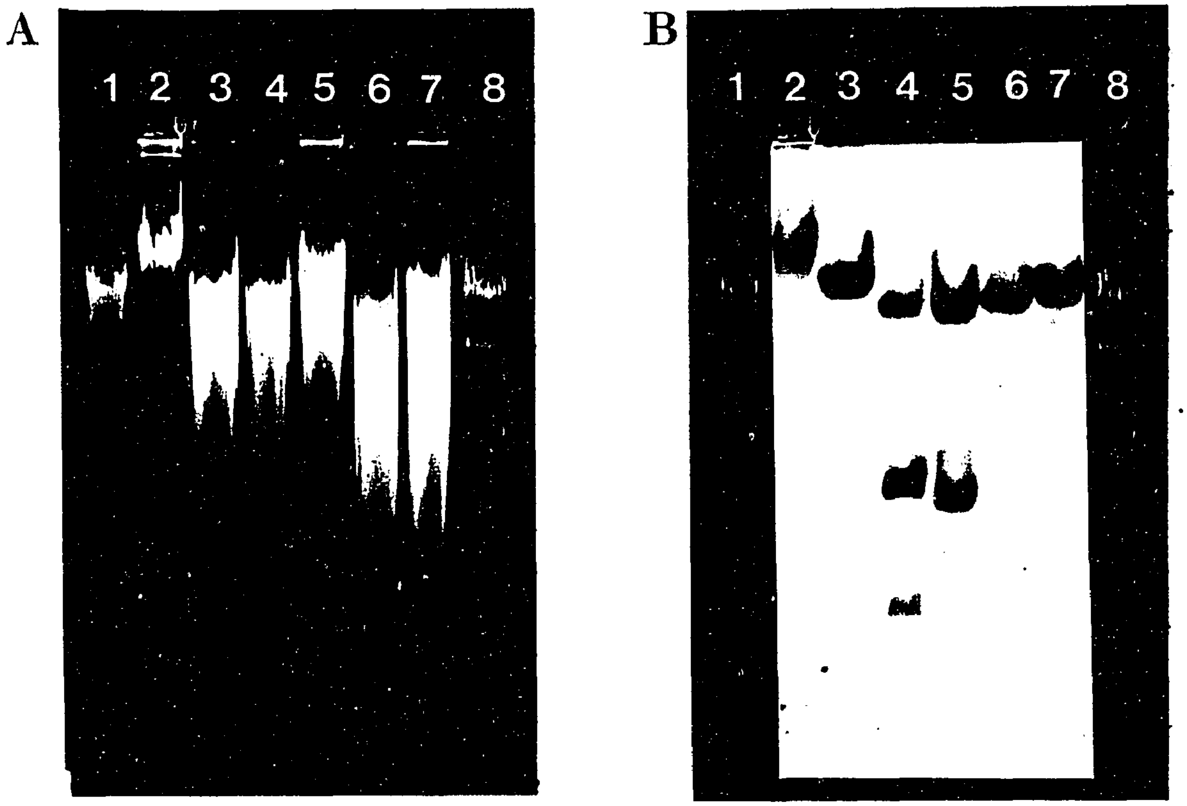


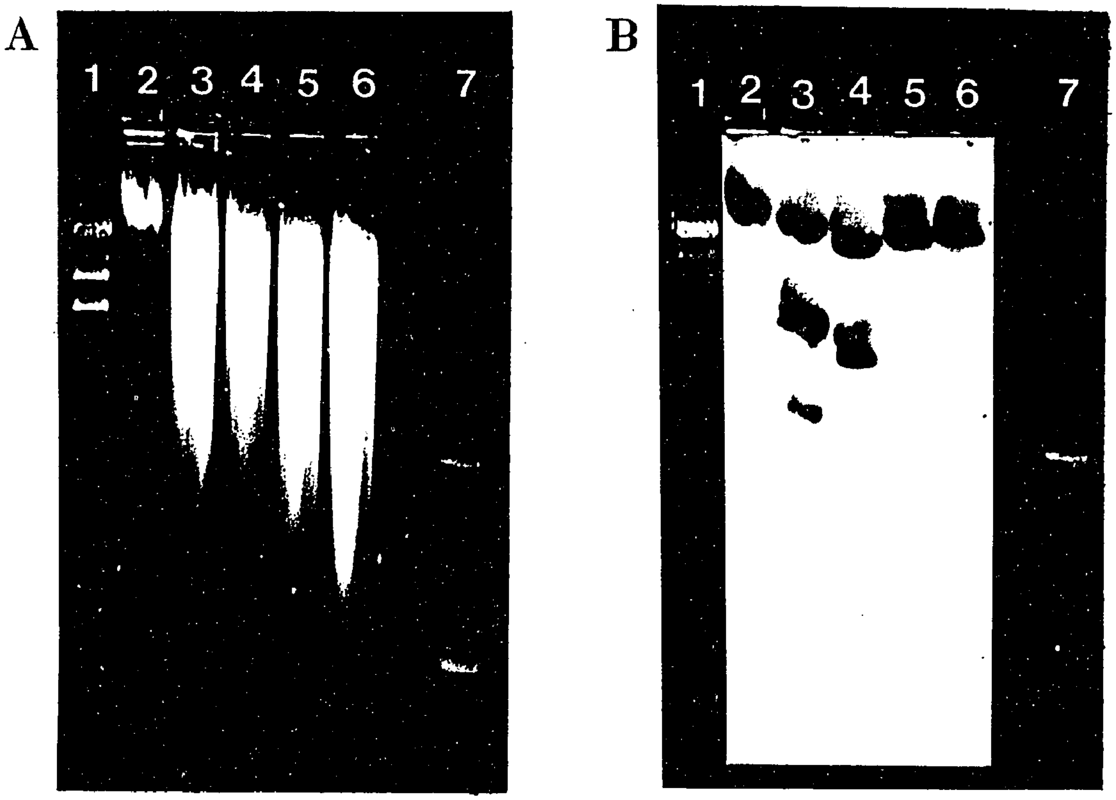
Figure 38. Proposed restriction enzyme map of sarpunti ribosomal DNA. B, *Bam*H I; E, *Eco*R I; H, *Hin*d III; S, *Pst* I; V, *Pvu* II; NTS, nontranscribed spacer; TR, transcribed region. *position 2 Kb away. Relative positions of the respective restriction enzymes are shown in the map in approximate scale.

Esox americanus americanus
Redfin pickerel



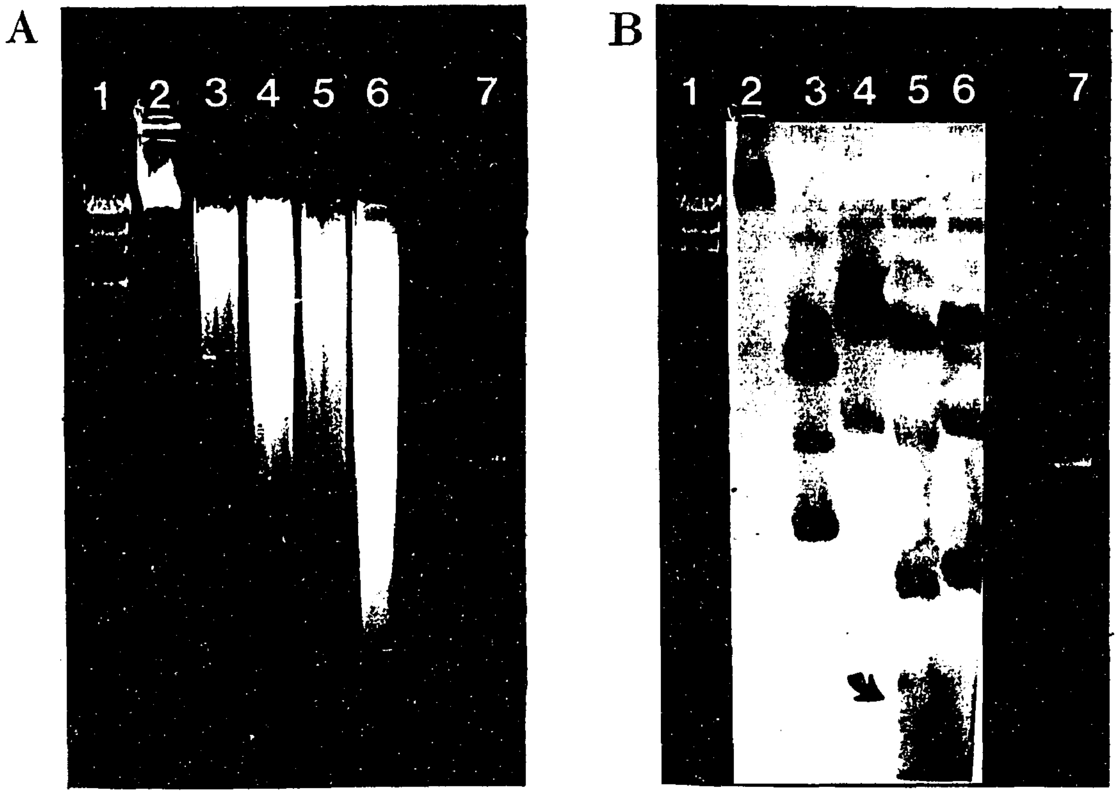
***Esox americanus americanus*, redfin pickerel, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 39. *Esox americanus americanus*, redfin pickerel, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-7) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, High Molecular Weight DNA Markers, BRL; 2, undigested; 3, *Hin*d III digest; 4, *Eco*R I digest; 5, *Bam*H I digest; 6, *Pvu* II digest; 7, *Pst* I digest; 8, λ DNA/*Hin*d III Fragments. Both A and B plates represent their actual sizes.



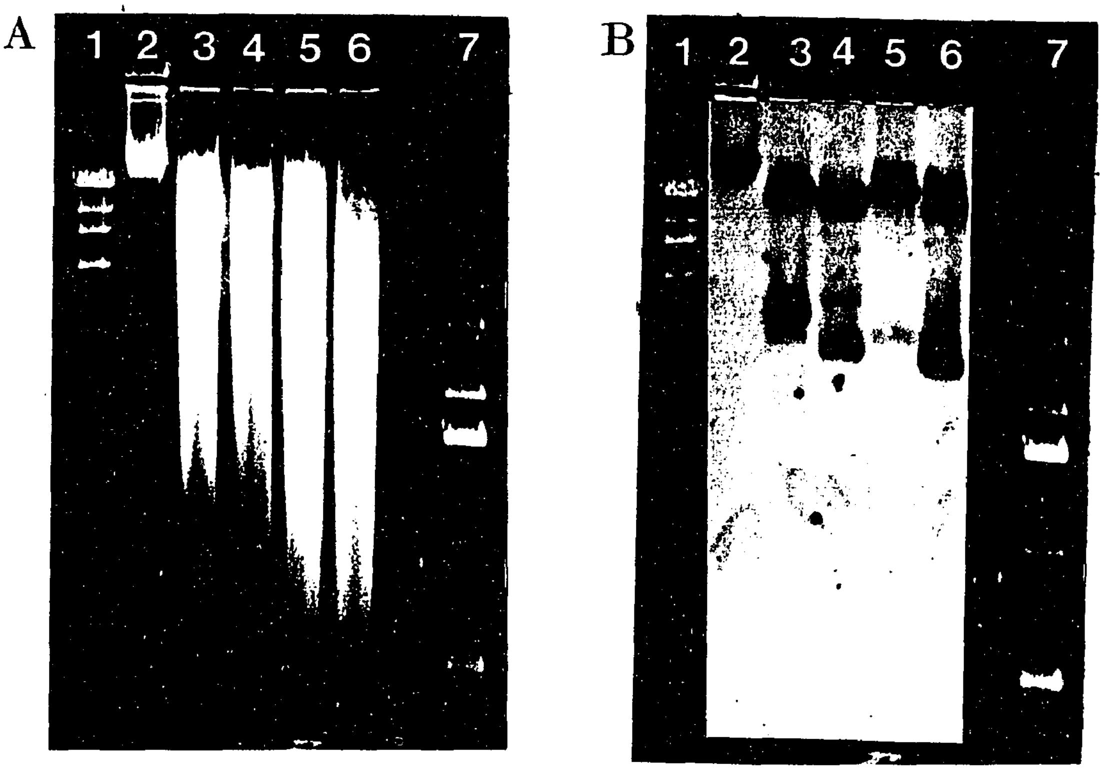
***Esox americanus americanus*, redfin pickerel, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 40. *Esox americanus americanus*, redfin pickerel, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Hin*d III + *Eco*R I digest; 4, *Hin*d III + *Bam*H I digest; 5, *Hin*d III + *Pvu* II digest; 6, *Hin*d III + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes.



***Esox americanus americanus*, redfin pickerel, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 41. *Esox americanus americanus*, redfin pickerel, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Eco*R I + *Bam*H I digest; 4, *Eco*R I + *Pvu* II digest; 5, *Eco*R I + *Pst* I digest; 6, *Eco*R I + *Pvu* II + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes. Large arrow, not hybrid bands.



Esox americanus americanus, redfin pickerel, nrDNA hybridized with _Xenopus laevis_ nrDNA

Figure 42. *Esox americanus americanus*, redfin pickerel, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Bam*H I + *Pvu* II digest; 4, *Bam*H I + *Pst* I digest; 5, *Pvu* II + *Pst* I digest; 6, *Bam*H I + *Pvu* II + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes.

Table 15

***Esox americanus americanus*, Redfin pickerel, rDNA fragments generated by restriction endonuclease digestion.**

Restriction Endonuclease	Fragment sizes in kilobases
*Hin*d III	26.0
*Eco*R I	1.85; 3.5; 17.0
*Bam*H I	3.4; 3.7; 11.7
Pvu II	17.9
Pst I	19.0
*Hin*d III + *Eco*R I	1.85; 3.5; 17.0
*Hin*d III + *Bam*H I	3.2; 3.7; 11.7
*Hin*d III + *Pvu* II	17.9
*Hin*d III + *Pst* I	17.9
*Eco*R I + *Bam*H I	1.2; 1.7, 2.2; 3.75; (8.5)
*Eco*R I + *Pvu* II	1.8; 3.5; 4.1; (9.9)
*Eco*R I + *Pst* I	0.9; 1.85; 3.5; (14.5)
*Eco*R I + *Pvu* II + *Pst* I	0.9; 1.85; 3.1; 3.5; (13.8)
*Bam*H I + *Pvu* II	2.9; 3.4; 11.7
*Bam*H I + *Pst* I	3.1§; 3.7; 11.7
Pvu II + *Pst* I	3.2; 14.8
*Bam*H I + *Pvu* II + *Pst* I	2.8§-3.1§*; 11.7

§fragment lengths were adjusted to match corresponding fragments; *co-migrated; fragments indicated by their length in parenthesis are partial digests.

RESTRICTION ENZYME MAP OF REDFIN PICKEREL, *ESOX AMERICANUS AMERICANUS*, RIBOSOMAL DNA

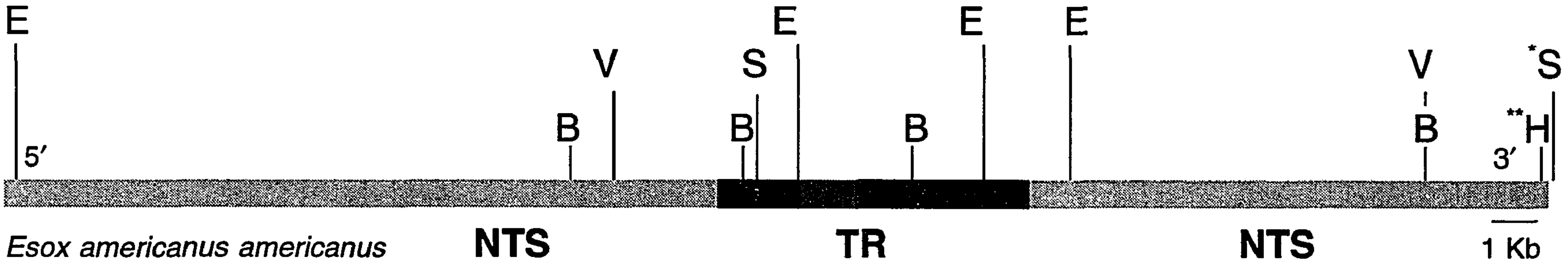
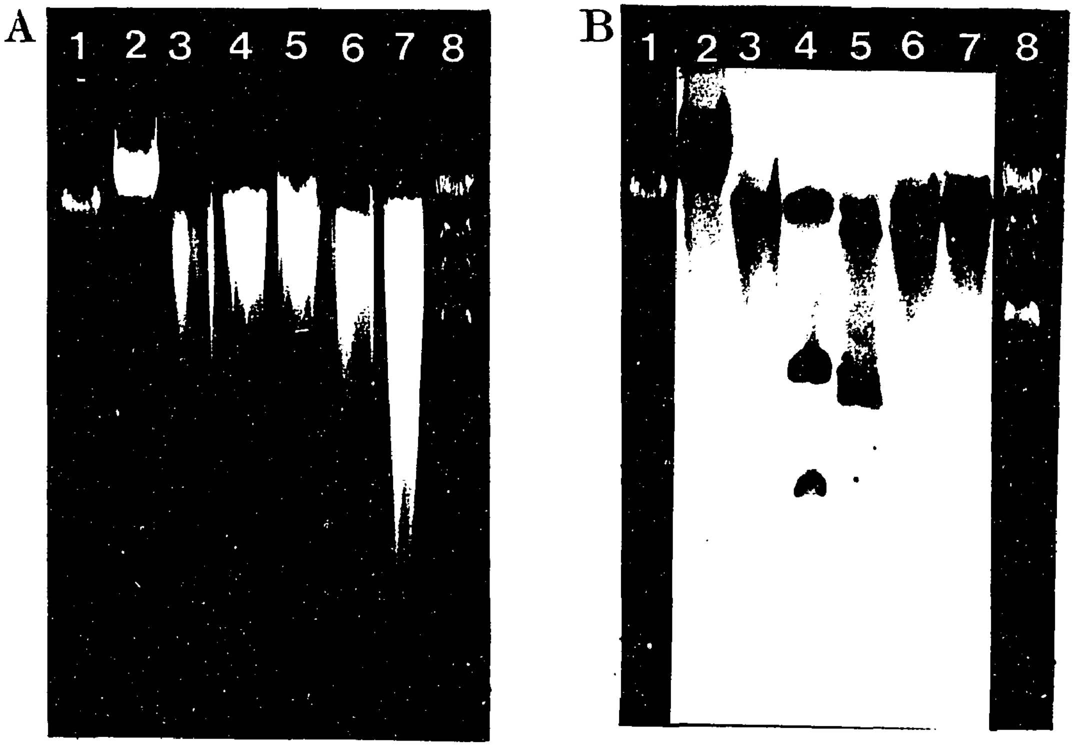


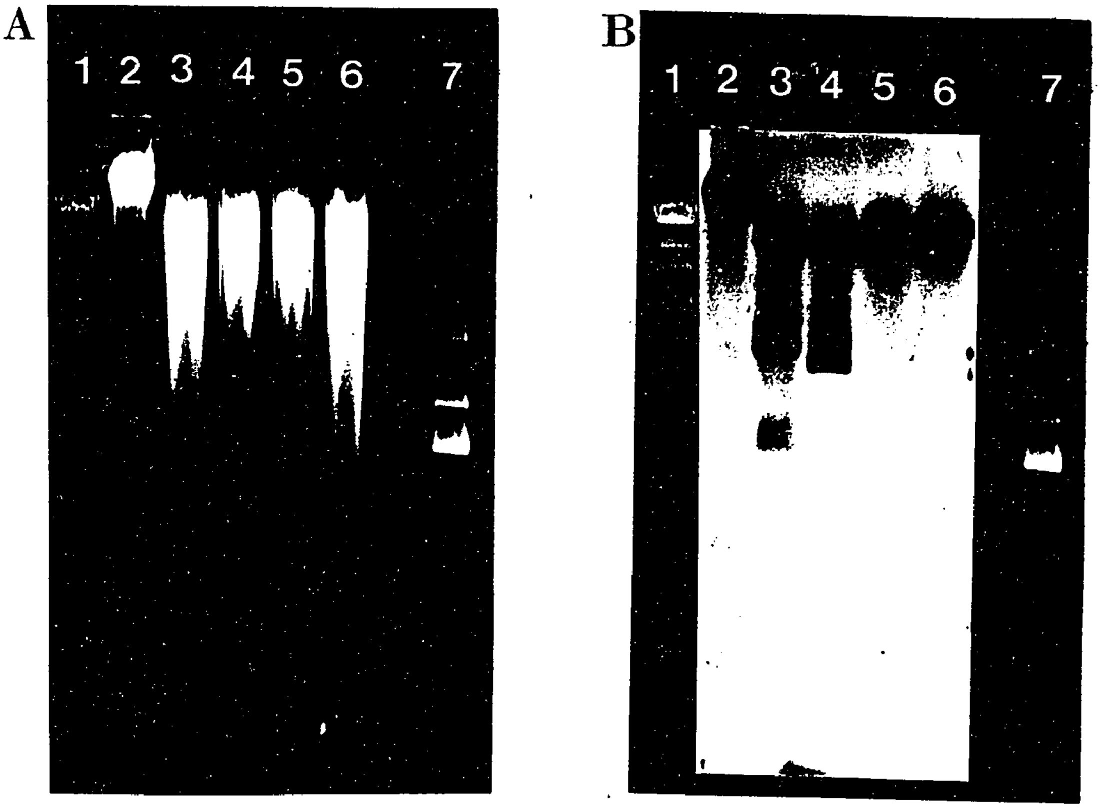
Figure 43. Proposed restriction enzyme map of redfin pickerel ribosomal DNA. B, *Bam*H I; E, *Eco*R I; H, *Hin*d III; S, *Pst* I; V, *Pvu* II; NTS, nontranscribed spacer; TR, transcribed region. *1.6 Kb out-side; **0.5 Kb out-side. Relative positions of the respective restriction enzymes are shown in the map in approximate scale.

Esox niger
Chain pickerel



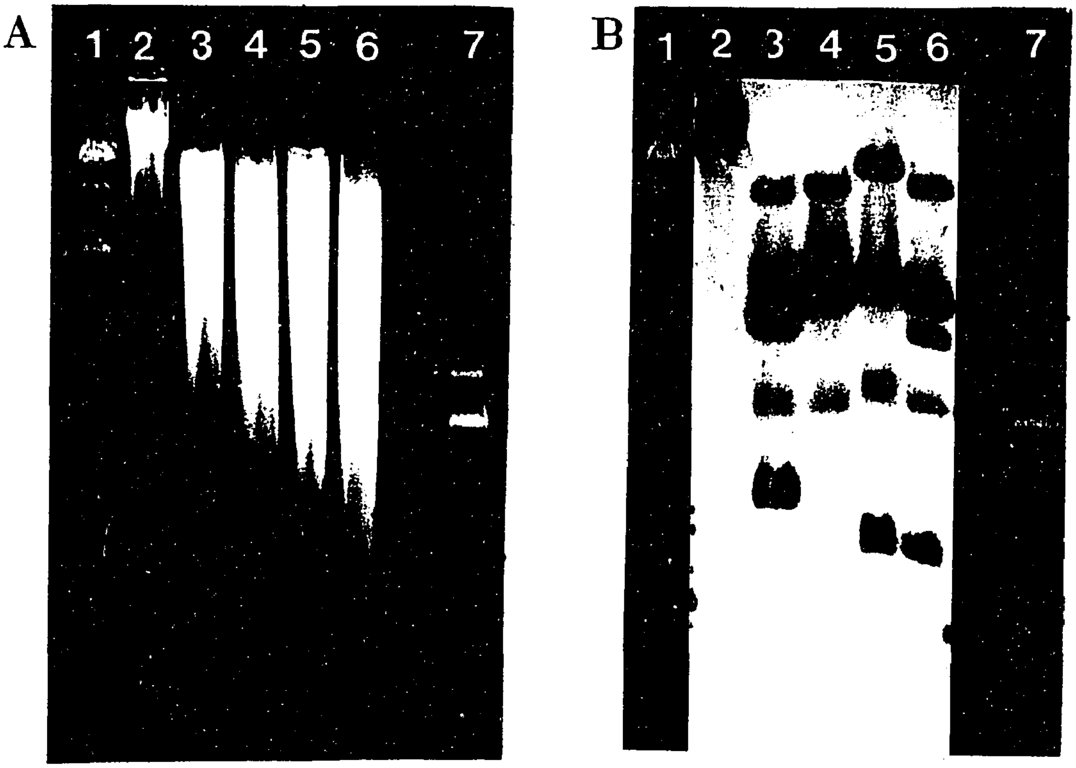
***Esox niger*, chain pickerel, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 44. *Esox niger*, chain pickerel, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-7) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, High Molecular Weight DNA Markers, BRL; 2, undigested; 3, *Hin*d III digest; 4, *Eco*R I digest; 5, *Bam*H I digest; 6, *Pvu* II digest; 7, *Pst* I digest; 8, λ DNA/*Hin*d III Fragments. Both A and B plates represent their actual sizes.



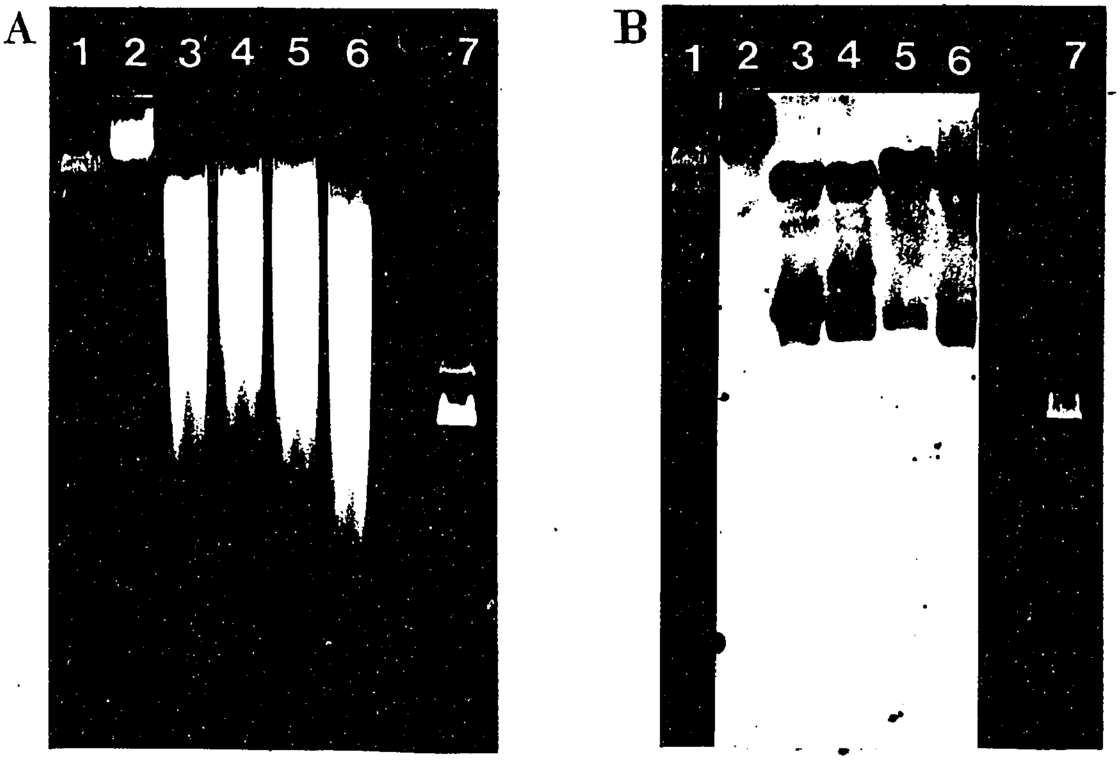
***Esox niger*, chain pickerel, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 45. *Esox niger*, chain pickerel, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Hin*d III + *Eco*R I digest; 4, *Hin*d III + *Bam*H I digest; 5, *Hin*d III + *Pvu* II digest; 6, *Hin*d III + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes.



***Esox niger*, chain pickerel, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 46. *Esox niger*, chain pickerel, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Eco*R I + *Bam*H I digest; 4, *Eco*R I + *Pvu* II digest; 5, *Eco*R I + *Pst* I digest; 6, *Eco*R I + *Pvu* II + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes.



Esox niger, chain pickerel, nrDNA hybridized with Xenopus laevis nrDNA

Figure 47. *Esox niger*, chain pickerel, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Bam*H I + *Pvu* II digest; 4, *Bam*H I + *Pst* I digest; 5, *Pvu* II + *Pst* I digest; 6, *Bam*H I + *Pvu* II + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes.

Table 16

Esox niger, Chain pickerel, rDNA fragments generated by restriction endonuclease digestion.

Restriction Endonuclease	Fragment sizes in kilobases
Hind III	≈ 26.0[*]
*Eco*R I	1.8; 3.5; 14.0
*Bam*H I	3.4; 3.7, 11.7; (15.1)
Pvu II	≈ 17.2[*]
Pst I	19.0
Hind III + *Eco*R I	1.8; 3.5; 14.0
Hind III + *Bam*H I	3.4[**]; 11.7
Hind III + *Pvu* II	17.0[***]
Hind III + *Pst* I	17.0[***]
*Eco*R I + *Bam*H I	1.2; 1.8; 2.3; 3.75; (8.5)
*Eco*R I + *Pvu* II	1.8; 3.5[§]; 4.1; (8.5)
*Eco*R I + *Pst* I	0.9; 1.8; 3.5[§]; (13.5)
*Eco*R I + *Pvu* II + *Pst* I	0.9; [1.1]; 1.8; 2.8[§]; 3.5[§]; (8.5)
*Bam*H I + *Pvu* II	2.4[‡]; 2.8[‡]; 11.7
*Bam*H I + *Pst* I	2.45[‡]; 3.4[‡]; 11.7
Pvu II + *Pst* I	2.5[‡]; 14.0[*]
*Bam*H I + *Pvu* II + *Pst* I	2.1[**,‡]; 11.7

[*]variable; [**]merger of bands; [***]major fragments; [§]length adjusted; fragment lengths in parenthesis are partial digests; fragment length in [ ], mobility delayed; [‡]length adjusted in map.

RESTRICTION ENZYME MAP OF CHAIN PICKEREL, *ESOX NIGER*, RIBOSOMAL DNA

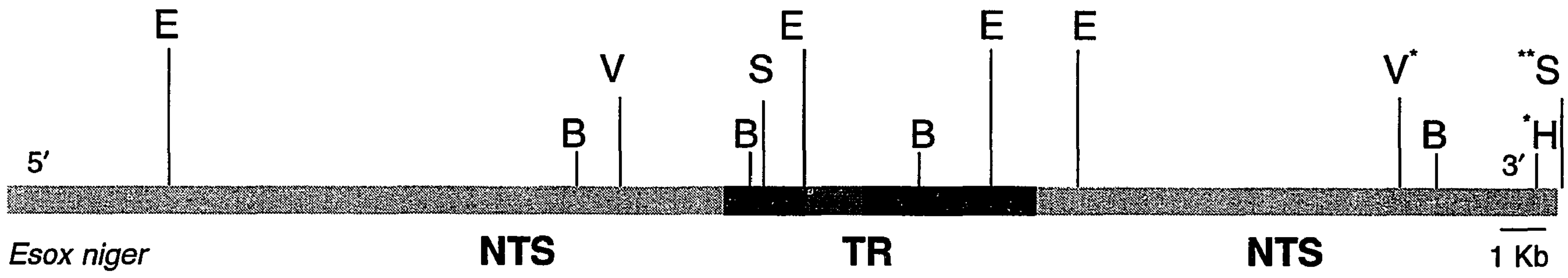
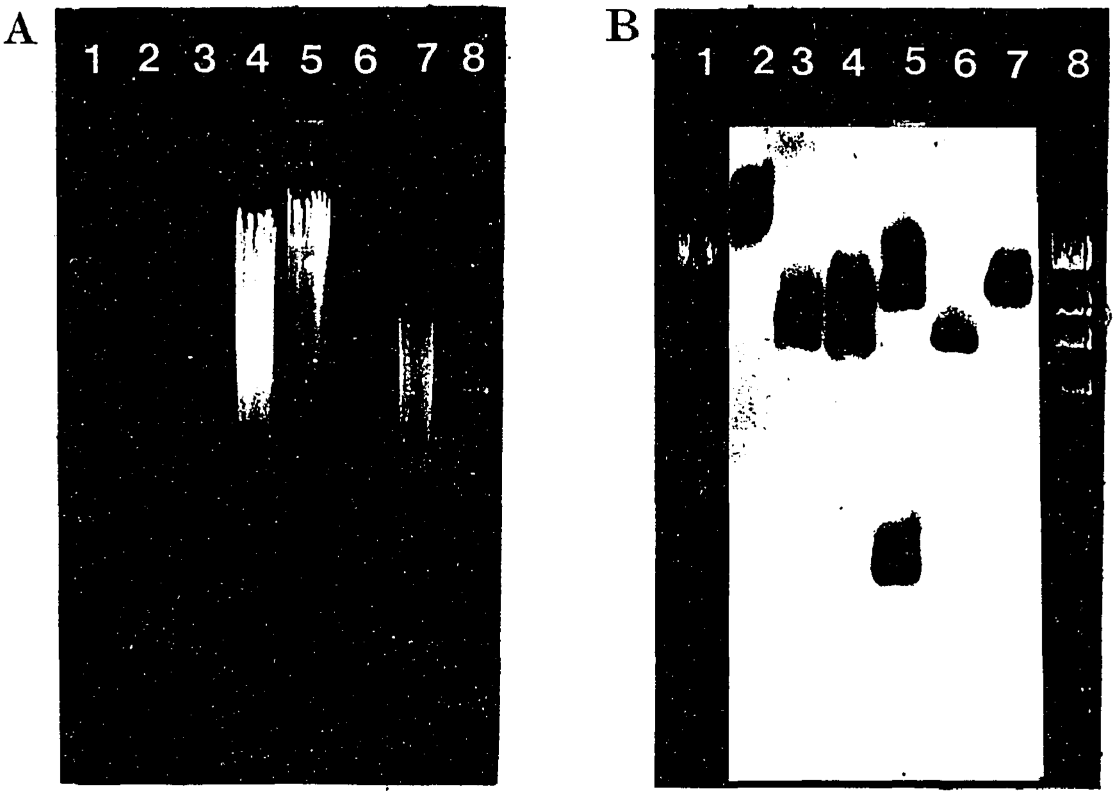


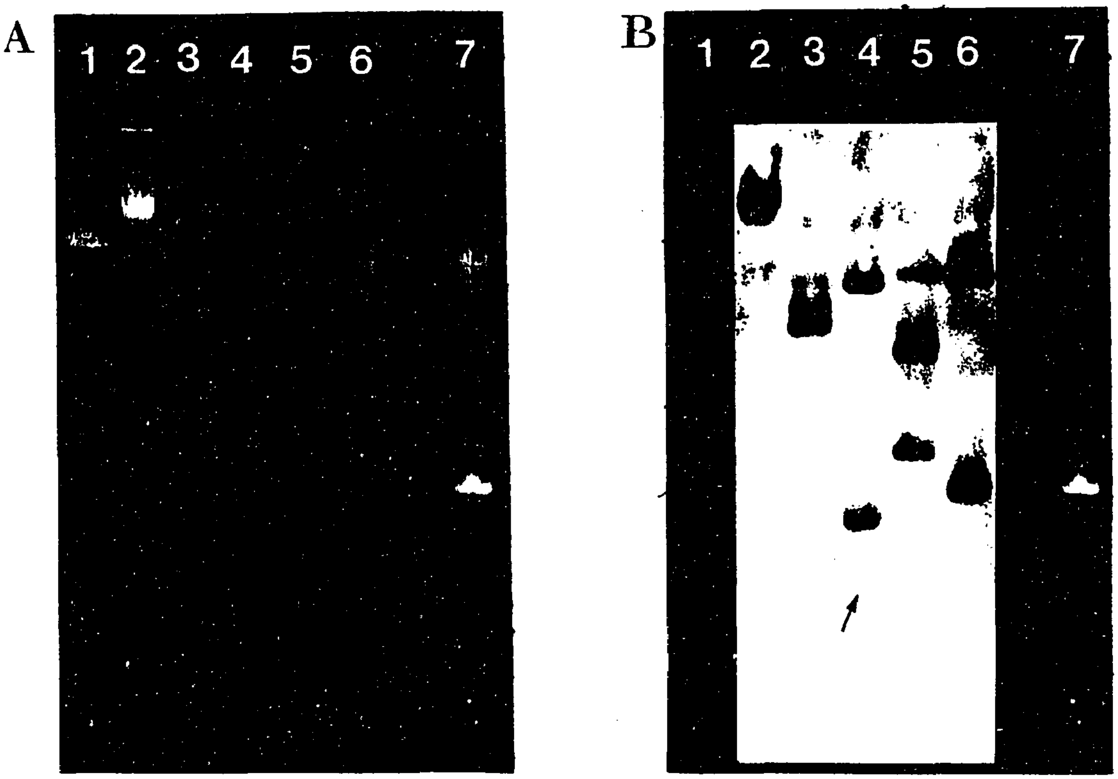
Figure 48. Proposed restriction enzyme map of chain pickerel ribosomal DNA. B, *Bam*H I; E, *Eco*R I; H, *Hind* III; S, *Pst* I; V, *Pvu* II; NTS, nontranscribed spacer; TR, transcribed region. *variable; **1.4 Kb out-side. Relative positions of the respective restriction enzymes are shown in the map in approximate scale.

Umbra pygmaea
Eastern mudminnow,



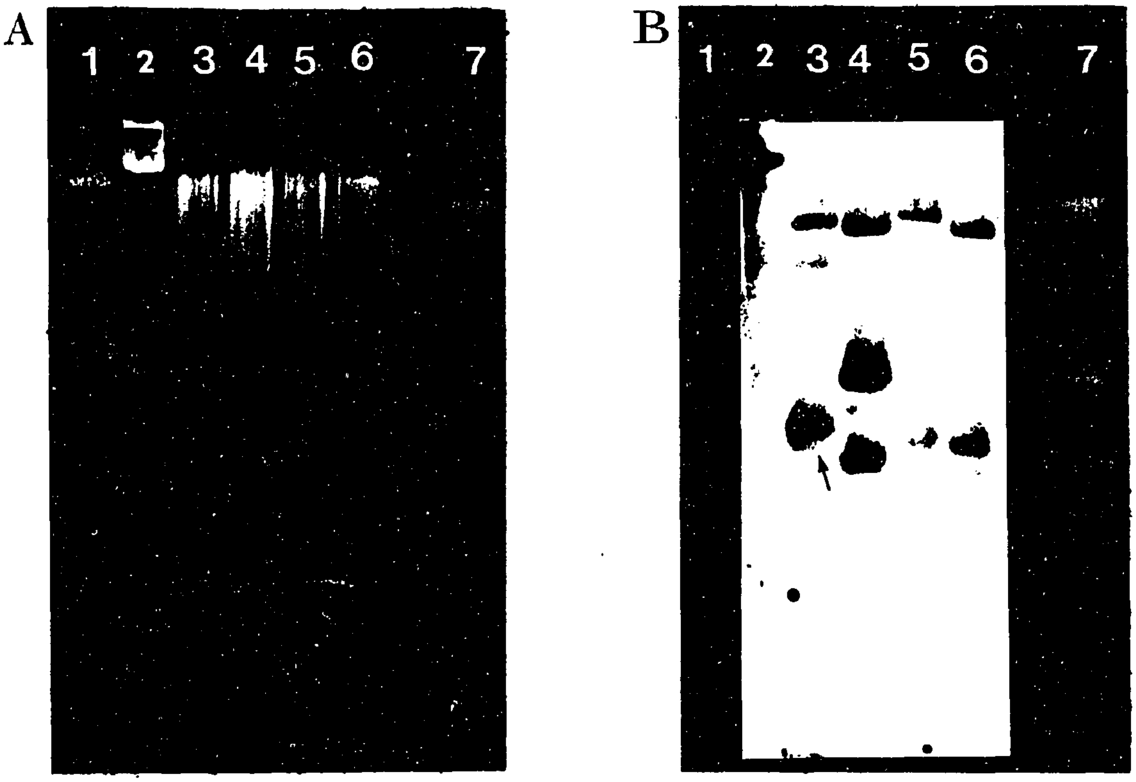
Umbra pygmaea, Eastern mudminnow, nrDNA hybridized with _Xenopus laevis_ nrDNA

Figure 49. *Umbra pygmaea*, eastern mudminnow, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-7) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, High Molecular Weight DNA Markers, BRL; 2, undigested; 3, *Hin*d III digest; 4, *Eco*R I digest; 5, *Bam*H I digest; 6, *Pvu* II digest; 7, *Pst* I digest; 8, λ DNA/*Hin*d III Fragments. Both A and B plates represent their actual sizes.



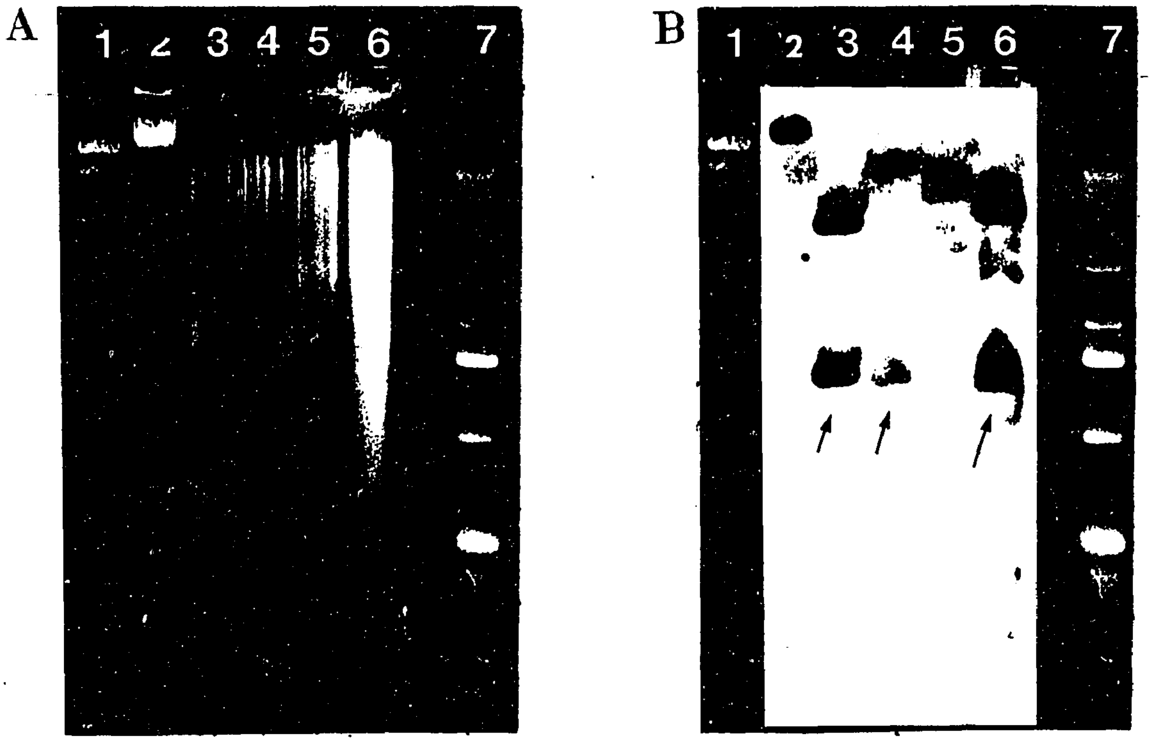
***Umbra pygmaea*, Eastern mudminnow, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 50. *Umbra pygmaea*, eastern mudminnow, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Hin*d III + *Eco*R I digest; 4, *Hin*d III + *Bam*H I digest; 5, *Hin*d III + *Pvu* II digest; 6, *Hin*d III + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates are shown in their actual sizes. Thin arrow points to a light hybrid band.



***Umbra pygmaea*, Eastern mudminnow, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 51. *Umbra pygmaea*, eastern mudminnow, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hind* III Fragments; 2, undigested; 3, *Eco*R I + *Bam*H I digest; 4, *Eco*R I + *Pvu* II digest; 5, *Eco*R I + *Pst* I digest; 6, *Eco*R I + *Pvu* II + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates are shown in their actual sizes. Thin arrow, points to a light hybrid band.



***Umbra pygmaea*, Eastern mudminnow, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 52. *Umbra pygmaea*, eastern mudminnow, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Bam*H I + *Pvu* II digest; 4, *Bam*H I + *Pst* I digest; 5, *Pvu* II + *Pst* I digest; 6, *Bam*H I + *Pvu* II + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates are shown in their actual sizes. Thin arrows point to light hybrid bands.

Table 17

Umbra pygmaea, Eastern mudminnow, rDNA fragments generated by restriction endonucleases

Restriction Endonuclease	Fragment sizes in kilobases
Hind III	8*; 11.8
*Eco*R I	5.7; 13**
*Bam*H I	1.1; 1.5; 14
Pvu II	6.2
Pst I	13
Hind III + *Eco*R I	4.5; 5.7
Hind III + *Bam*H I	1.1; 1.5; 6.0
Hind III + *Pvu* II	2.2; 3.3; 6.2
Hind III + *Pst* I	1.6*; 6.5*
*Eco*R I + *Bam*H I	1.0; 1.25; 5.0***,[1]
*Eco*R I + *Pvu* II	0.9; 1.6; 4.8
*Eco*R I + *Pst* I	1.2; 5.7
*Eco*R I + *Pvu* II + *Pst* I	1.0; 1.2; 4.8
*Bam*H I + *Pvu* II	1.1; 1.5; 3.5
*Bam*H I + *Pst* I	1.1; 1.5; 14
Pvu II + *Pst* I	6.1
*Bam*H I + *Pvu* II + *Pst* I	1.1; 1.5; 4.5***,[2]

*Observed star activity; **variable; ***mobility was possibly affected by bound RE proteins; [1]4.5 Kb is the expected size; [2]3.5 Kb is the expected size.

RESTRICTION ENZYME MAP OF EASTERN MUDMINNOW, *UMBRA PYGMAEA*, RIBOSOMAL DNA

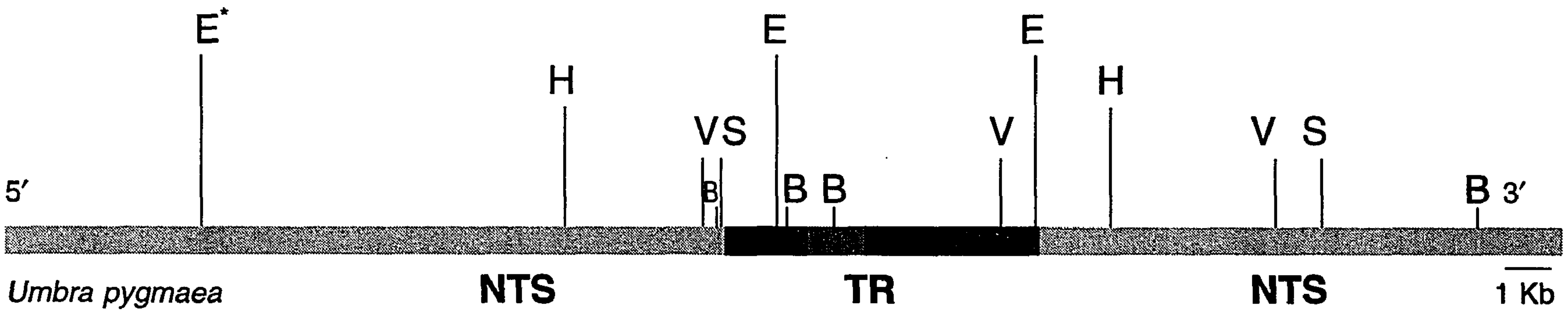
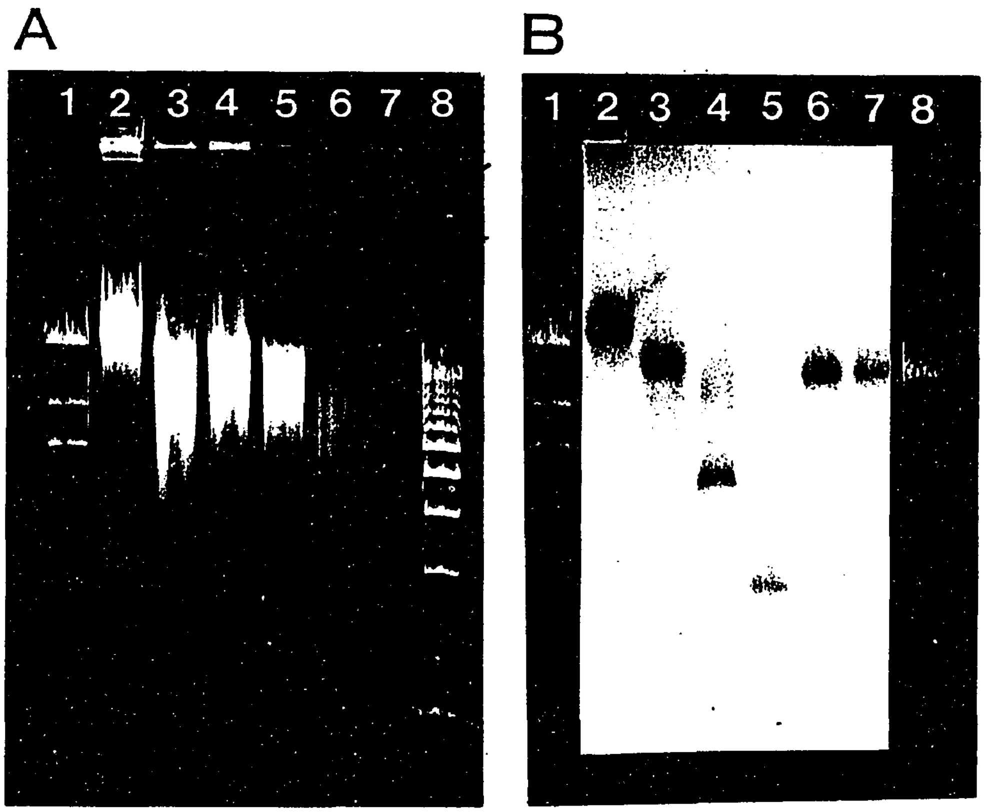


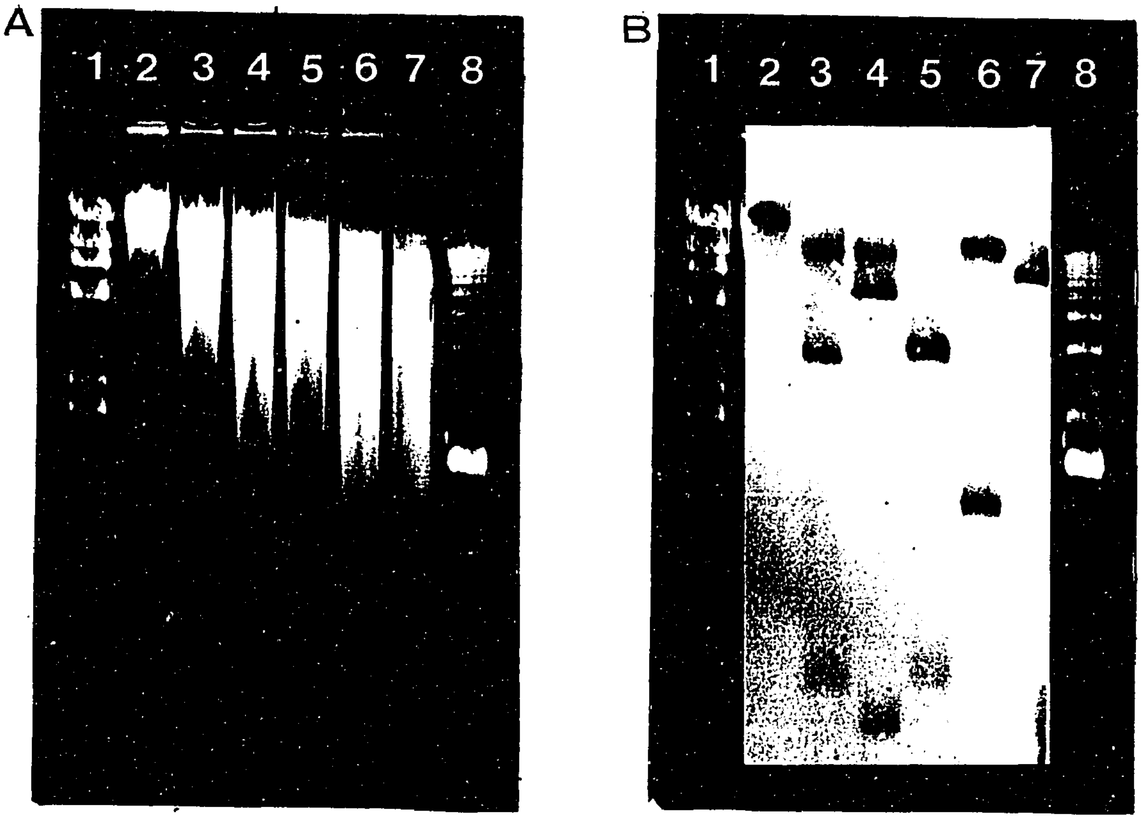
Figure 53. Proposed restriction enzyme map of eastern mudminnow ribosomal DNA. B, *Bam*H I; E, *Eco*R I; H, *Hin*d III; S, *Pst* I; V, *Pvu* II; NTS, nontranscribed spacer; TR, transcribed region; *variable. Relative positions of the respective restriction enzymes are shown in the map in approximate scale.

Osmerus mordax
Rainbow smelt



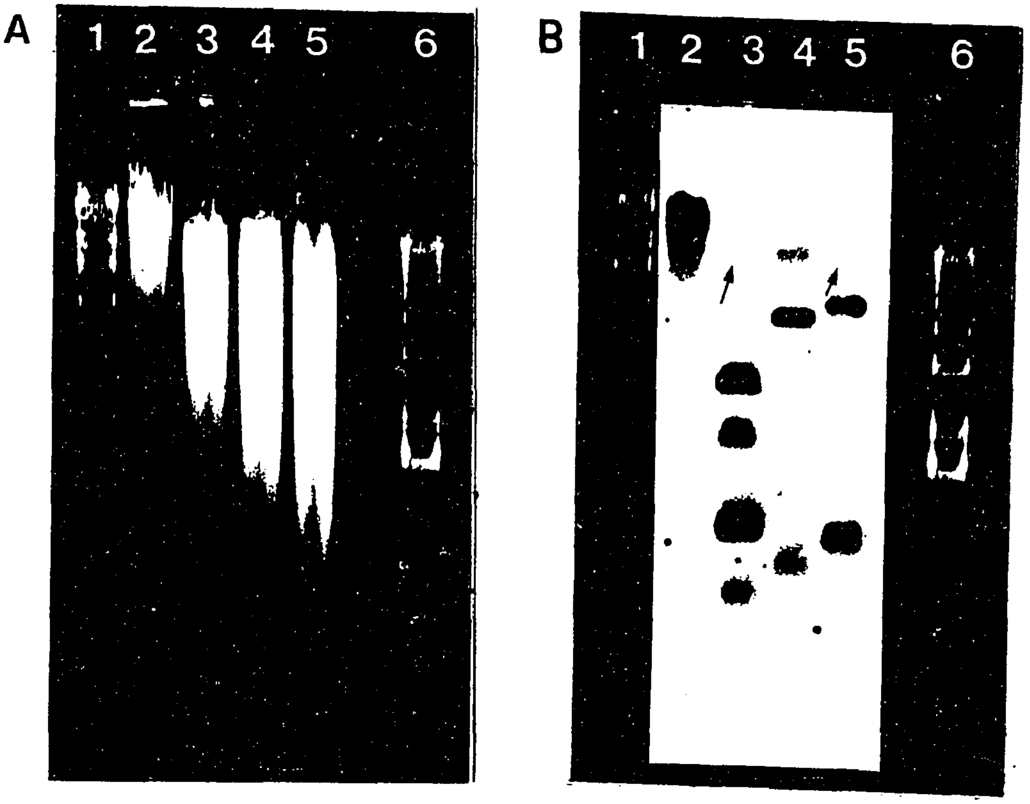
Osmerus mordax, rainbow smelt, nrDNA hybridized with _Xenopus laevis_ nrDNA

Figure 54. *Osmerus mordax*, rainbow smelt, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of rainbow smelt DNA (lanes 2-7) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ/*Hin*d III DNA Fragments; 2, undigested; 3, *Hin*d III digest; 4, *Eco*R I digest; 5, *Bam*H I digest; 6, *Pvu* II digest; 7, *Pst* I digest; 8, 1Kb DNA Ladder. Both A and B plates represent their actual sizes.



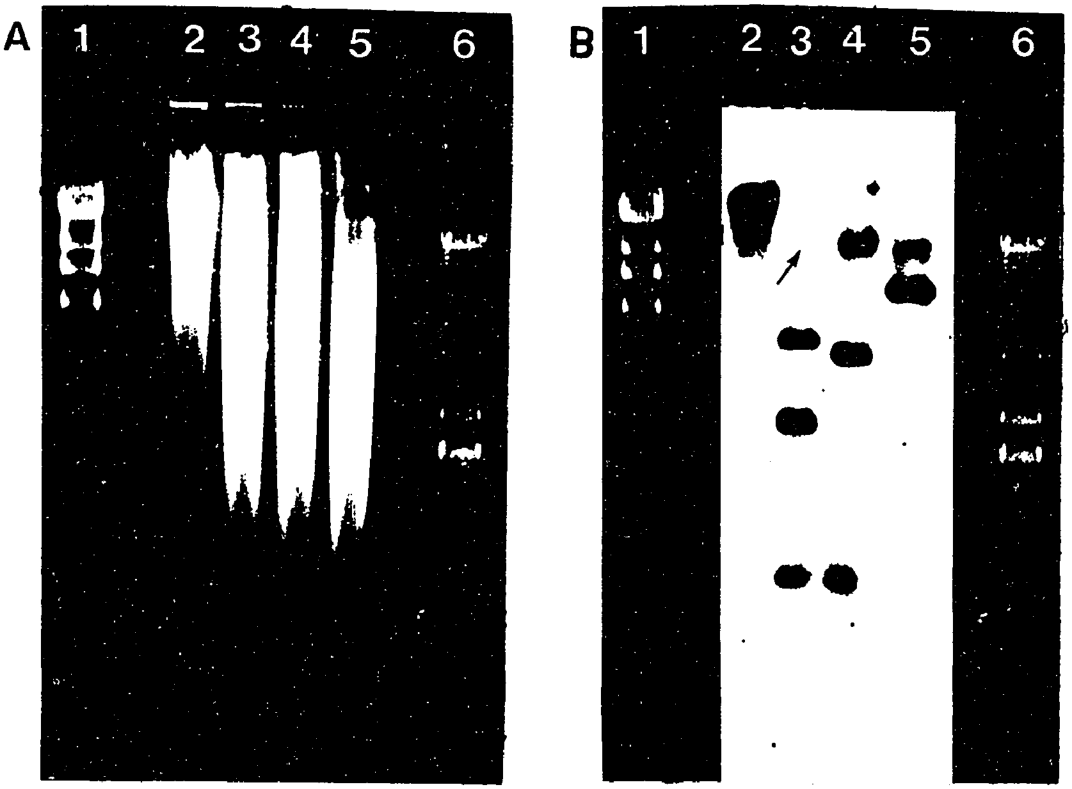
***Osmerus mordax*, rainbow smelt, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 55. *Osmerus mordax*, rainbow smelt, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of rainbow smelt DNA (lanes 2-7) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Bam*H I digest; 4, *Hin*d III + *Eco*R I digest; 5, *Hin*d III + *Bam*H I digest; 6, *Hin*d III + *Pvu* II digest; 7, *Hin*d III + *Pst* I digest; 8, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes.



***Osmerus mordax*, rainbow smelt, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 56. *Osmerus mordax*, rainbow smelt, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of rainbow smelt DNA (lanes 2-5) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Eco*R I + *Bam*H I digest; 4, *Eco*R I + *Pvu* II digest; 5, *Eco*R I + *Pst* I digest; 6, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes. Thin arrows point to light hybrid bands.



Osmerus mordax, rainbow smelt, nrDNA hybridized with *Xenopus laevis* nrDNA

Figure 57. *Osmerus mordax*, rainbow smelt, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of rainbow smelt DNA (lanes 2-5) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Bam*H I + *Pvu* II digest; 4, *Bam*H I + *Pst* I digest; 5, *Pvu* II + *Pst* I digest; 6, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes. Thin arrow points to a light hybrid band.

Table 18

Osmerus mordax, Rainbow smelt, rDNA fragments generated by restriction endonuclease digestion.

Restriction Endonuclease	Fragment sizes in kilobases
*Hin*d III	15.2
*Eco*R I	4.6; ≥ 9.4
*Bam*H I	0.6; 2.9; 9.1
Pvu II	13.0
Pst I	13.2
*Hin*d III + *Eco*R I	0.5; 4.6; 9.4
*Hin*d III + *Bam*H I	0.6; 3.1; 2.9
*Hin*d III + *Pvu* II	1.4; 13.0
*Hin*d III + *Pst* I	6.1
*Eco*R I + *Bam*H I	0.6; 1.2; 1.8, 2.2*; 8.5
*Eco*R I + *Pvu* II	0.8; 3.8*; 9.5
*Eco*R I + *Pst* I	1.0; 4.6; 8.3
*Bam*H I + *Pvu* II	0.6; 1.4*; 2.9*; (10.5)
*Bam*H I + *Pst* I	0.6; 2.8; 10.5
Pvu II + *Pst* I	4.9; 9.1

*length adjusted; fragment length in parenthesis is a partial digest.

RESTRICTION ENZYME MAP OF RAINBOW SMELT, *OSMERUS MORDAX*, RIBOSOMAL DNA

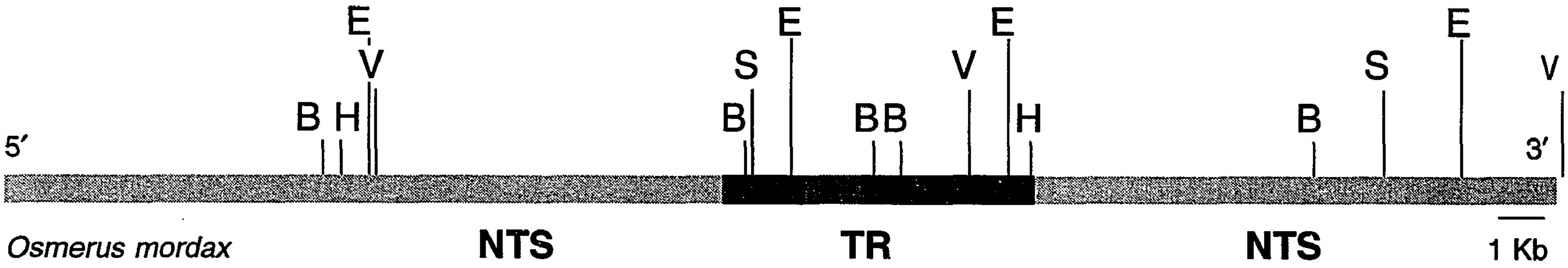
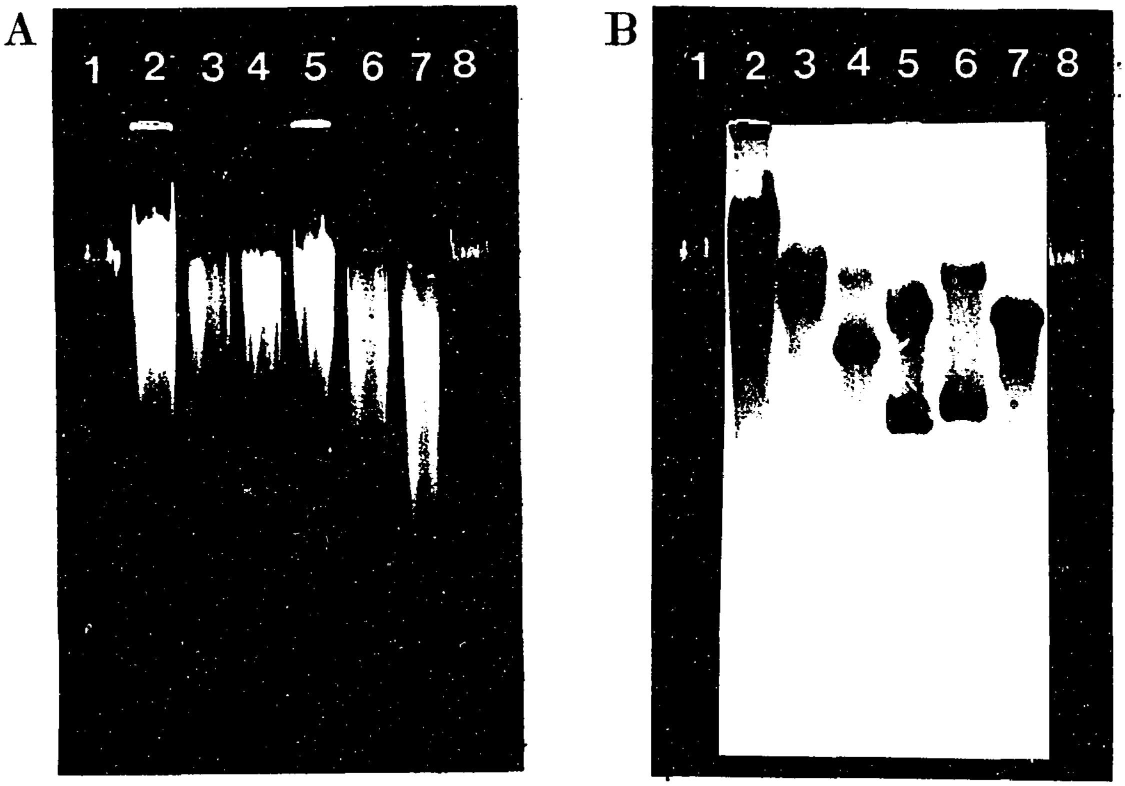


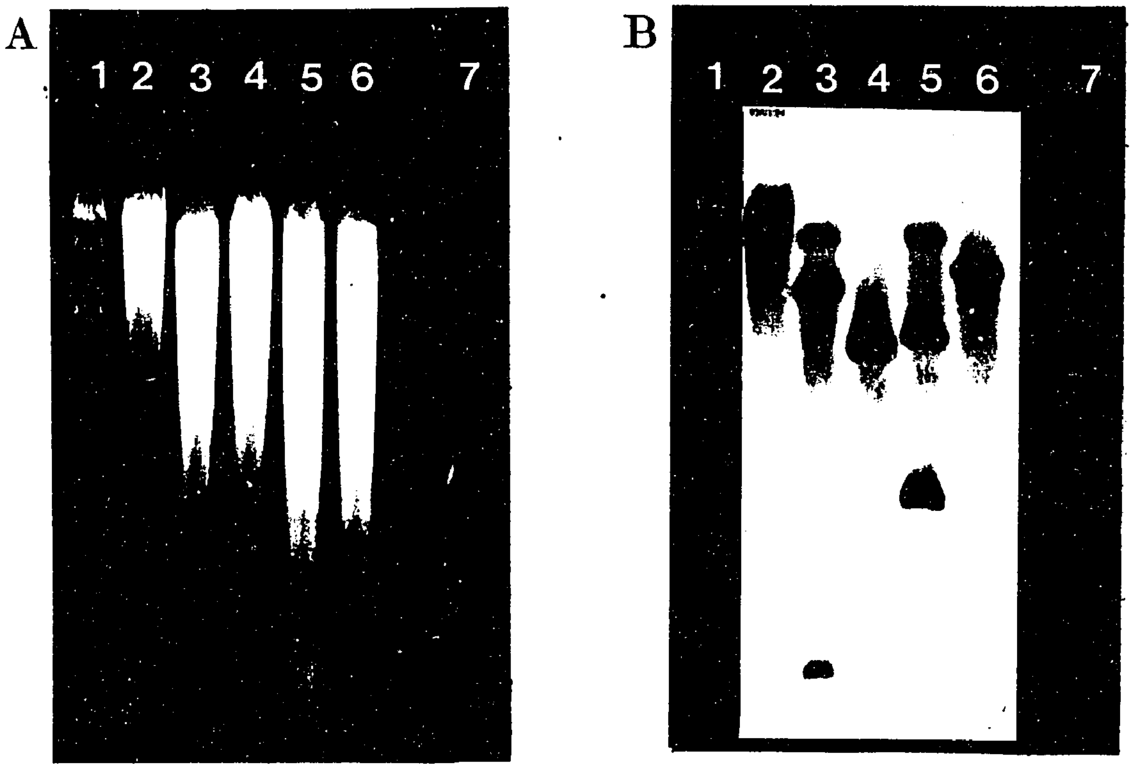
Figure 58. Proposed restriction enzyme map of rainbow smelt ribosomal DNA. B, *Bam*H I; E, *Eco*R I; H, *Hind* III; S, *Pst* I; V, *Pvu* II; NTS, nontranscribed spacer; TR, transcribed region. Relative positions of the respective restriction enzymes are shown in the map in approximate scale.

Coregonus clupeaformis
Lake whitefish



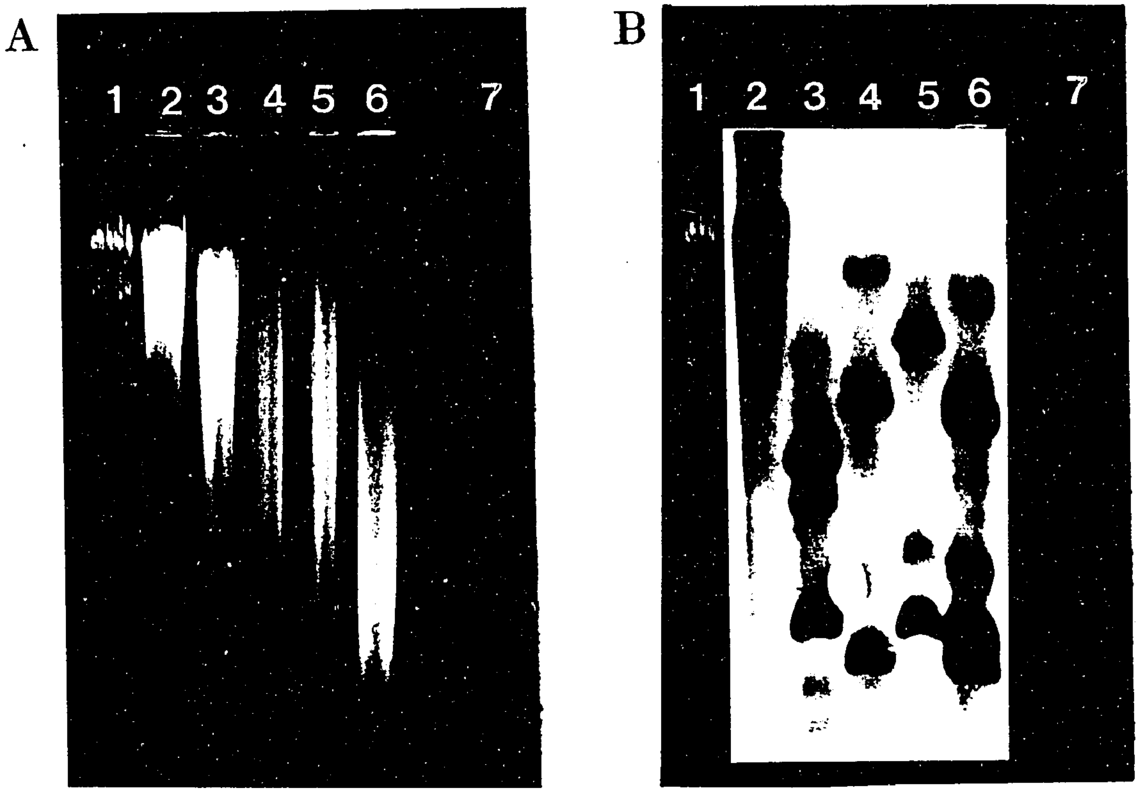
***Coregonus clupeaformis*, lake whitefish, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 59. *Coregonus clupeaformis*, lake whitefish, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-7) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, High Molecular Weight DNA Markers, BRL; 2, undigested; 3, *Hin*d III digest; 4, *Eco*R I digest; 5, *Bam*H I digest; 6, *Pvu* II digest; 7, *Pst* I digest; 8, λ DNA/*Hin*d III Fragments. Both A and B plates represent their actual sizes.



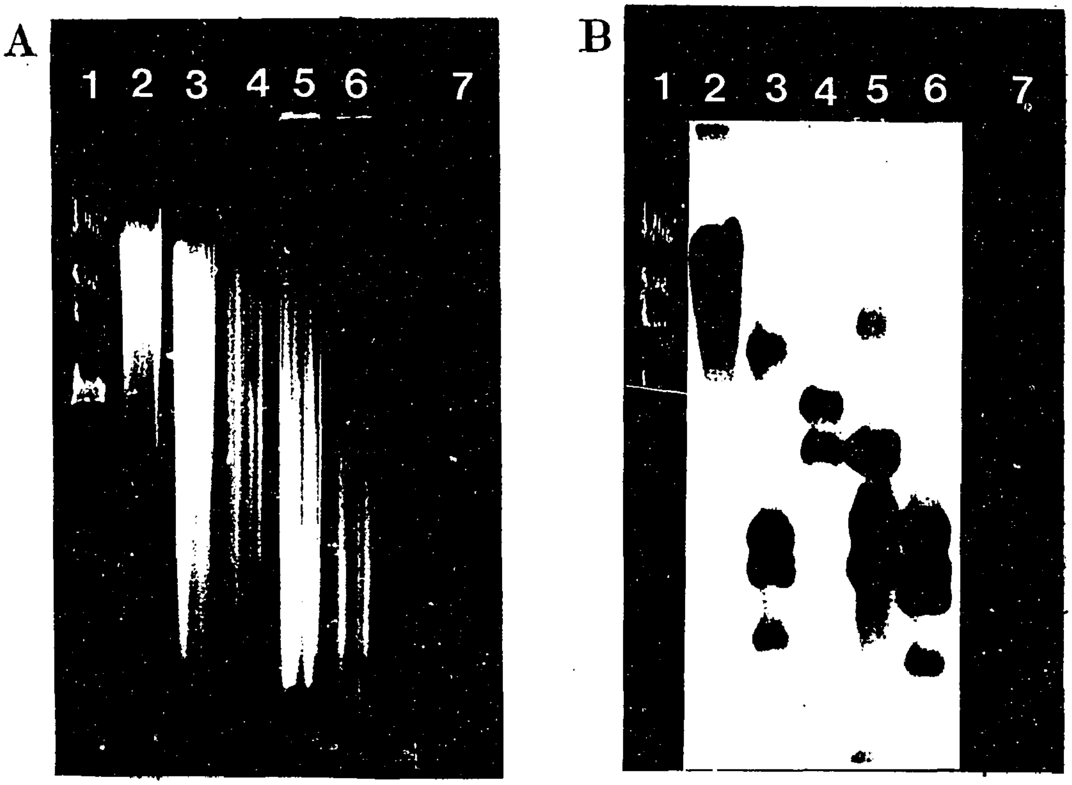
***Coregonus clupeaformis*, lake whitefish, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 60. *Coregonus clupeaformis*, lake whitefish, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Hin*d III + *Eco*R I digest; 4, *Hin*d III + *Bam*H I digest; 5, *Hin*d III + *Pvu* II digest; 6, *Hin*d III + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes.



Coregonus clupeaformis, lake whitefish, nrDNA hybridized with *Xenopus laevis* nrDNA

Figure 61. *Coregonus clupeaformis*, lake whitefish, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hind* III Fragments; 2, undigested; 3, *Eco*R I + *Bam*H I digest; 4, *Eco*R I + *Pvu* II digest; 5, *Eco*R I + *Pst* I digest; 6, *Eco*R I + *Pvu* II + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes.



***Coregonus clupeaformis*, lake whitefish, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 62. *Coregonus clupeaformis*, lake whitefish, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hind* III Fragments; 2, undigested; 3, *Bam*H I + *Pvu* II digest; 4, *Bam*H I + *Pst* I digest; 5, *Pvu* II + *Pst* I digest; 6, *Bam*H I + *Pvu* II + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes.

Table 19

Coregonus clupeaformis, Lake whitefish, rDNA fragments generated by restriction endonucleases

Restriction Endonuclease	Fragment sizes in kilobases
*Hin*d III	23.0*
*Eco*R I	5.8; 18.0
*Bam*H I	3.5; 8.5
Pvu II	3.7; 19.0
Pst I	7.9
*Hin*d III + *Eco*R I	0.7; 5.8; 18.0
*Hin*d III + *Bam*H I	0.7; 3.5
*Hin*d III + *Pvu* II	1.5; 3.7; 17.0
*Hin*d III + *Pst* I	7.0
*Eco*R I + *Bam*H I	0.7; 0.9; 1.15; 1.9**; 2.3; 2.7; (3.65); 5.1
*Eco*R I + *Pvu* II	0.9; 1.0; 3.7; 13.5
*Eco*R I + *Pst* I	0.9; 1.15; 1.65; 5.8
*Eco*R I + *Pvu* II + *Pst* I	0.9; 1.15; 1.65; 3.7; (8.0)
*Bam*H I + *Pvu* II	1.35; 1.9; 2.15; (6.0)
*Bam*H I + *Pst* I	3.1; 4.3
Pvu II + *Pst* I	1.75; 2.4; 3.75; (8.0)
*Bam*H I + *Pvu* II + *Pst* I	1.3; 1.75; 1.9; 2.4

*Variable; **Possibly due to star activity; values in parenthesis are partial digests.

RESTRICTION ENZYME MAP OF LAKE WHITEFISH, *COREGONUS CLUPEAFORMIS*, RIBOSOMAL DNA

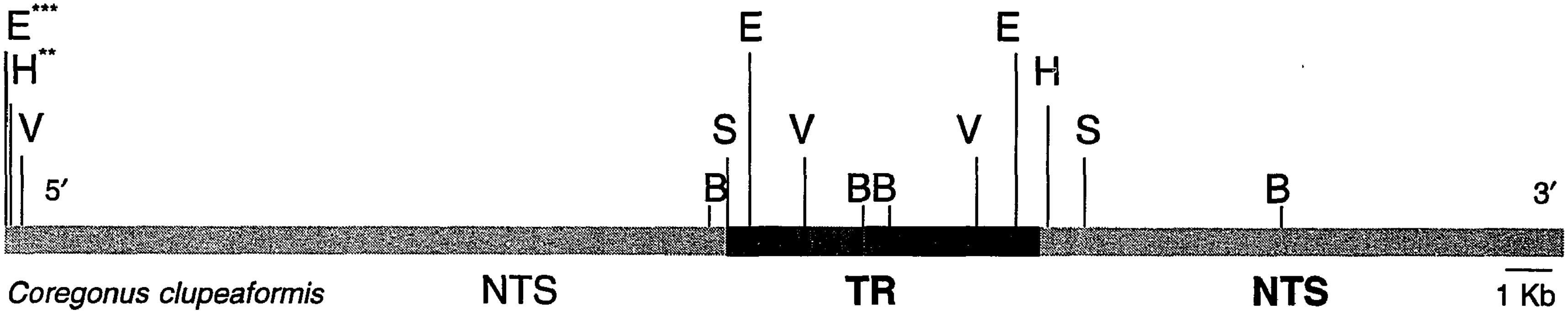
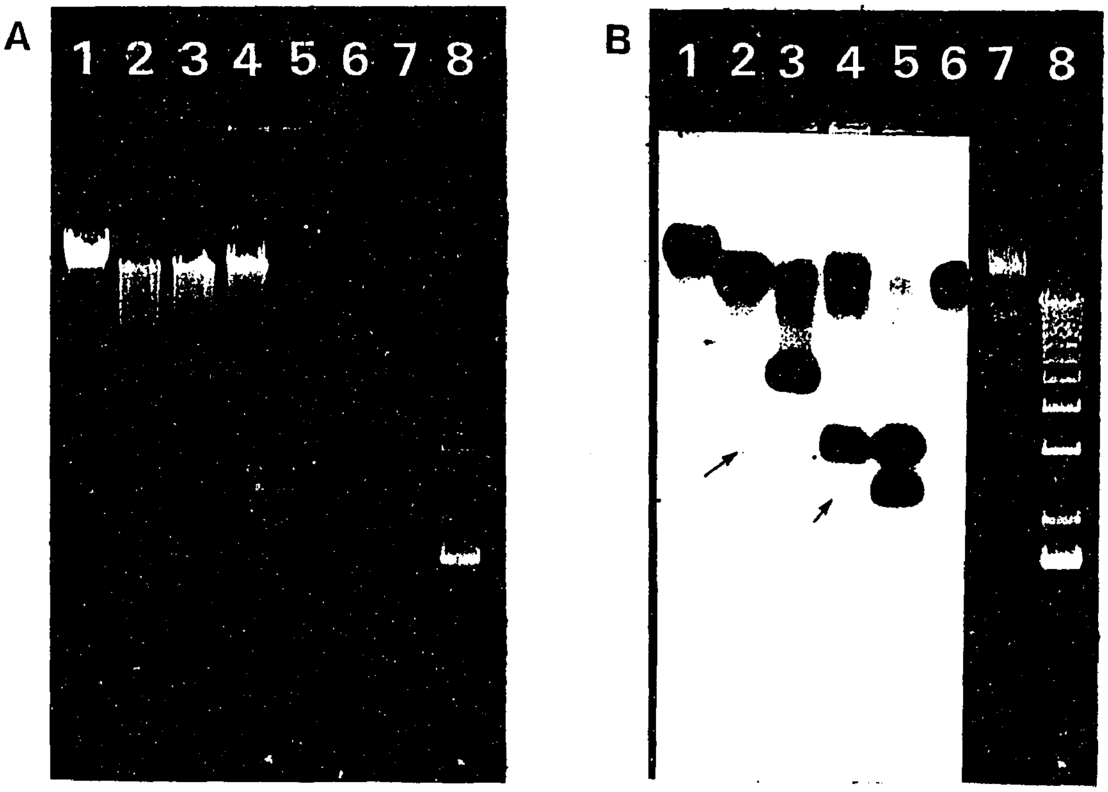


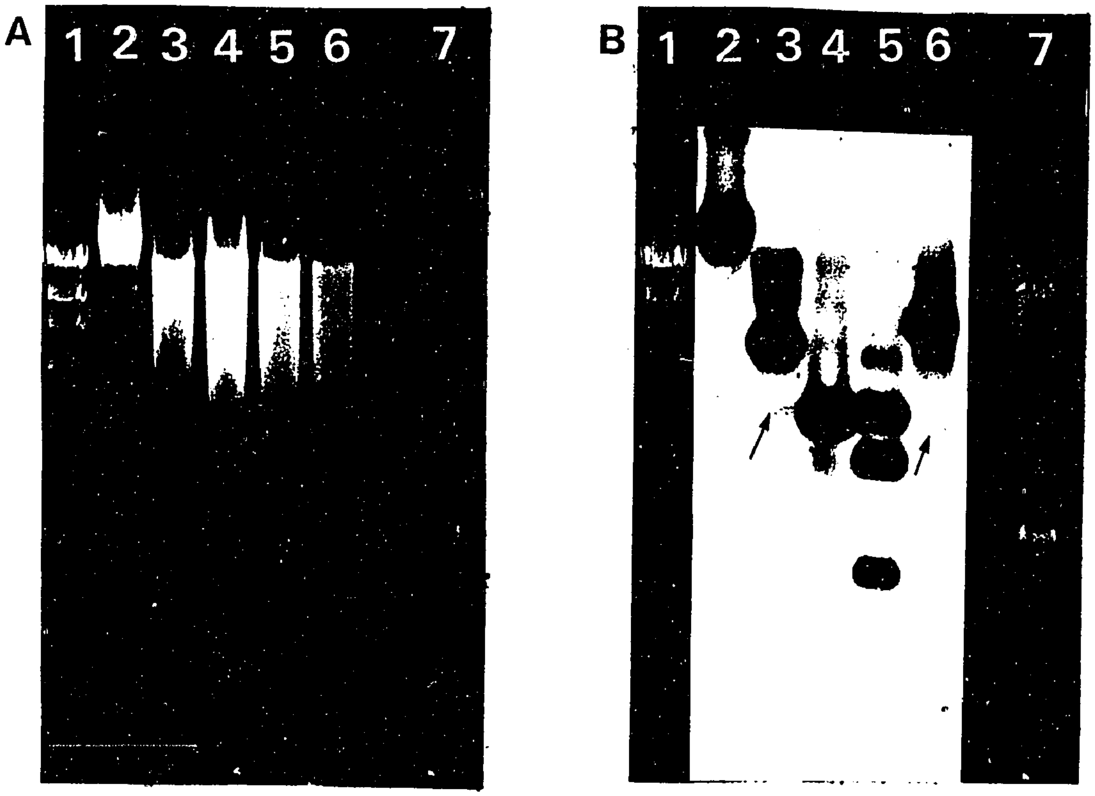
Figure 63. Proposed restriction enzyme map of lake whitefish ribosomal DNA. B, *Bam*H I; E, *Eco*R I; H, *Hin*d III; S, *Pst* I; V, *Pvu* II; NTS, nontranscribed spacer; TR, transcribed region; **should be 0.5 Kb out-side; ***should be 2.5 Kb out-side. Relative positions of the respective restriction enzymes are shown in the map in approximate scale.

Oncorhynchus mykiss
Rainbow trout



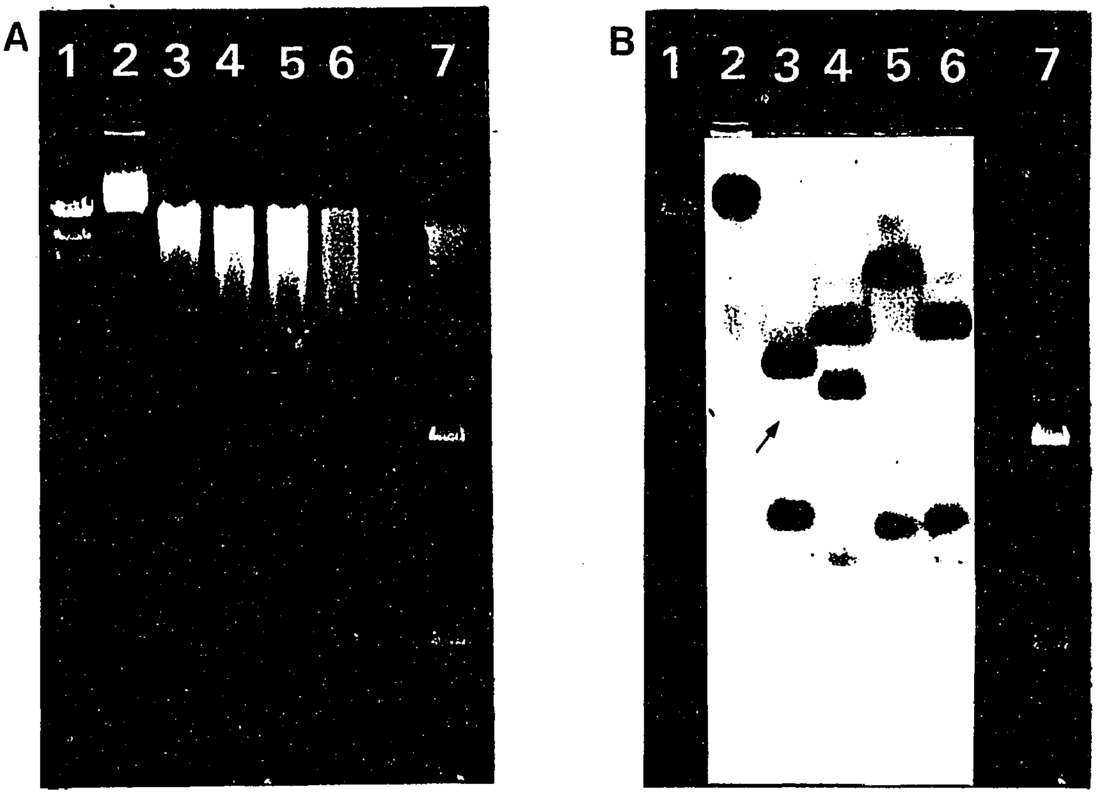
***Oncorhynchus mykiss*, rainbow trout, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 64. *Oncorhynchus mykiss*, rainbow trout, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 1-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, Undigested; 2, *Hin*d III digest; 3, *Eco*R I digest; 4, *Bam*H I digest; 5, *Pvu* II digest; 6, *Pst* I digest; 7, λ DNA/*Hin*d III Fragments; 8, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes. Each thin arrow points to a light hybrid band.



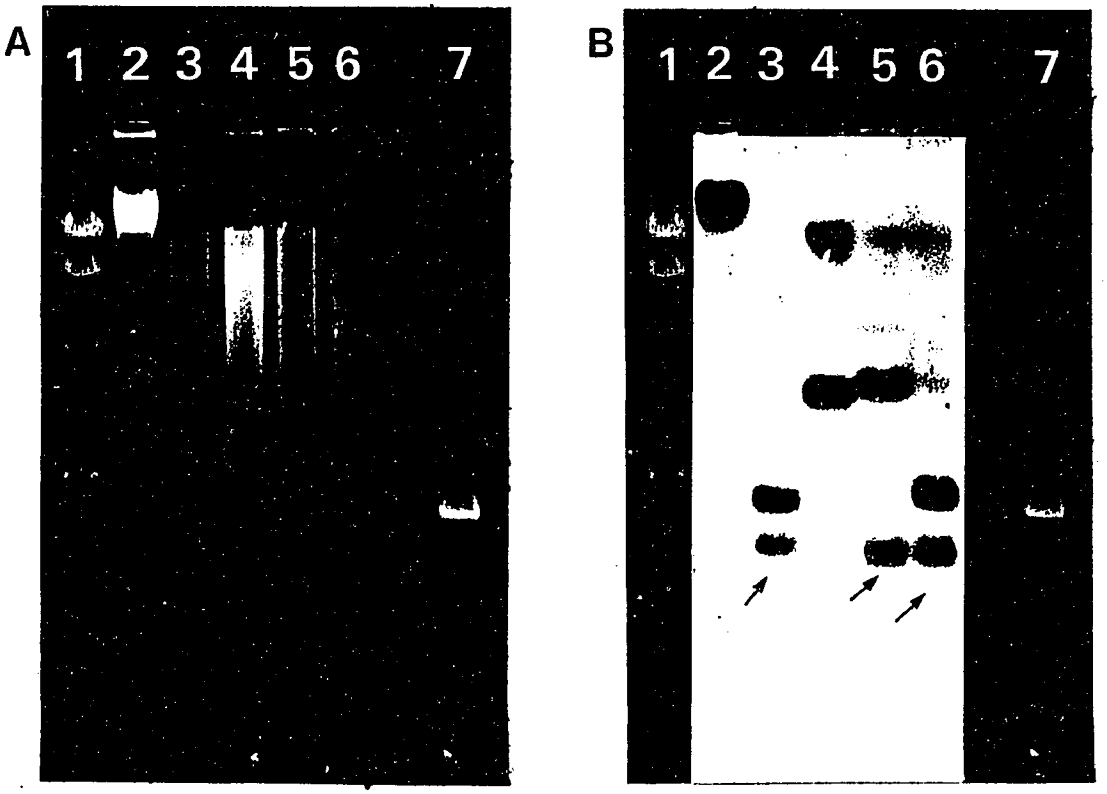
***Oncorhynchus mykiss*, rainbow trout, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 65. *Oncorhynchus mykiss*, rainbow trout, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Hin*d III + *Eco*R I digest; 4, *Hin*d III + *Bam*H I digest; 5, *Hin*d III + *Pvu* II digest; 6, *Hin*d III + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes. Each thin arrow points to a light hybrid band.



Oncorhynchus mykiss, rainbow trout, nrDNA hybridized with _Xenopus laevis_ nrDNA

Figure 66. *Oncorhynchus mykiss*, rainbow trout, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Eco*R I + *Bam*H I digest; 4, *Eco*R I + *Pvu* II digest; 5, *Eco*R I + *Pst* I digest; 6, *Eco*R I + *Pvu* II + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes. Thin arrow points to a light hybrid band.



***Oncorhynchus mykiss*, rainbow trout, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 67. *Oncorhynchus mykiss*, rainbow trout, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Bam*H I + *Pvu* II digest; 4, *Bam*H I + *Pst* I digest; 5, *Pvu* II + *Pst* I digest; 6, *Bam*H I + *Pvu* II + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes. Each thin arrow points to a light hybrid band.

Table 20

***Oncorhynchus mykiss*, Rainbow trout, rDNA fragments generated by restriction endonuclease digestion.**

Restriction Endonuclease	Fragment sizes in kilobases
Hind III	2.8[*]; 19.5
*Eco*R I	4.5; 19.5[**,§]
*Bam*H I	2.3[*]; 3.0; 21.0[**,§]
Pvu II	2.3; 3.2; (4.5); 21[*,**]
Pst I	17.0
Hind III + *Eco*R I	2.8[*]; 4.5; 19.5[**,§]
Hind III + *Bam*H I	2.3; 3.0
Hind III + *Pvu* II	1.3; 2.3; 3.2; (4.8)
Hind III + *Pst* I	2.8[*]; 5.8
*Eco*R I + *Bam*H I	1.1; 1.8[*]; 2.5
*Eco*R I + *Pvu* II	0.8; 2.0; 3.2
*Eco*R I + *Pst* I	0.95; 4.5
*Eco*R I + *Pvu* II + *Pst* I	0.8; 1.0; 3.2
*Bam*H I + *Pvu* II	1.0[*]; 1.3; 1.7
*Bam*H I + *Pst* I	2.8; 21.0[**]
Pvu II + *Pst* I	1.15[*]; 1.2; 3.2; 19[*,**,§]
*Bam*H I + *Pvu* II + *Pst* I	1.0[*]; 1.2; 1.8; (3.2); 19[*,**,§]

[*]very light band; [**]variable; [§]major band; fragment lengths in parenthesis are partial digests.

RESTRICTION ENZYME MAP OF RAINBOW TROUT, *ONCORHYNCHUS MYKISS*, RIBOSOMAL DNA

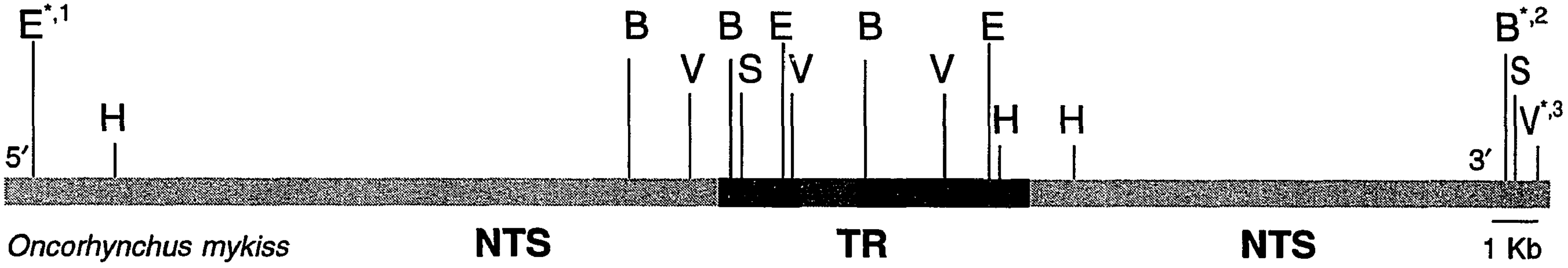
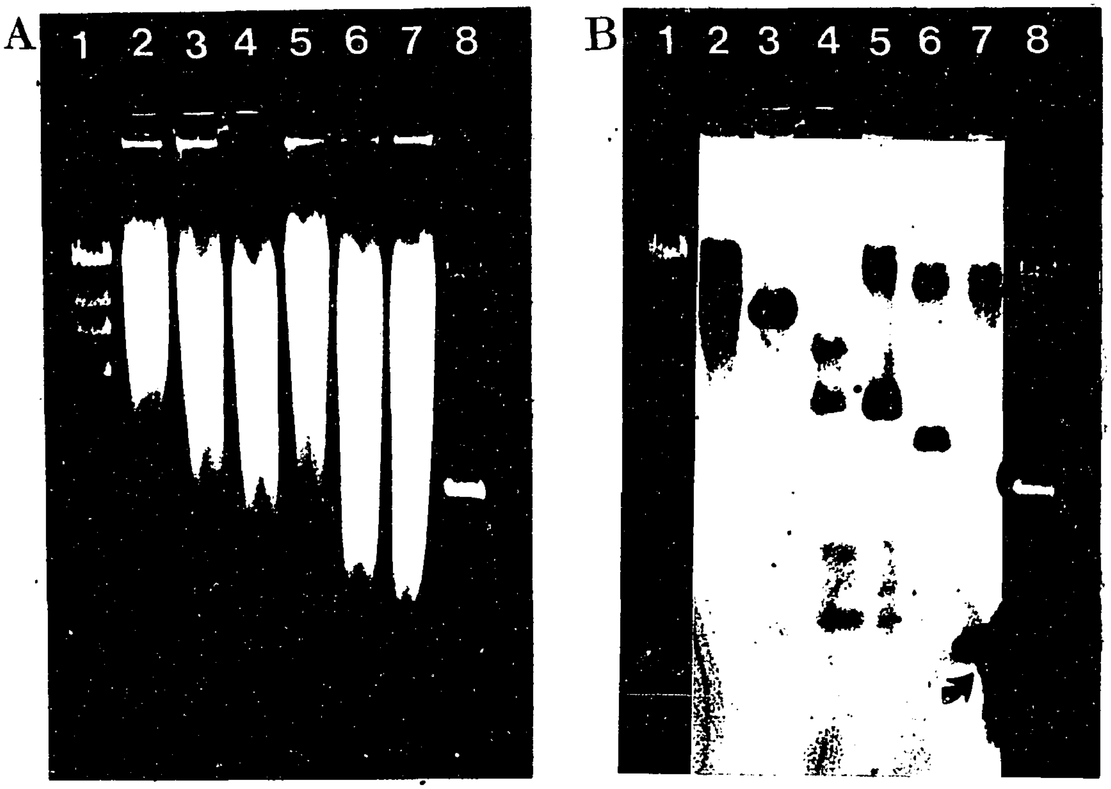


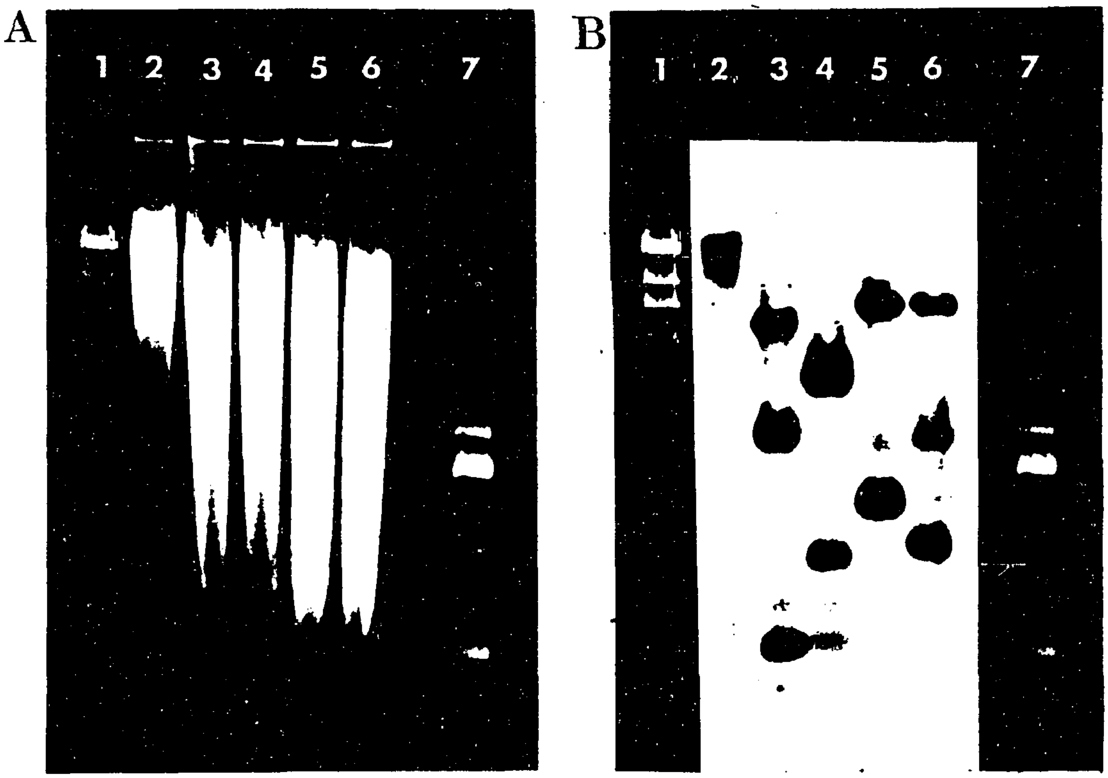
Figure 68. Proposed restriction enzyme map of rainbow trout ribosomal DNA. B, *Bam*H I; E, *Eco*R I; H, *Hin*d III; S, *Pst* I; V, *Pvu* II; NTS, nontranscribed spacer; TR, transcribed region; *variable; [1]major band 3 Kb out-side; [2]major band 7 Kb out-side; [3]major band 8 Kb out-side; Relative positions of the respective restriction enzymes are shown in the map in approximate scale.

Salmo trutta
Brown trout



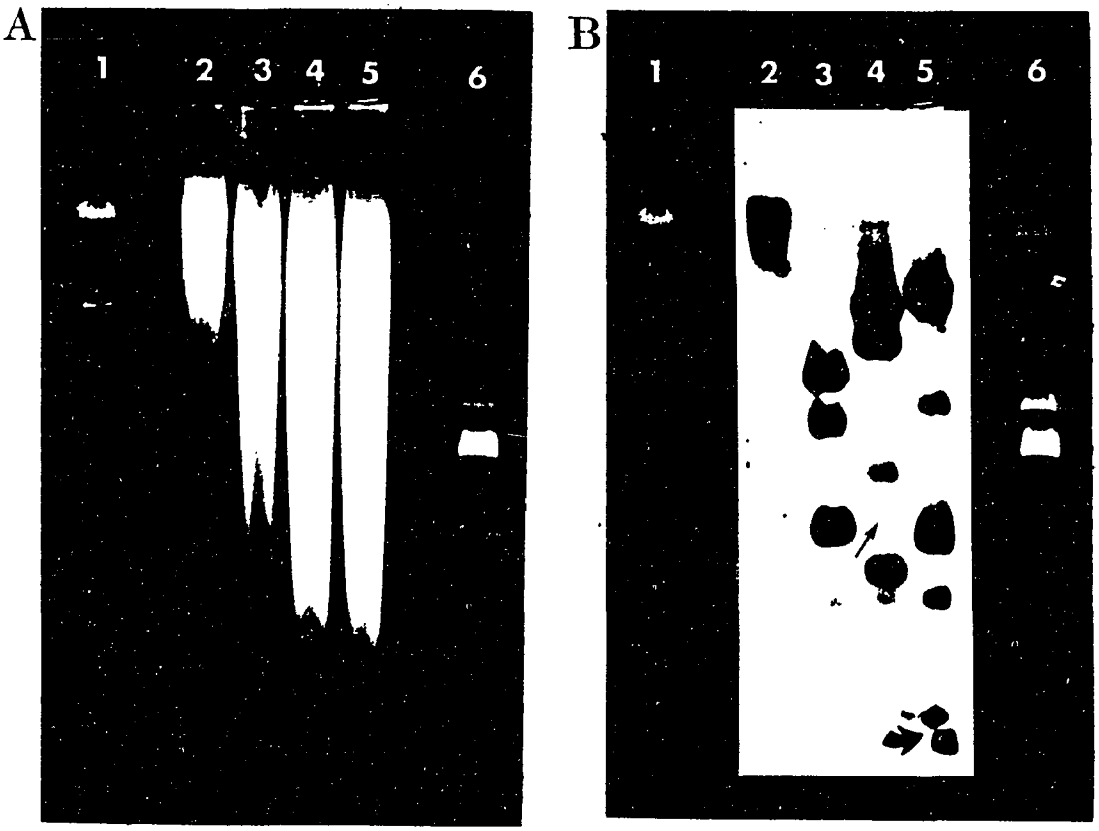
***Salmo trutta*, brown trout, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 69. *Salmo trutta*, brown trout, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-7) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Hin*d III digest; 4, *Eco*R I digest; 5, *Bam*H I digest; 6, *Pvu* II digest; 7, *Pst* I digest; 8, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes. Thick arrow points to is not a hybrid band.



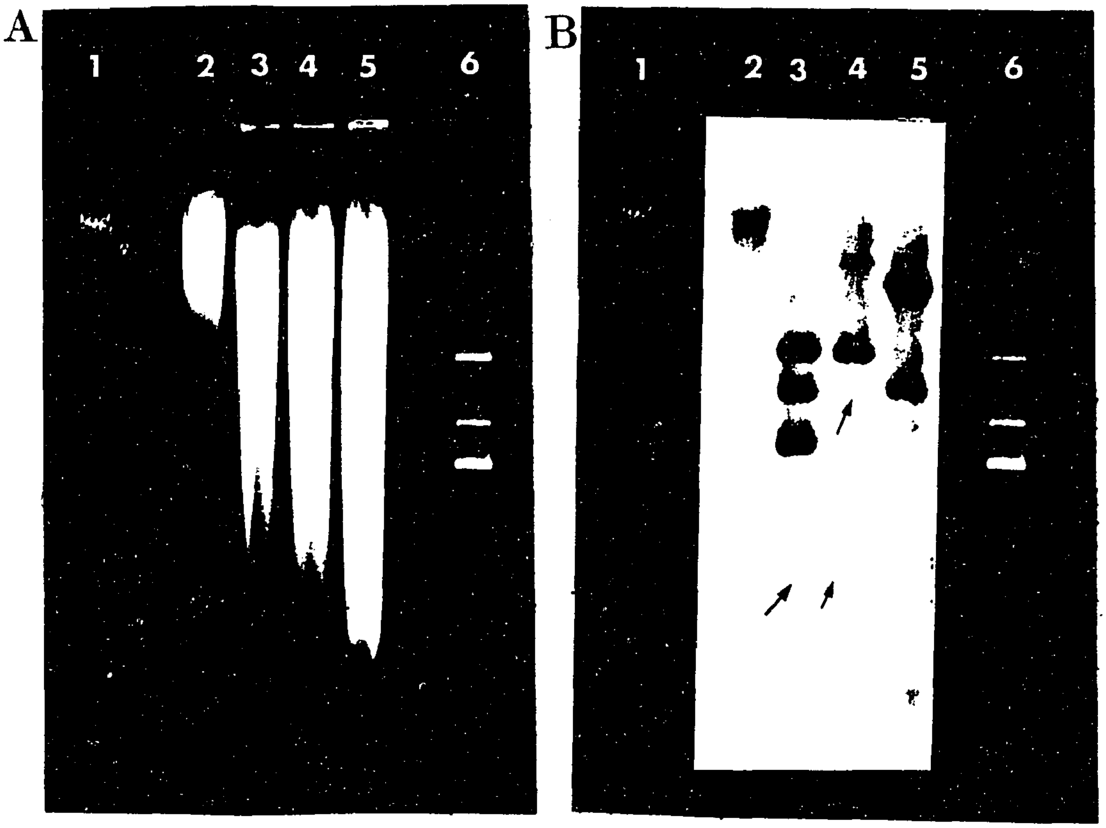
***Salmo trutta*, brown trout, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 70. *Salmo trutta*, brown trout, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hind* III Fragments; 2, undigested; 3, *Hind* III + *Eco*R I digest; 4, *Hind* III + *Bam*H I digest; 5, *Hind* III + *Pvu* II digest; 6, *Hind* III + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes.



***Salmo trutta*, brown trout, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 71. *Salmo trutta*, brown trout, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-5) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Eco*R I + *Bam*H I digest; 4, *Eco*R I + *Pvu* II digest; 5, *Eco*R I + *Pst* I digest; 6, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes. Thin arrow points to a light hybrid band; thick arrow points to not a hybrid band.



Salmo trutta, brown trout, nrDNA hybridized with Xenopus laevis nrDNA

Figure 72. *Salmo trutta*, brown trout, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-5) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hind* III Fragments; 2, undigested; 3, *Bam*H I + *Pvu* II digest; 4, *Bam*H I + *Pst* I digest; 5, *Pvu* II + *Pst* I digest; 6, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes. Each thin arrow points to a light hybrid band.

Table 21

***Salmo trutta*, Brown trout, rDNA fragments generated by restriction endonuclease digestion.**

Restriction Endonuclease	Fragment sizes in kilobases
*Hin*d III	6.7
*Eco*R I	0.7; 1.0; 2.7; 4.2
*Bam*H I	0.7; 2.65; $19.0^{*,\S}$
Pvu II	2.1; $9.6^{*,\S}$
Pst I	$7.9^{*,\S}$
*Hin*d III + *Eco*R I	0.56; 0.7; 1.9; 4.2
*Hin*d III + *Bam*H I	0.56; 0.7; 0.9; 2.65
*Hin*d III + *Pvu* II	1.3; (2.1^{**}); 5.6
*Hin*d III + *Pst* I	0.95; 1.9; 6.0
*Eco*R I + *Bam*H I	0.7; 1.1; 1.7; 2.1
*Eco*R I + *Pvu* II	0.7; 0.9; (1.2^{L}); (1.4); 2.7; 3.65
*Eco*R I + *Pst* I	0.7; 1.0; 1.8; 4.2
*Bam*H I + *Pvu* II	0.56; $1.5^{**,D}$; 2.1^{**}; 2.7; (4.8^{L})
*Bam*H I + *Pst* I	0.56; 2.7^{L}; (3.1); (5.5^{D})
Pvu II + *Pst* I	2.1^{*}; 4.7

*variable; **length adjusted; §major fragment; ^{L}light band; ^{D}mobility delayed; fragment lengths in parenthesis are partial digests.

RESTRICTION ENZYME MAP OF BROWN TROUT, *SALMO TRUTTA*, RIBOSOMAL DNA

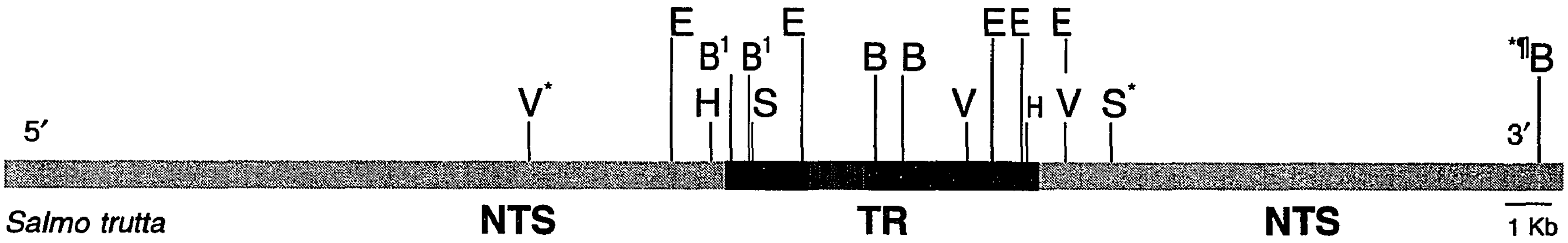
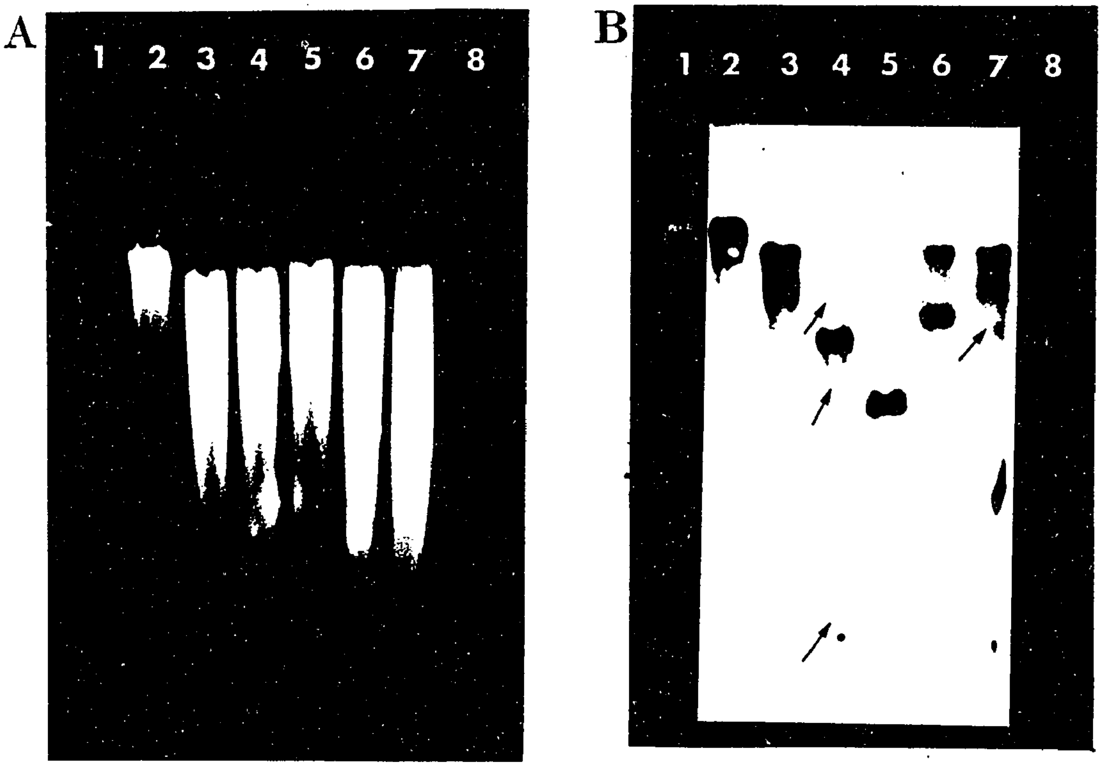


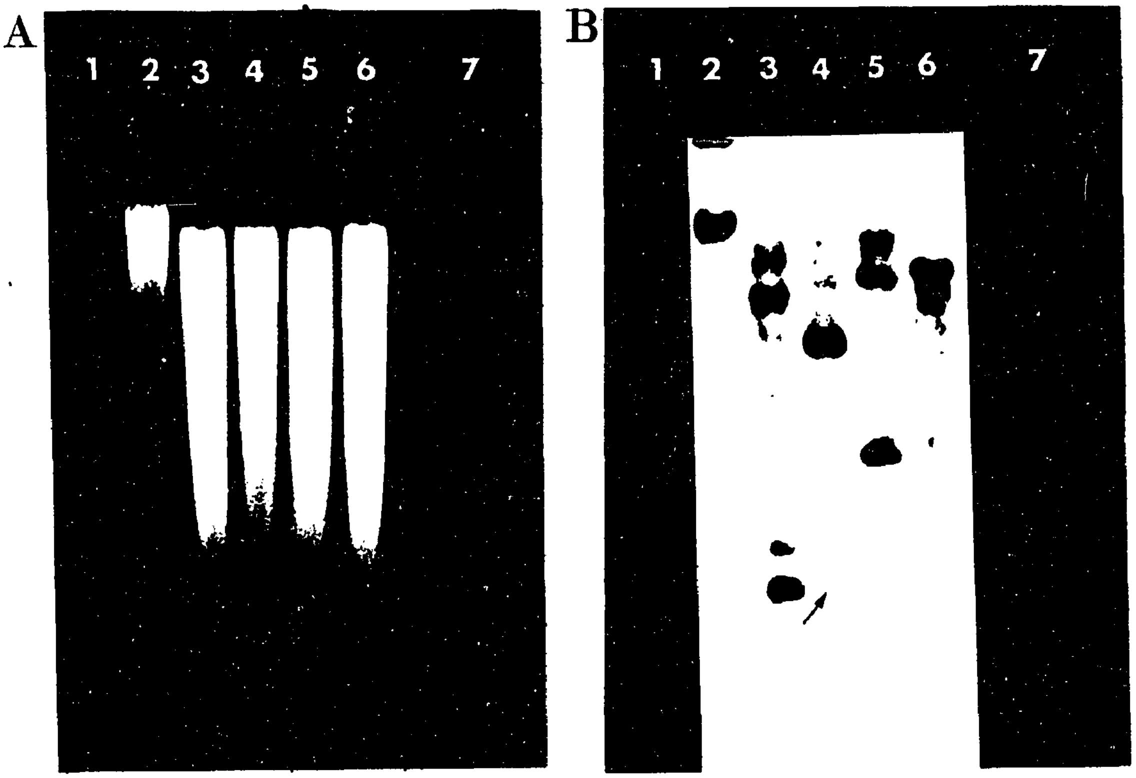
Figure 73. Proposed restriction enzyme map of brown trout ribosomal DNA. B, *Bam*H I; E, *Eco*R I; H, *Hind* III; S, *Pst* I; V, *Pvu* II; NTS, nontranscribed spacer; TR, transcribed region; ^{1}alternate forms; *variable; ¶major position 5.1 Kb outside. Relative positions of the respective restriction enzymes are shown in the map in approximate scale.

Salvelinus fontinalis
Brook trout



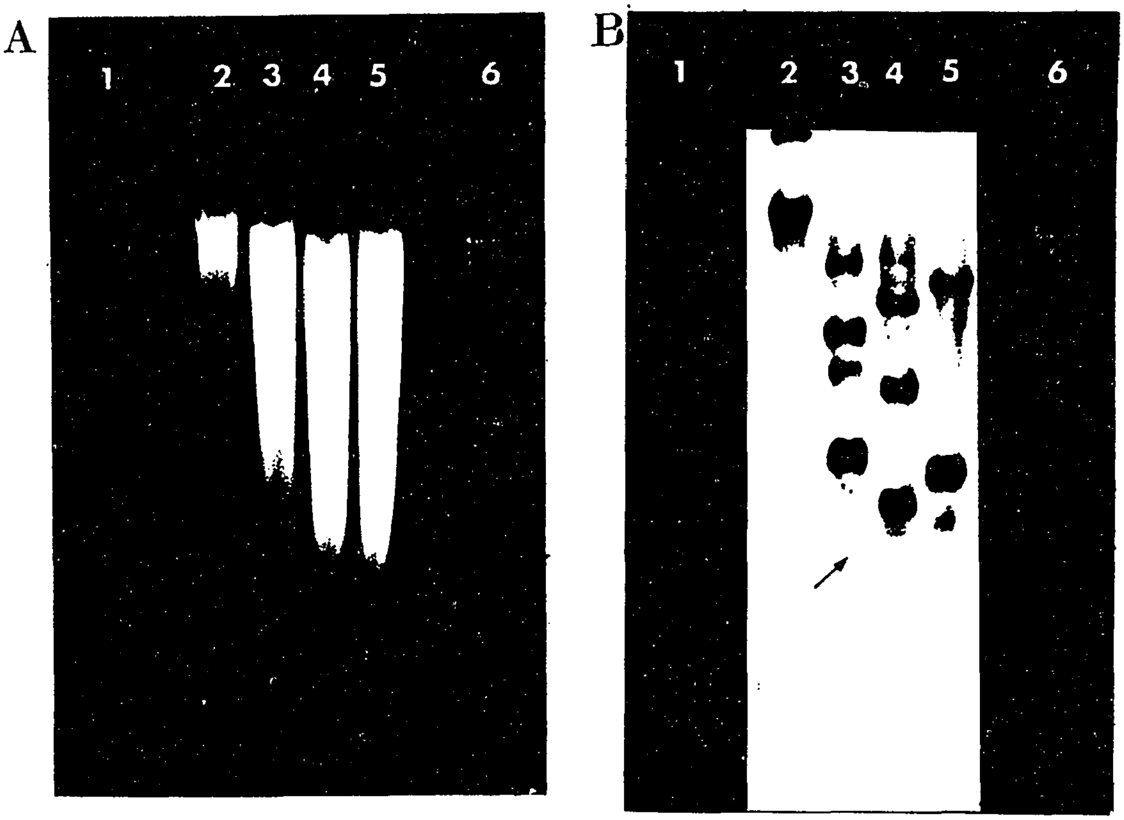
***Salvelinus fontinalis*, brook trout, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 74. *Salvelinus fontinalis*, brook trout, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-7) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Hin*d III digest; 4, *Eco*R I digest; 5, *Bam*H I digest; 6, *Pvu* II digest; 7, *Pst* I digest; 8, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes. Each thin arrow points to a light hybrid band.



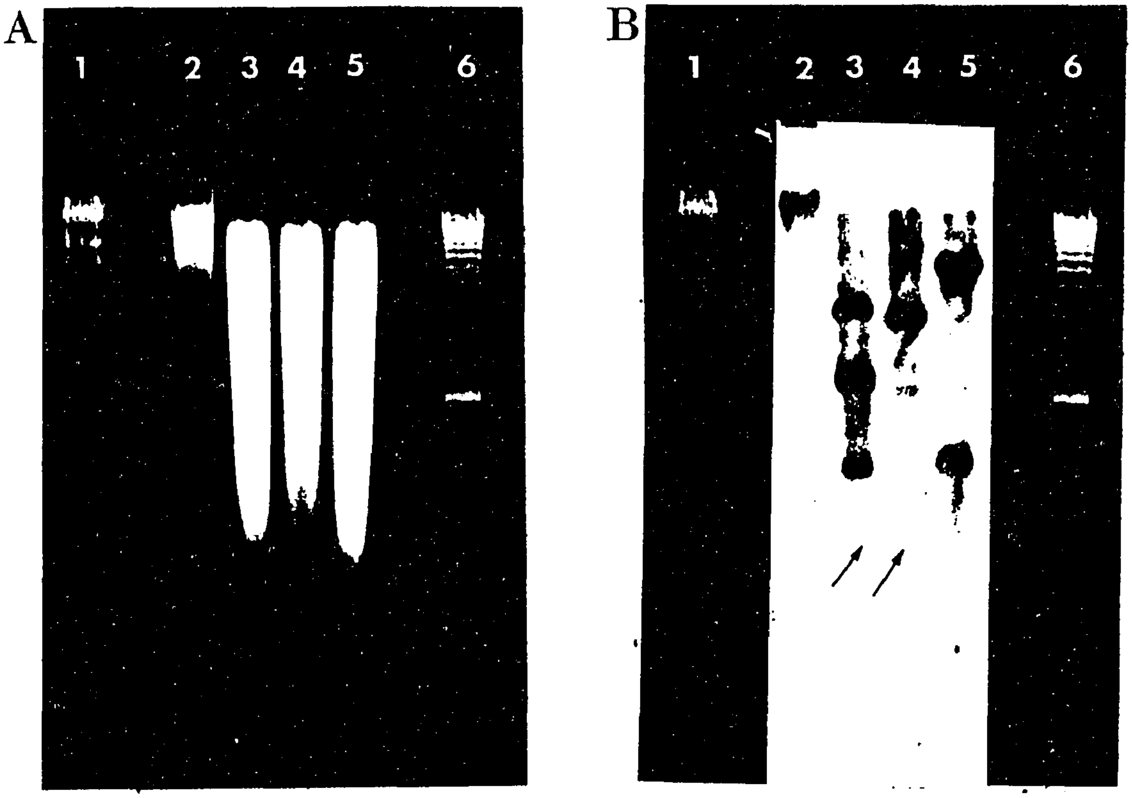
Salvelinus fontinalis*, brook trout, nrDNA hybridized with *Xenopus laevis* nrDNA

Figure 75. *Salvelinus fontinalis*, brook trout, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Hin*d III + *Eco*R I digest; 4, *Hin*d III + *Bam*H I digest; 5, *Hin*d III + *Pvu* II digest; 6, *Hin*d III + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes. Thin arrow points to a light hybrid band.



Salvelinus fontinalis, brook trout, nrDNA hybridized with _Xenopus laevis_ nrDNA

Figure 76. *Salvelinus fontinalis*, brook trout, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-5) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hind* III Fragments; 2, undigested; 3, *Eco*R I + *Bam*H I digest; 4, *Eco*R I + *Pvu* II digest; 5, *Eco*R I + *Pst* I digest; 6, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes. Thin arrow points to a light hybrid band.



***Salvelinus fontinalis*, brook trout, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 77. *Salvelinus fontinalis*, brook trout, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-5) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Bam*H I + *Pvu* II digest; 4, *Bam*H I + *Pst* I digest; 5, *Pvu* II + *Pst* I digest; 6, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes. Each thin arrow points to a light hybrid band.

Table 22

Salvelinus fontinalis, Brook trout, rDNA fragments generated by restriction endonuclease digestion.

Restriction Endonuclease	Fragment sizes in kilobases
Hind III	7.2; (13)
*Eco*R I	0.7; 3.25; 4.6; (7.6)
*Bam*H I	2.8
Pvu II	6.0; 13.0[*,§]
Pst I	6.0[**,§]; 8.0[*,§]; 13.0[*,§]
Hind III + *Eco*R I	0.6; (0.7); (3.25); 4.6; (7.2)
Hind III + *Bam*H I	0.7; 2.8; (5.8)
Hind III + *Pvu* II	1.3; 5.9
Hind III + *Pst* I	6.0
*Eco*R I + *Bam*H I	0.65; 1.4[**]; 1.9[**]; 2.5; (6.2)
*Eco*R I + *Pvu* II	0.7; 0.8; 2.0; 3.8; (6.7)
*Eco*R I + *Pst* I	0.7; 1.2; 4.6
*Bam*H I + *Pvu* II	0.7; 1.0; 1.7; 2.8
*Bam*H I + *Pst* I	0.7; 2.7
Pvu II + *Pst* I	1.0; 5.0

[*] variable; [**] length adjusted; [§] major fragment; fragment lengths in parenthesis are partial digests.

RESTRICTION ENZYME MAP OF BROOK TROUT, *SALVELINUS FONTINALIS*, RIBOSOMAL DNA

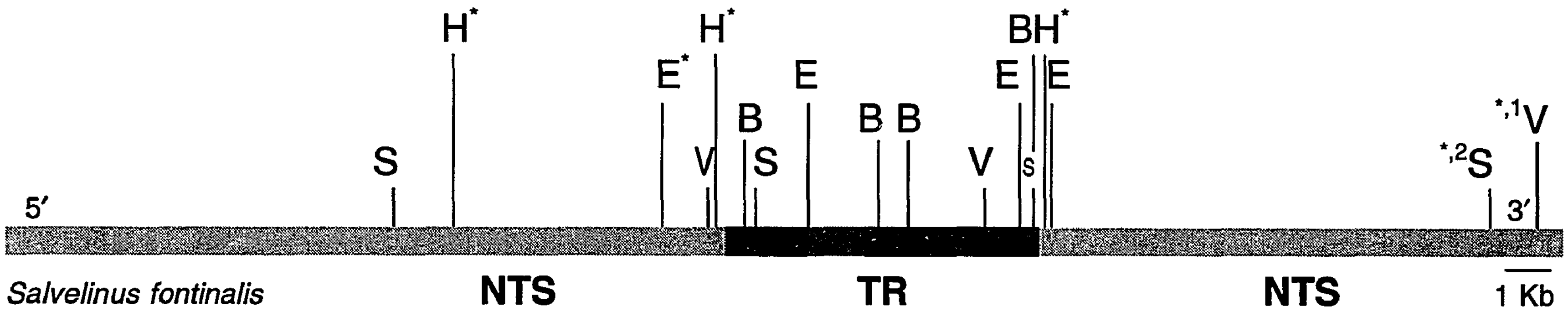
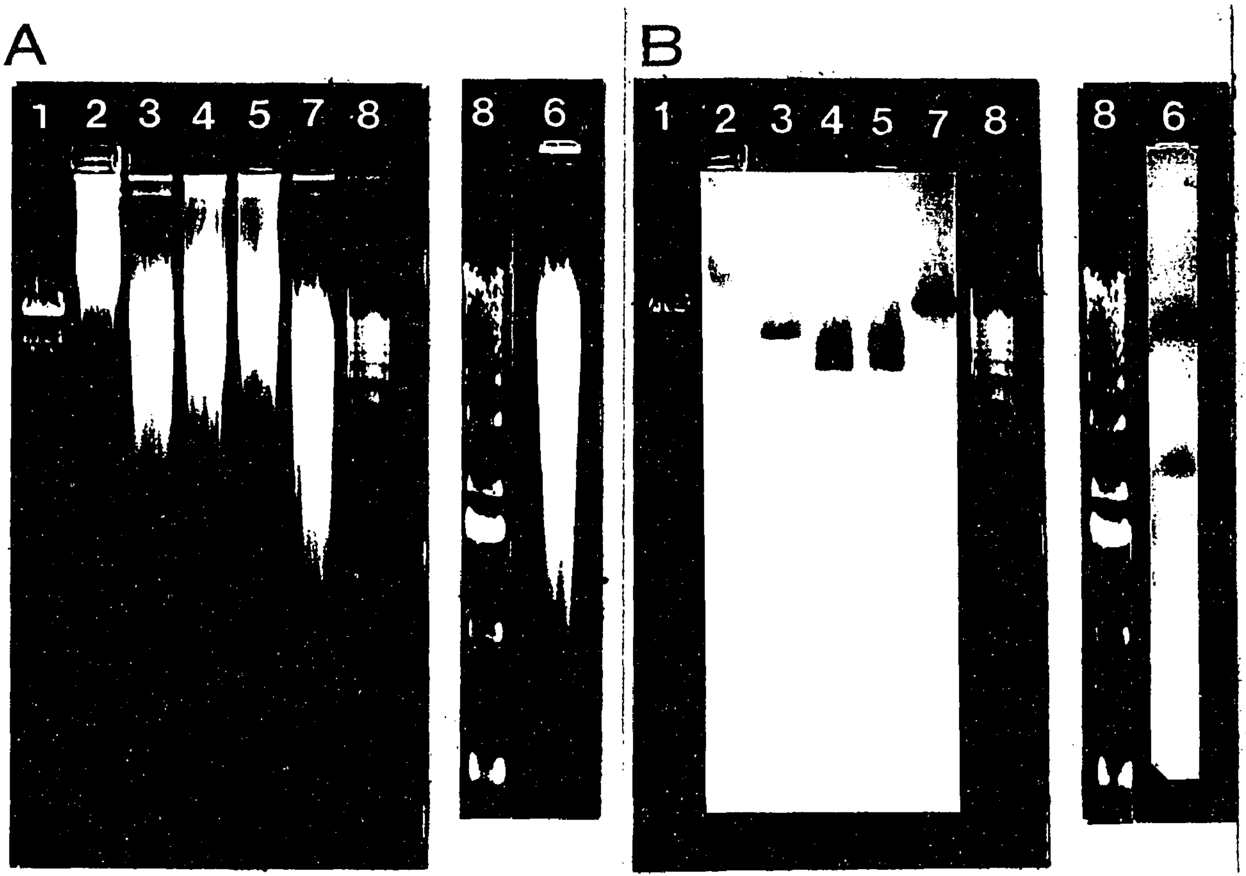


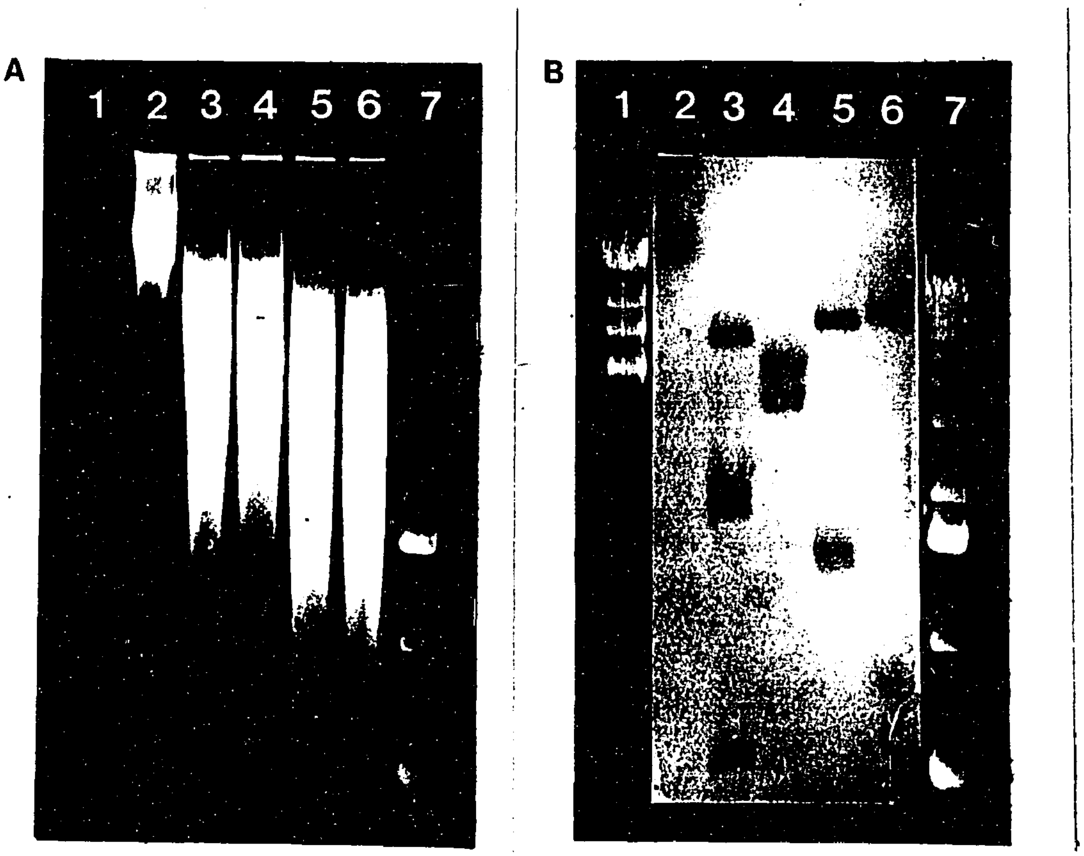
Figure 78. Proposed restriction enzyme map of brook trout ribosomal DNA. B, *Bam*H I; E, *Eco*R I; H, *Hin*d III; S, *Pst* I; V, *Pvu* II; NTS, nontranscribed spacer; TR, transcribed region; *variable; [1]major position 1 Kb out-side; [2]major position 3 Kb out-side. Relative positions of the respective restriction enzymes are shown in the map in approximate scale.

Morone saxatilis
Striped bass



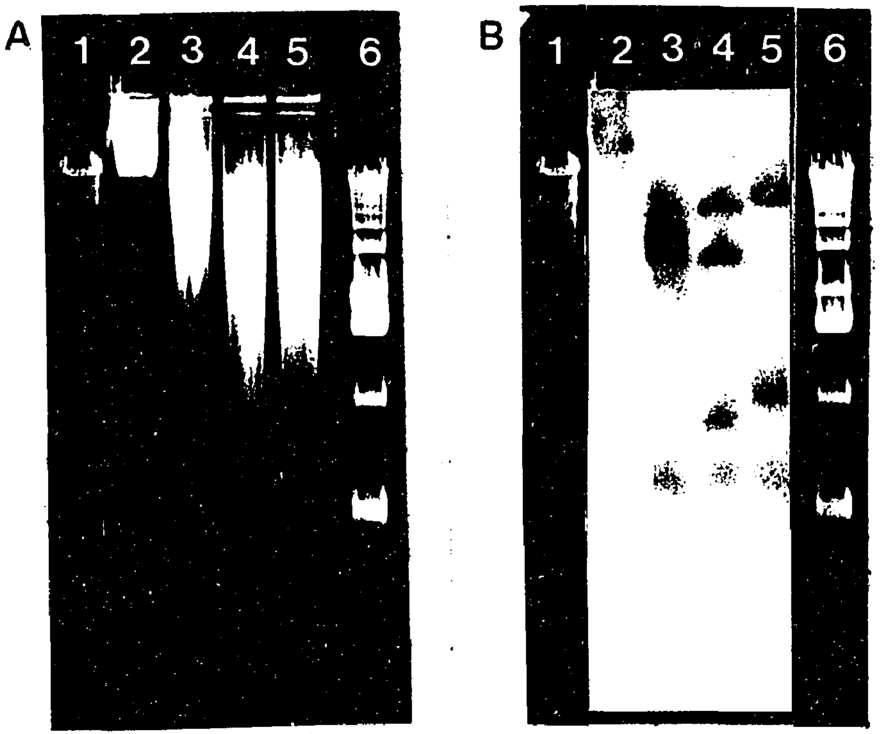
***Morone saxatilis*, striped bass, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 79. *Morone saxatilis*, striped bass, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of striped bass DNA (lanes 2-7) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ/*Hin*d III DNA Fragments; 2, undigested; 3, *Hin*d III digest; 4, *Eco*R I digest; 5, *Bam*H I digest; 6, *Pvu* II digest; 7, *Pst* I digest; 8, 1Kb DNA Ladder. Both A and B plates represent their actual sizes.



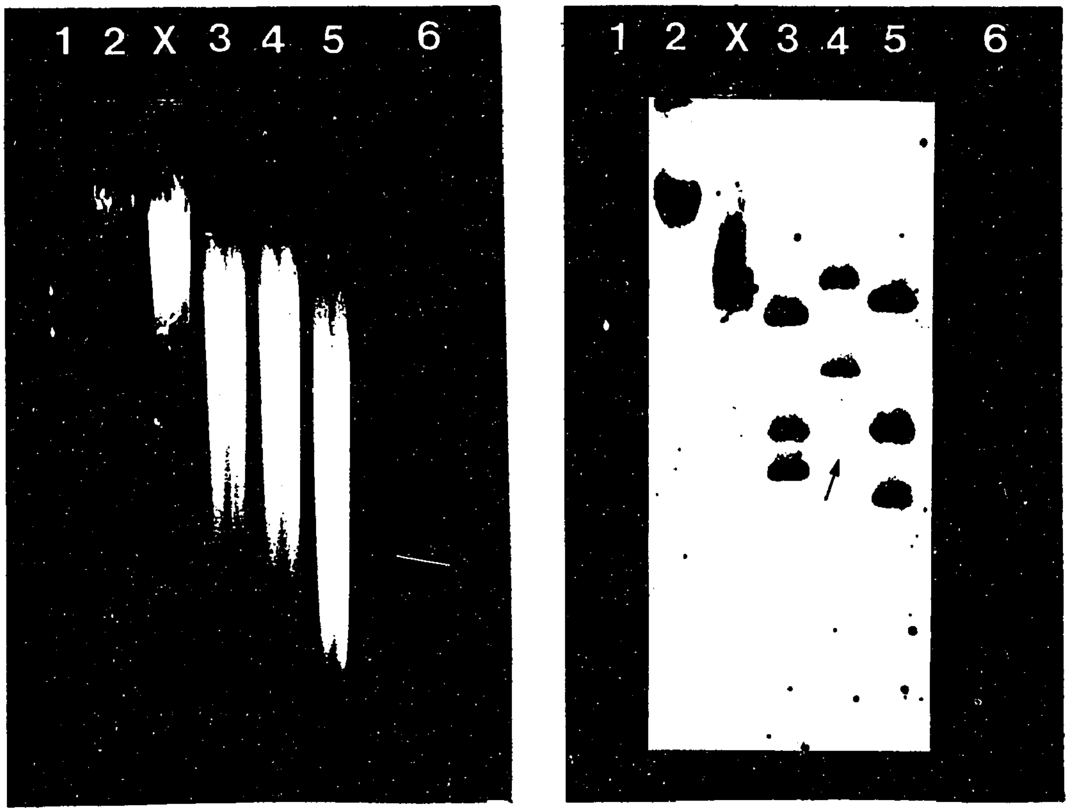
***Morone saxatilis*, striped bass, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 80. *Morone saxatilis*, striped bass, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of striped bass DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Hin* d III + *Eco*R I digest; 4, *Hin*d III + *Bam*H I digest; 5, *Hin*d III + *Pvu* II digest; 6, *Hin*d III + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes. Thin arrow points to a light hybrid band.



***Morone saxatilis*, striped bass, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 81. *Morone saxatilis*, striped bass, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of striped bass DNA (lanes 2-5) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hind* III Fragments; 2, undigested; 3, *Eco*R I + *Bam*H I digest; 4, *Eco*R I + *Pvu* II digest; 5, *Eco*R I + *Pst* I digest; 6, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes.



***Morone saxatilis*, striped bass, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 82. *Morone saxatilis*, striped bass, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of striped bass DNA (lanes 2-5) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; X, *Bam*H I digest; 3, *Bam*H I + *Pvu* II digest; 4, *Bam*H I + *Pst* I digest; 5, *Pvu* II + *Pst* I digest; 6, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes. Thin arrow points to a light hybrid band.

Table 23

Morone saxatilis, Striped bass, rDNA fragments generated by restriction endonuclease digestion.

Restriction Endonuclease	Fragment sizes in kilobases
*Hin*d III	8.0
*Eco*R I	5.6
*Bam*H I	5.0; 7.0
Pvu II	2.2; 7.0
Pst I	23.0
*Hin*d III + *Eco*R I	0.6; 1.7; 5.6
*Hin*d III + *Bam*H I	0.6; 3.2; 4.2
*Hin*d III + *Pvu* II	1.5; 7.0
*Hin*d III + *Pst* I	1.0[*]; 7.0
*Eco*R I + *Bam*H I	0.6; 2.5[**]; 2.7[§]; 4.4[§]
*Eco*R I + *Pvu* II	0.7; 1.4; 2.2; 4.8
*Eco*R I + *Pst* I	0.6; 1.0[‡]; 5.6
*Bam*H I + *Pvu* II	1.8; 2.2; 4.7
*Bam*H I + *Pst* I	2.0; 3.1; 7.0
Pvu II + *Pst* I	1.6[*]; 2.2; 5.4

[*] length adjusted; [**] light band; [§] strong hybridization overlapped the bands;
[‡] mobility delayed; fragment lengths in parenthesis are partial digests.

RESTRICTION ENZYME MAP OF STRIPED BASS, *MORONE SAXATILIS*, RIBOSOMAL DNA

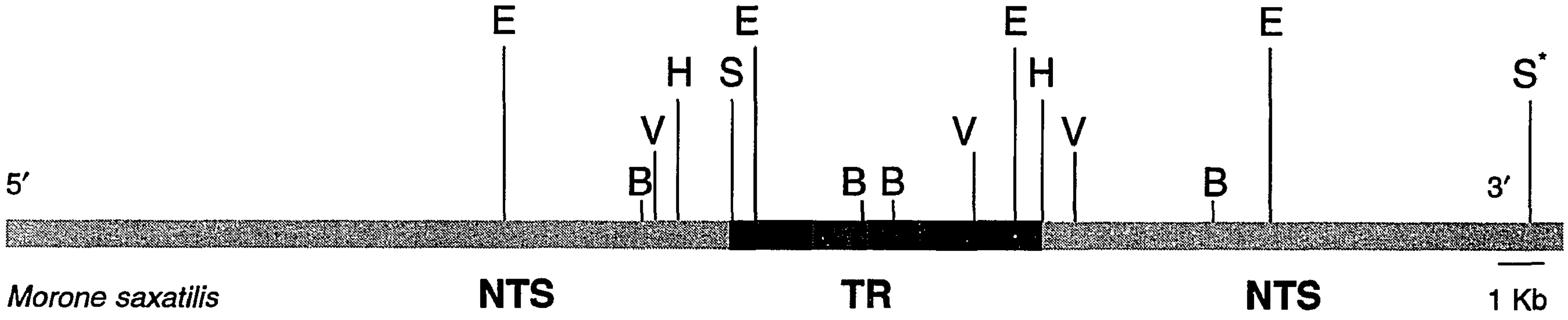
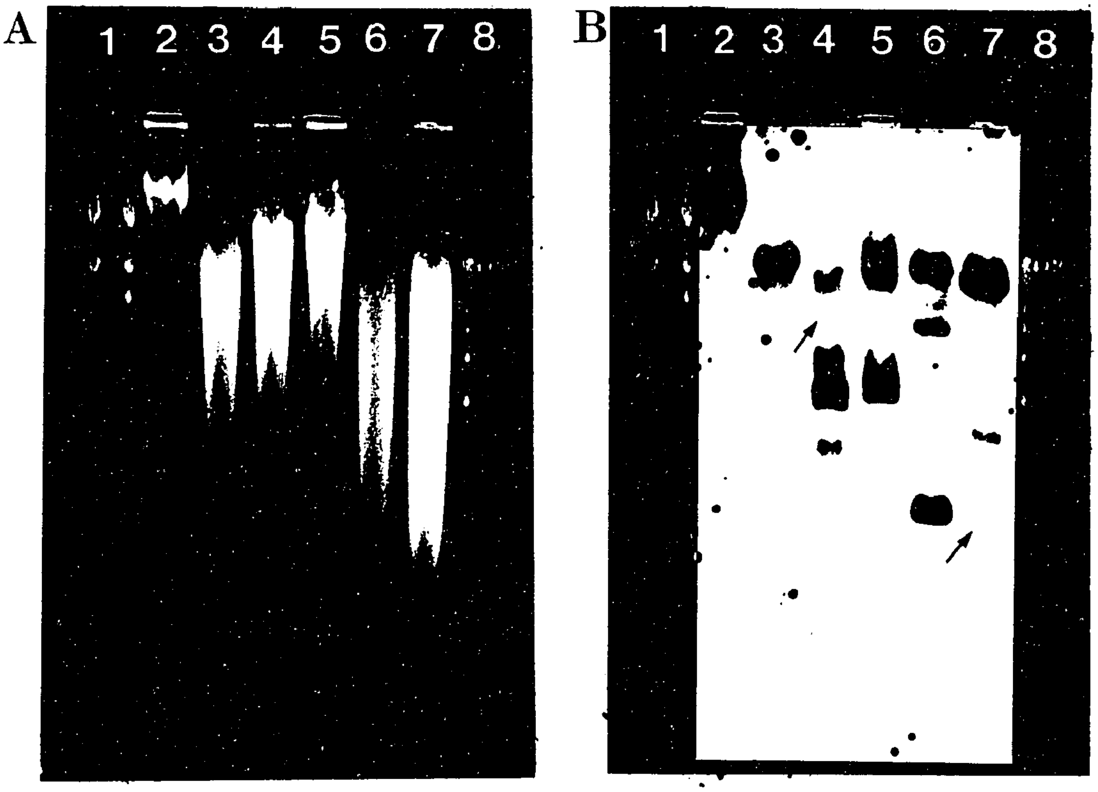


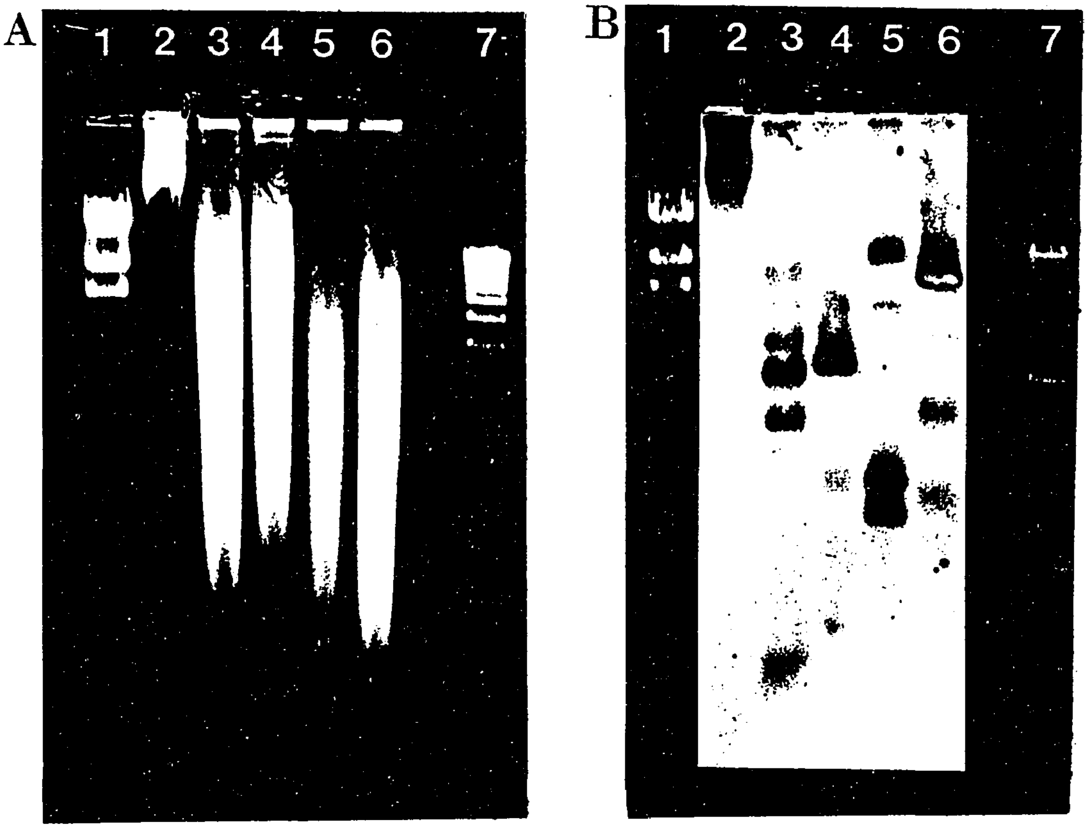
Figure 83. Proposed restriction enzyme map of striped bass ribosomal DNA. B, *Bam*H I; E, *Eco*R I; H, *Hind* III; S, *Pst* I; V, *Pvu* II; NTS, nontranscribed spacer; TR, transcribed region. *should be 5.5 Kb out-side. Relative positions of the respective restriction enzymes are shown in the map in approximate scale.

Oreochromis aureus
White tilapia



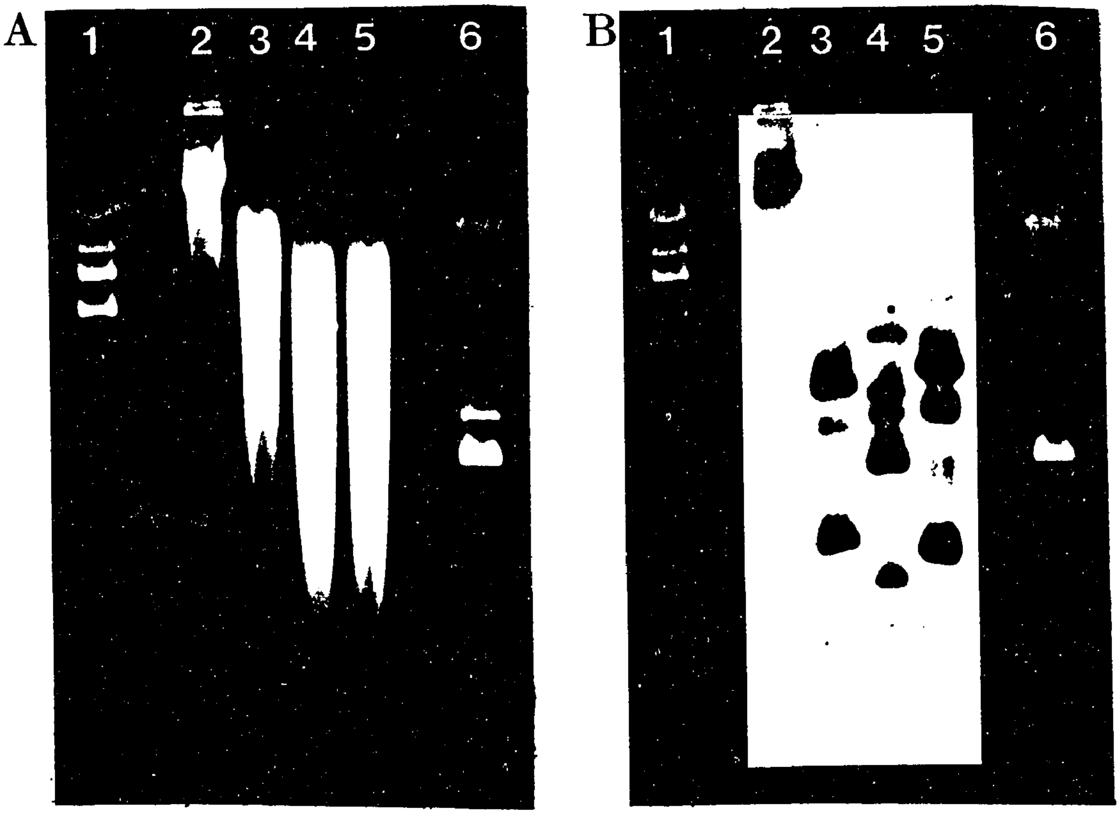
***Oreochromis aureus*, white tilapia, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 84. *Oreochromis aureus*, white tilapia, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of striped bass DNA (lanes 2-7) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ/*Hind* III DNA Fragments; 2, undigested; 3, *Hind* III digest; 4, *Eco*R I digest; 5, *Bam*H I digest; 6, *Pvu* II digest; 7, *Pst* I digest; 8, 1Kb DNA Ladder. Both A and B plates represent their actual sizes. Each thin arrow points to a light hybrid band.



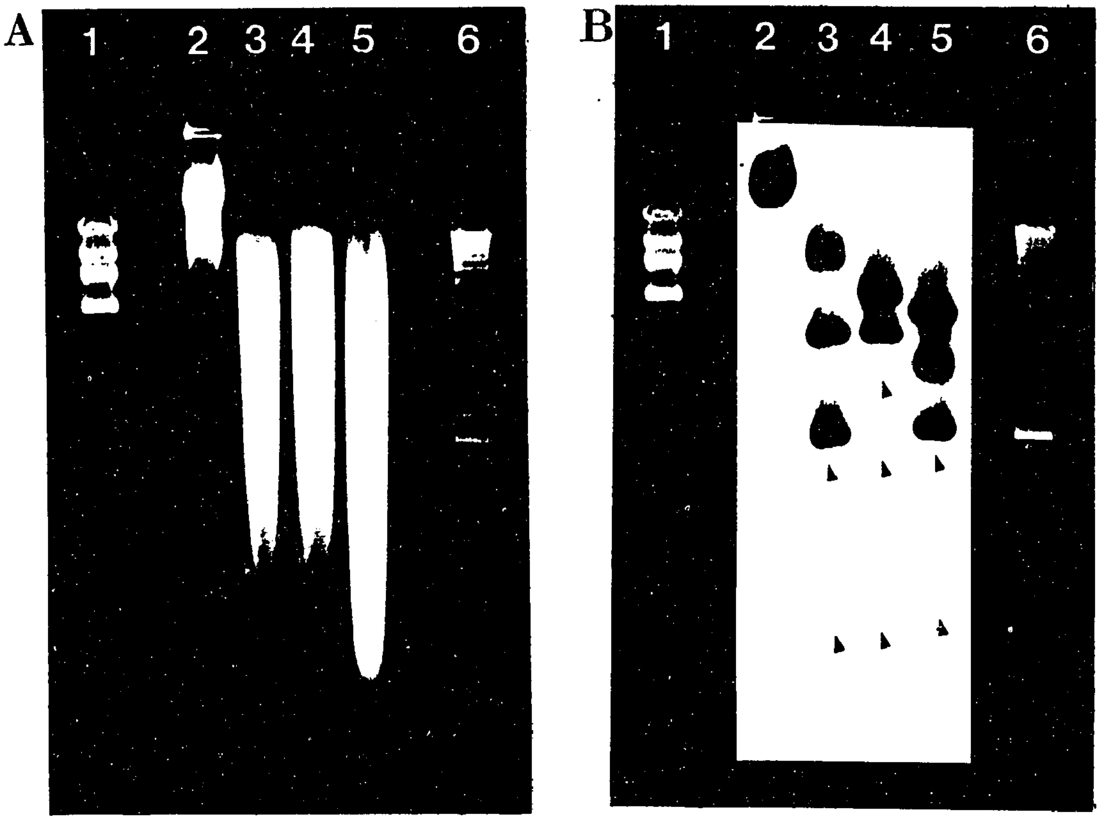
***Oreochromis aureus*, white tilapia, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 85. *Oreochromis aureus*, white tilapia, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of striped bass DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Hin*d III + *Eco*R I digest; 4, *Hin*d III + *Bam*H I digest; 5, *Hin*d III + *Pvu* II digest; 6, *Hin*d III + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes.



***Oreochromis aureus*, white tilapia, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 86. *Oreochromis aureus*, white tilapia, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of striped bass DNA (lanes 2-5) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragme s; 2, undigested; 3, *Eco*R I + *Bam*H I digest; 4, *Eco*R I + *Pvu* II digest; 5, *Eco*R I + *Pst* I digest; 6, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes.



***Oreochromis aureus*, white tilapia, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 87. *Oreochromis aureus*, white tilapia, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of striped bass DNA (lanes 2-5) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; X, *Bam*H I digest; 3, *Bam*H I + *Pvu* II digest; 4, *Bam*H I + *Pst* I digest; 5, *Pvu* II + *Pst* I digest; 6, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes. Each thin arrow head points to a light hybrid band.

Table 24

Oreochromis aureus, White tilapia, rDNA fragments generated by restriction endonucleases

Restriction Endonuclease	Fragment sizes in kilobases
*Hin*d III	9.6
*Eco*R I	2.3; 2.85; 4.0; (6.2); (7.0)
*Bam*H I	3.1; 7.0
Pvu II	1.6; 5.0; (6.5); 10.0
Pst I	1.6; 2.5; 4.7; (7.0)
*Hin*d III + *Eco*R I	0.6; 2.2; 2.85; 4.0; (6.2); (7.0)
*Hin*d III + *Bam*H I	1.7*; 2.5
*Hin*d III + *Pvu* II	1.7; 1.4; 5.0; (10.0)
*Hin*d III + *Pst* I	1.6; 2.6; (4.3); (7.0)
*Eco*R I + *Bam*H I	1.0; 2.0; 2.3; 2.5
*Eco*R I + *Pvu* II	0.8; 1.6; 2.3; 2.6; (3.1); (7.0)
*Eco*R I + *Pst* I	1.0; 1.6; 2.1; (2.8); (4.0)
*Bam*H I + *Pvu* II	0.5; 1.2; 1.6; 2.6; 5.5
*Bam*H I + *Pst* I	0.6; 1.25; (2.4); (3.0); 4.5
Pvu II + *Pst* I	0.7; 1.3; 1.6; 2.3; 3.4

*band due to star activity; values in parenthesis are partial digests.

RESTRICTION ENZYME MAP OF WHITE TILAPIA, *OREOCHROMIS AUREUS*, RIBOSOMAL DNA

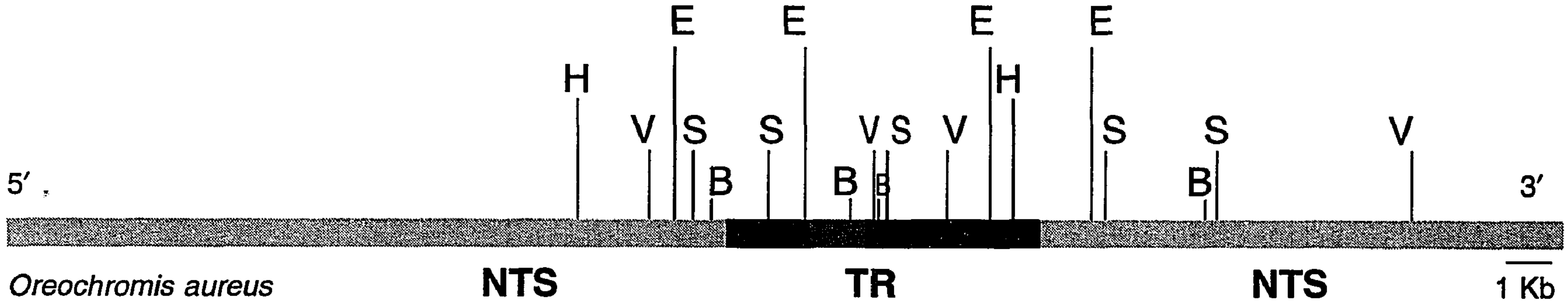
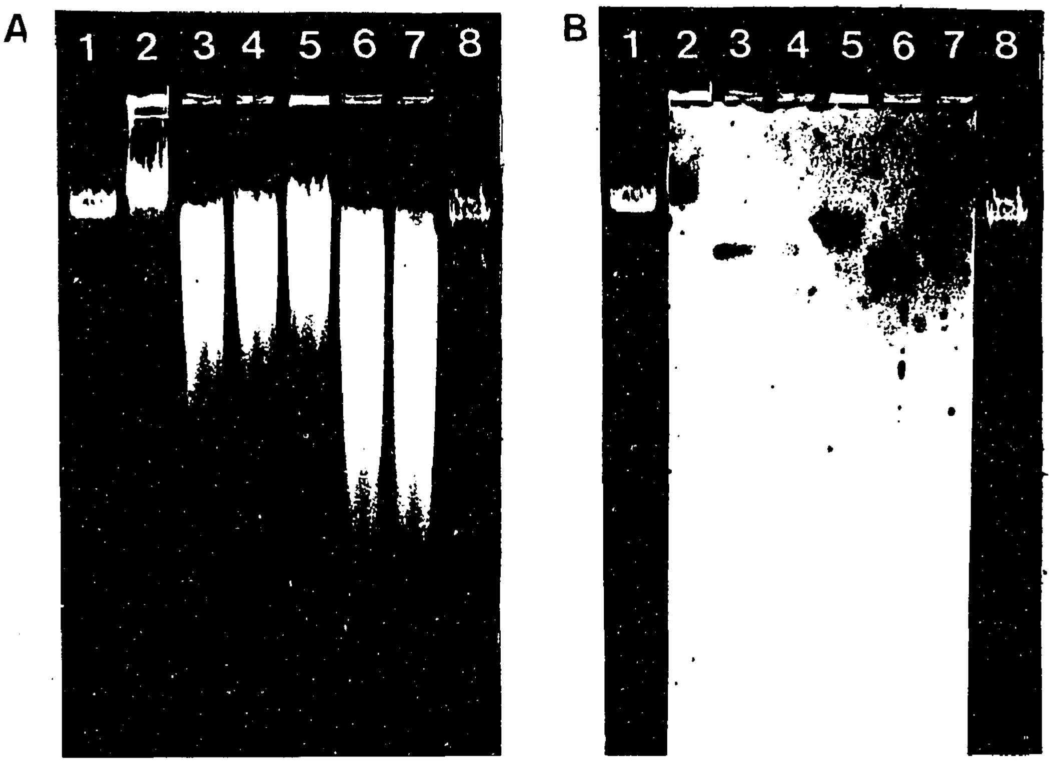


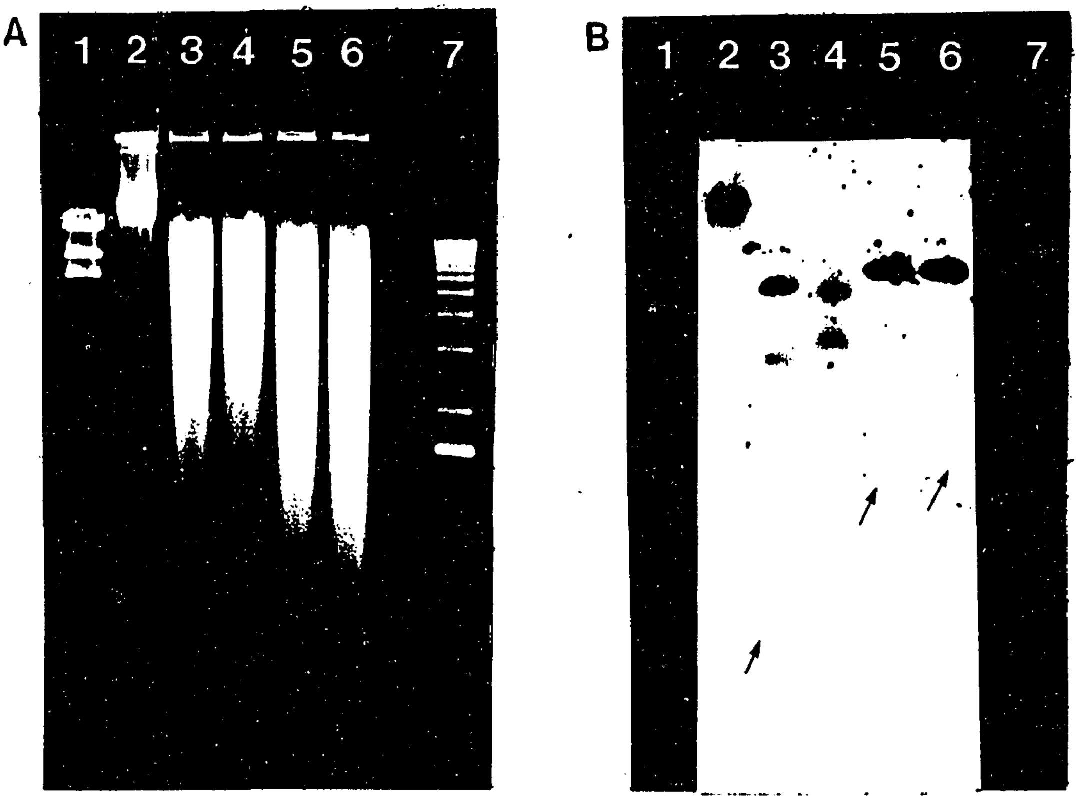
Figure 88. Proposed restriction enzyme map of white tilapia ribosomal DNA. B, *Bam*H I; E, *Eco*R I; H, *Hin*d III; S, *Pst* I; V, *Pvu* II; NTS, nontranscribed spacer; TR, transcribed region. Relative positions of the respective restriction enzymes are shown in the map in approximate scale.

Perca flavescens
Yellow perch



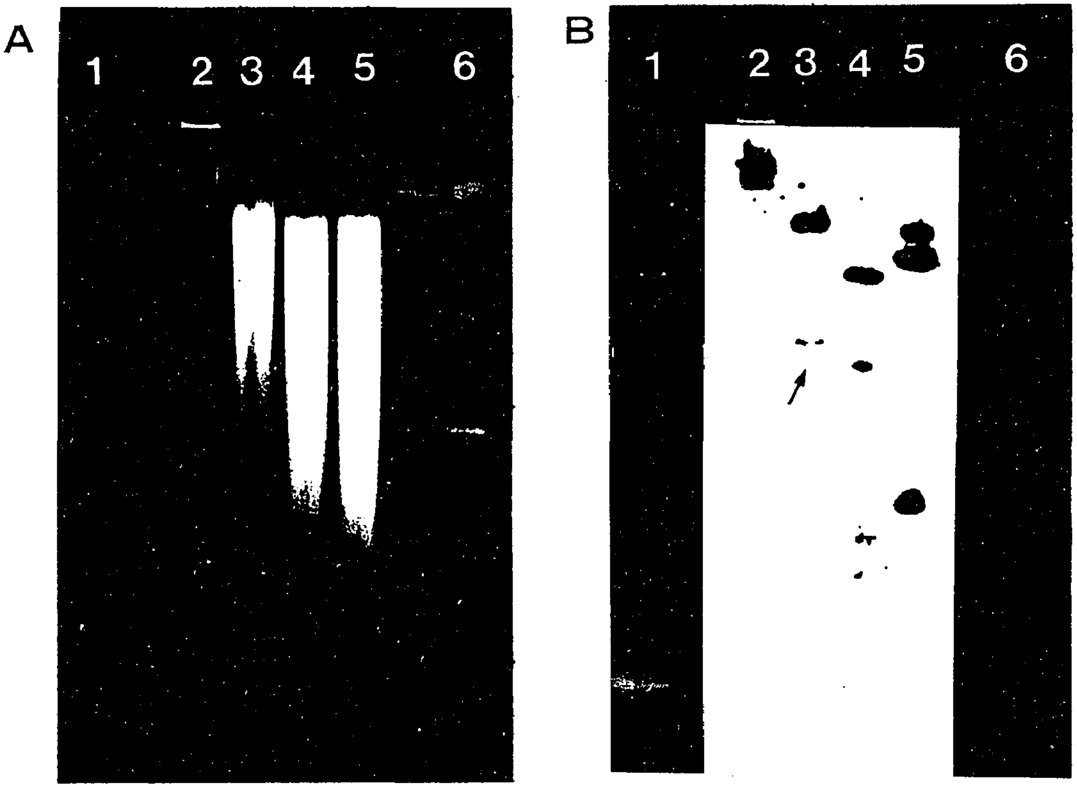
***Perca flavescens*, yellow perch, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 89. *Perca flavescens*, yellow perch, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-7) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Hin*d III digest; 4, *Eco*R I digest; 5, *Bam*H I digest; 6, *Pvu* II digest; 7, *Pst* I digest; 8, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes.



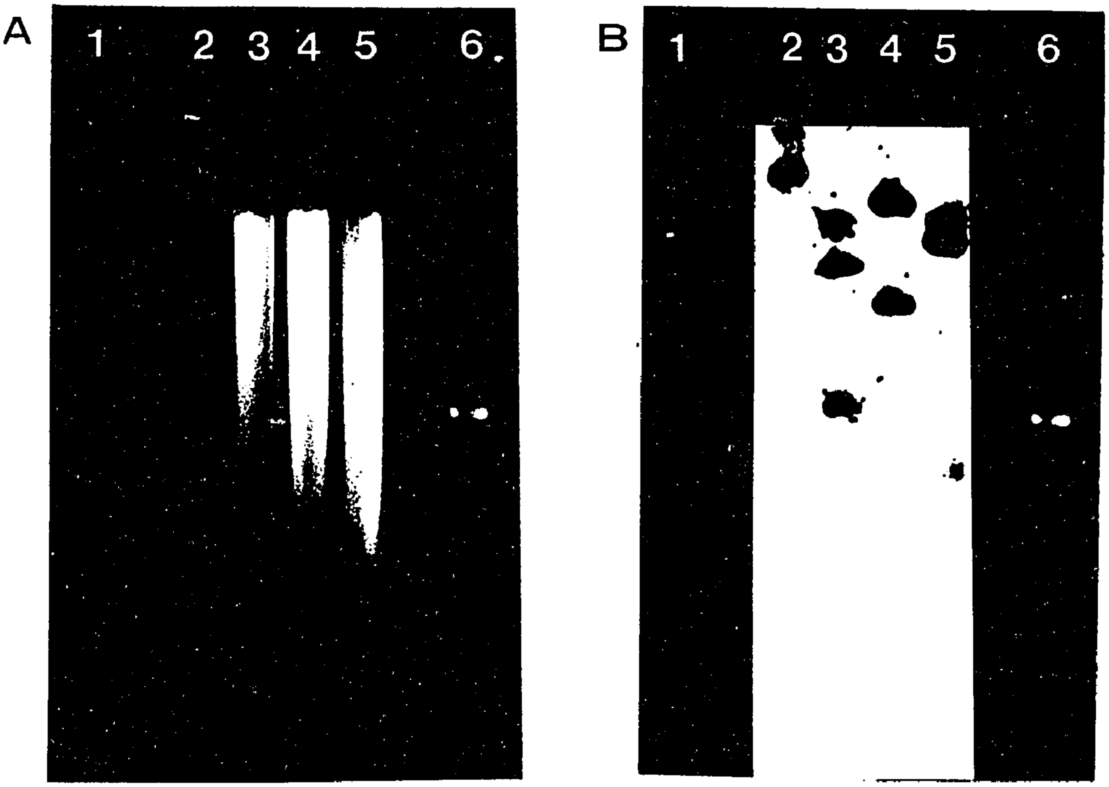
***Perca flavescens*, yellow perch, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 90. *Perca flavescens*, yellow perch, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Hin*d III + *Eco*R I digest; 4, *Hin*d III + *Bam*H I digest; 5, *Hin*d III + *Pvu* II digest; 6, *Hin*d III + *Pst* I digest; 7, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes. Each thin arrow points to a light hybrid band.



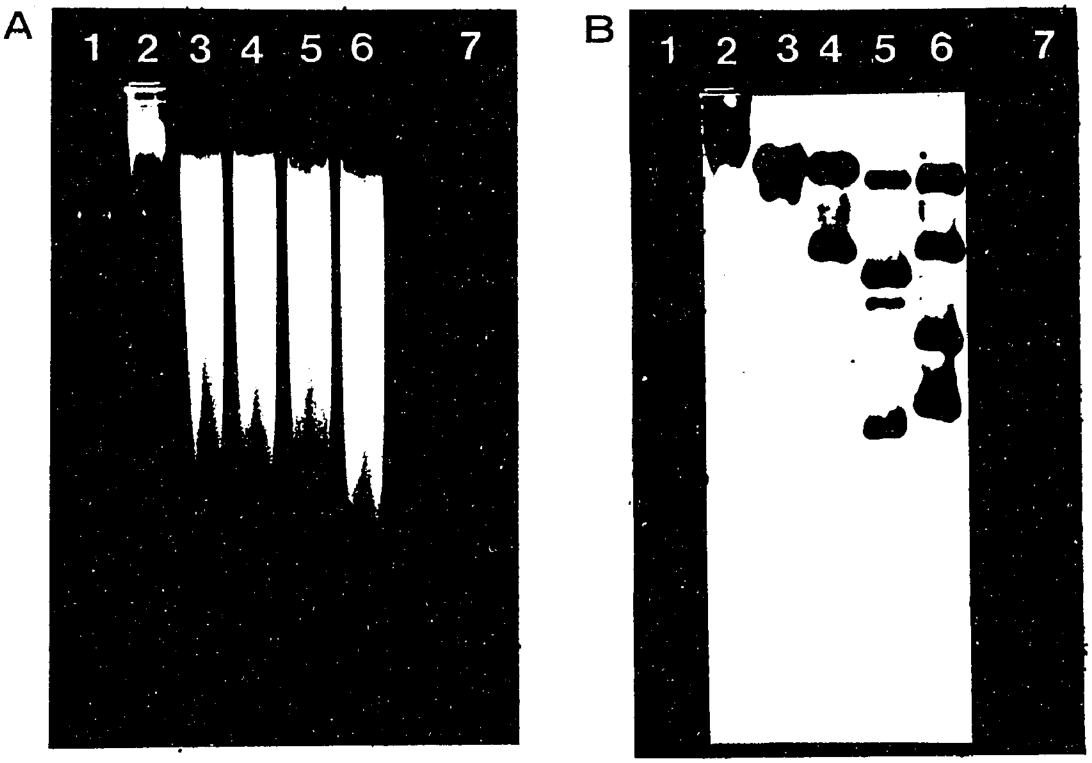
Perca flavescens, yellow perch, nrDNA hybridized with Xenopus laevis nrDNA

Figure 91. *Perca flavescens*, yellow perch, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-5) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Eco*R I + *Bam*H I digest; 4, *Eco*R I + *Pvu* II digest; 5, *Eco*R I + *Pst* I digest; 6, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes. Thin arrow points to a light hybrid band.



***Perca flavescens*, yellow perch, nrDNA hybridized with *Xenopus laevis* nrDNA**

Figure 92. *Perca flavescens*, yellow perch, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-5) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Bam*H I + *Pvu* II digest; 4, *Bam*H I + *Pst* I digest; 5, *Pvu* II + *Pst* I digest; 6, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes.



Perca flavescens, yellow perch, nrDNA hybridized with *Xenopus laevis* nrDNA

Figure 93. *Perca flavescens*, yellow perch, nrDNA hybridized with *Xenopus laevis* nrDNA. A, Electrophoretic separation in agarose gel of fish DNA (lanes 2-6) before and after digestion. B, Pattern of hybridization of rDNA after Southern transfer. Lanes: 1, λ DNA/*Hin*d III Fragments; 2, undigested; 3, *Pst* I digest; 4, *Pst* I + *Bam*H I digest; 5, *Pst* I + *Bam*H I + *Eco*R I digest; 6, *Pst* I + *Bam*H I + *Pvu* II digest; 7, 1 Kb DNA Ladder. Both A and B plates represent their actual sizes.

Table 25

Perca flavescens, Yellow perch, rDNA fragments generated by restriction endonuclease digestion.

Restriction Endonuclease	Fragment sizes in kilobases
*Hin*d III	7.6
*Eco*R I	5.05; 7.9
*Bam*H I	11.0[*]
Pvu II	6.5
Pst I	13.0
*Hin*d III + *Eco*R I	0.6[§]; 1.8; 5.05
*Hin*d III + *Bam*H I	3.3; 4.3
*Hin*d III + *Pvu* II	1.4; 6.2
*Hin*d III + *Pst* I	1.5[§,***]; 6.8
*Eco*R I + *Bam*H I	2.3[§]; 2.6; 7.9
*Eco*R I + *Pvu* II	0.8; 2.25; 4.2
*Eco*R I + *Pst* I	0.9; 5.05; 7.0
*Bam*H I + *Pvu* II	1.8; 4.6; 6.5
*Bam*H I + *Pst* I	3.3; 9.7
Pvu II + *Pst* I	1.3; 5.1; 6.5
*Bam*H I + *Eco*R I + *Pst* I	0.9; 2.3[§]; 2.5[**]; 7.0
*Bam*H I + *Pst* I *Pvu* II	1.3; 1.8; 3.3; 6.5

[*] variable; [**] length adjusted; [***] mobility delayed; [§] very light band.

RESTRICTION ENZYME MAP OF YELLOW PERCH, *PERCA FLAVESCENS*, RIBOSOMAL DNA

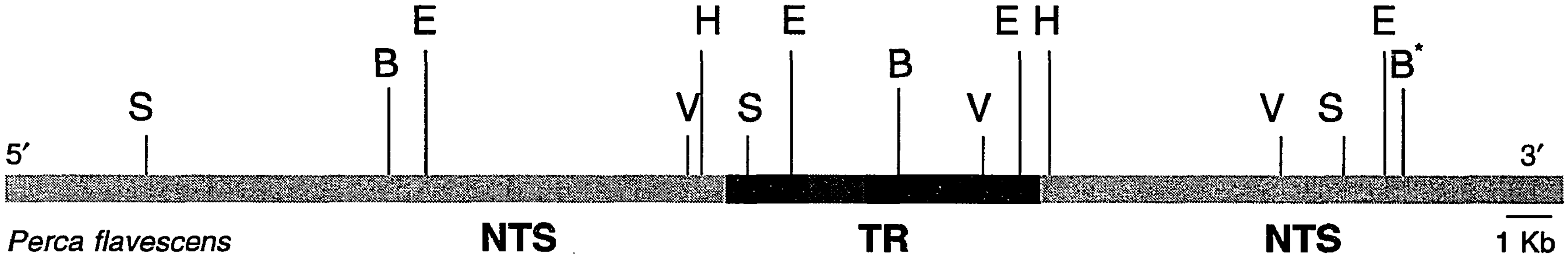


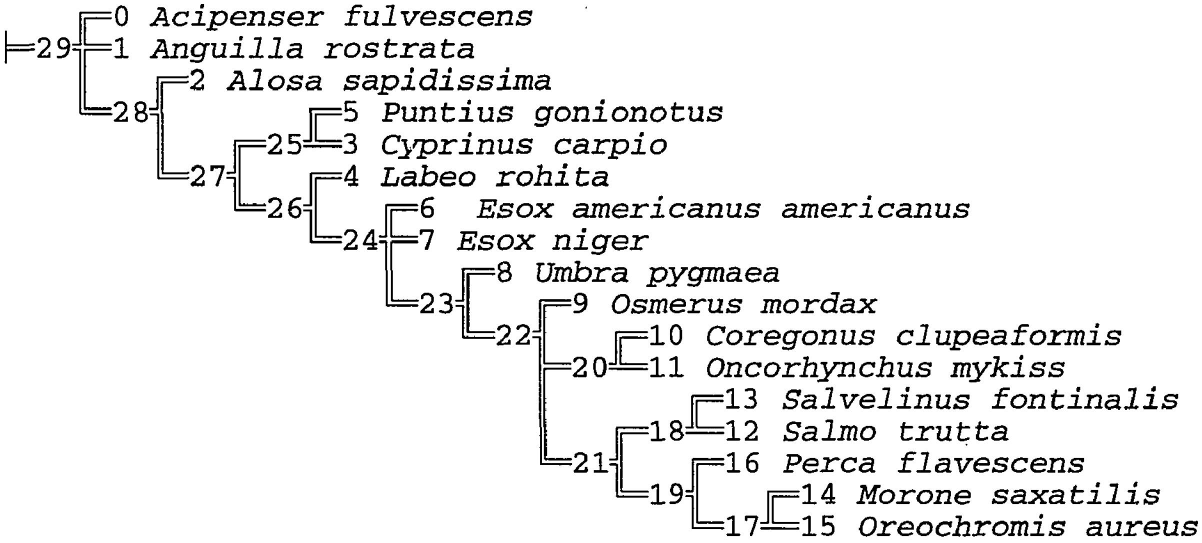
Figure 94. Proposed restriction enzyme map of yellow perch ribosomal DNA. B, *Bam*H I; E, *Eco*R I; H, *Hin*d III; S, *Pst* I; V, *Pvu* II; NTS, nontranscribed spacer; TR, transcribed region; *variable. Relative positions of the respective restriction enzymes are shown in the map in approximate scale.

Table 26

Molecular character matrix of the rDNA of 17 actinopterygian taxa based on five restriction endonuclease sites. Position of each character site is identified in Figure 8. 0 = absent; 1 = present. B, *Bam*H I; E, *Eco*R I; H, *Hin*d III; S, *Pst* I; V, *Pvu* II.

Taxa	Restriction endonuclease site characters																			
	B^1	B^2	B^3	B^4	B^5	E^1	E^2	E^3	E^4	H^1	H^2	S^1	S^2	S^3	V^1	V^2	V^3	V^4	V^5	V^6
Acipenser fulvescens	1	0	1	1	1	0	1	1	1	0	1	0	0	1	0	0	0	0	1	0
Anguilla rostrata	1	0	1	1	0	0	1	1	0	1	0	0	0	1	0	1	0	0	0	1
Alosa sapidissima	0	0	1	1	0	0	1	1	1	0	1	0	1	1	0	0	0	0	0	0
Cyprinus carpio	0	0	0	1	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0
Labeo rohita	0	0	1	1	0	0	1	1	0	0	0	0	1	0	0	0	1	0	0	0
Puntius gonionotus	0	0	0	1	0	1	1	0	1	0	0	0	1	0	0	0	1	0	0	0
Esox americanus americanus	0	1	0	1	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	0
Esox niger	0	1	0	1	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	0
Umbra pygmaea	1	1	1	0	0	0	1	1	0	0	0	0	1	0	1	0	0	0	1	0
Osmerus mordax	0	1	1	1	0	0	1	1	0	0	1	0	1	0	0	0	0	0	1	0
Coregonus clupeaformis	0	1	1	1	0	0	1	1	0	0	1	0	1	0	0	1	0	0	1	0
Oncorhynchus mykiss	0	1	1	0	0	0	1	1	0	0	1	0	1	0	1	1	0	0	1	0
Salmo trutta	0	1	1	1	0	0	1	1	1	1	1	0	1	0	0	0	0	0	1	0
Salvelinus fontinalis	0	1	1	1	1	0	1	1	1	1	**0**	0	1	1	1	0	0	0	1	0
Morone saxatilis	**1**	0	1	1	0	0	1	1	0	1	1	0	1	0	0	0	0	0	1	1
Oreochromis aureus	1	0	1	1	0	1	1	1	0	**1**	1	1	1	1	0	0	0	1	1	0
Perca flavescens	0	0	0	1	0	0	1	1	0	1	1	0	1	0	1	0	0	0	1	0

Numbers in bold represent adjusted sites.



Hennig length 43 ci 44 ri 55

Figure 95. Phylogeny of the actinopterygian fishes studied based on restriction endonuclease (RE) recognition sites in their rDNA regions as in Figure 8. The RE used are *Bam*H I, *Eco*R I, *Hin*d III, *Pst* I, and *Pvu* II. Numbers in the cladogram represent nodes. length, tree length; ci, consistency index; ri, retention index.

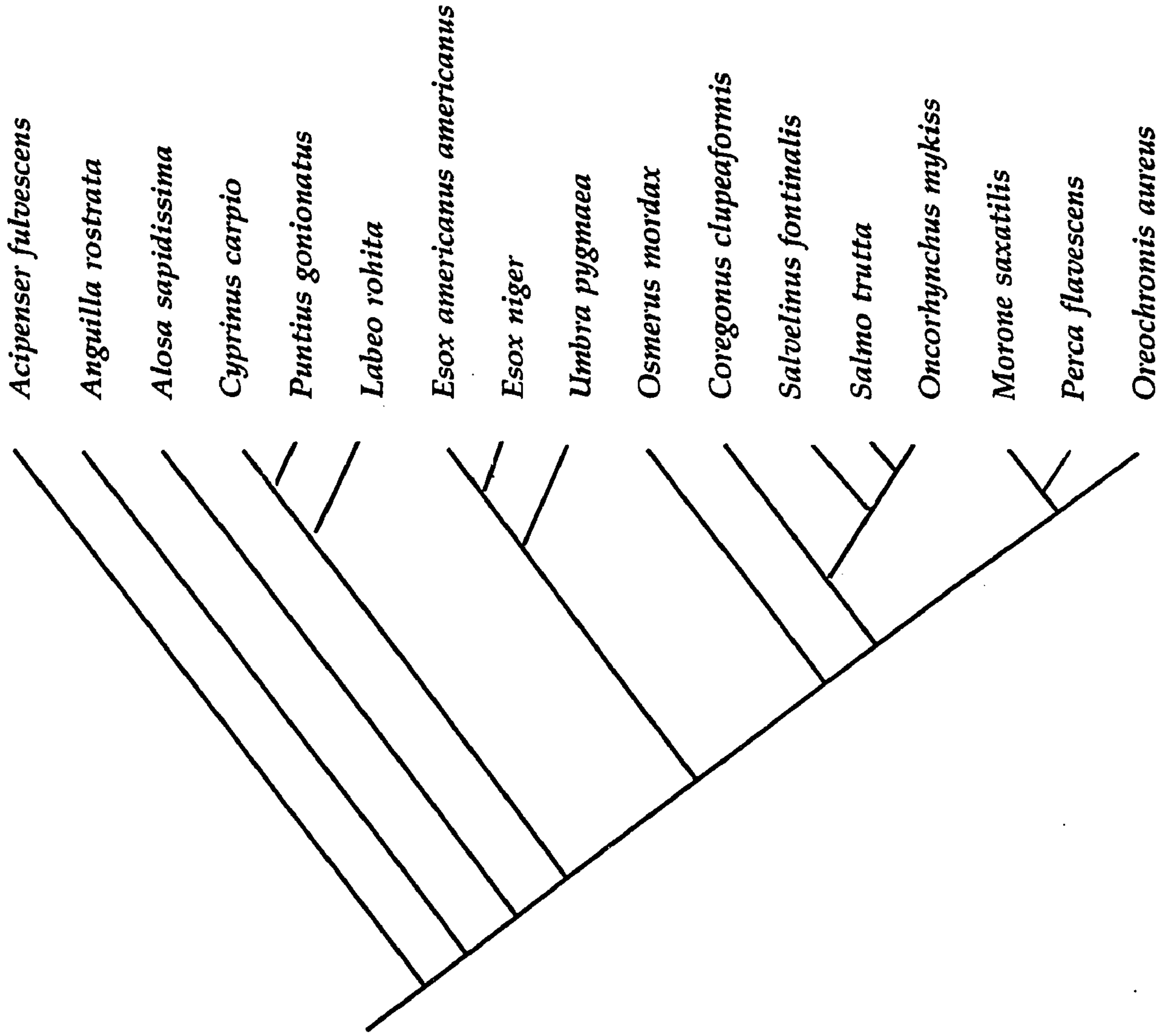


Figure 96. A hypothetical cladogram reconstruct of the actinopterygians included in this study based on morphological characters (Rosen, 1974; Lauder and Liem, 1983; Fink, 1984; Howes, 1991; Phillips and Pleyte, 1991; Bart and Page, 1992; Cavender and Coburn, 1992; Stearley, 1992; Wilson and Williams, 1992; Le *et al.*, 1993, Nelson, 1994). The relationships of the cyprinids (1) (*Cyprinus carpio; Puntius gonionotus; Labeo rohita*), (2) esocoids (*Esox*), (3) salmonids[§] (*Coregonus clupeaformis; Salvelinus fontinalis; Salmo trutta; Oncorhynchus mykiss*), and (4) percoideas (*Morone saxatilis; Perca flavescens; Oreochromis aureus*) are based on (1) Howes, 1991 and Cavender and Coburn, 1992, (2) Rosen, 1974, and Nelson, 1994, (3) Phillips and Pleyte, 1991 and Wilson and Williams, 1992, and (4) Bart and Page, 1992; Lauder and Liem, 1983, respectively. [§]Both morphology and karyotype were reported to be included (Phillips and Pleyte, 1991).

Table 27

Morphological character matrix of the 17 actinopterygian taxa based on published information. Characters are listed in Appendix 1. 0 = absent; 1 = present; 2 = double/developed; ? = unknown.

Taxa[1]	Transformation series																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Acipenser	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Anguilla	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Alosa	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cyprinus	1	1	1	1	?	1	1	1	0	0	0	0	0	0	0	?	0	0	0	0	0
Labeo	1	1	1	1	?	1	2	0	1	0	0	0	0	0	0	?	0	0	0	0	0
Puntius	1	1	1	1	?	1	1	1	0	0	0	0	0	0	0	?	0	0	0	0	0
Esox	1	1	1	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0
Esox[2]	1	1	1	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0
Umbra	1	1	1	0	1	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0
Osmerus	1	1	1	1	1	0	0	0	0	1	1	2	0	0	1	0	0	0	0	0	0
Coregonus	1	1	1	1	1	0	0	0	0	1	1	2	0	0	1	0	0	0	0	0	0
Oncorhynchus	1	1	1	1	1	0	0	0	0	1	1	2	0	0	0	1	1	0	1	0	0
Salmo	1	1	1	1	1	0	0	0	0	1	1	2	0	0	0	1	1	1	0	0	0
Salvelinus	1	1	1	1	1	0	0	0	0	1	1	2	0	0	0	1	1	2	0	0	0
Morone	1	1	1	?	1	0	0	0	0	1	1	2	0	0	0	0	0	0	0	1	0
Oreochromis	1	1	1	?	1	0	0	0	0	1	1	2	0	0	0	0	0	0	0	1	1
Perca	1	1	1	?	1	0	0	0	0	1	1	2	0	0	0	0	0	0	0	1	0

[1]represents taxa as in Table 26; [2]Represents the other species of *Esox niger*.

mhennig length 23 ci 95 ri 97 trees 3

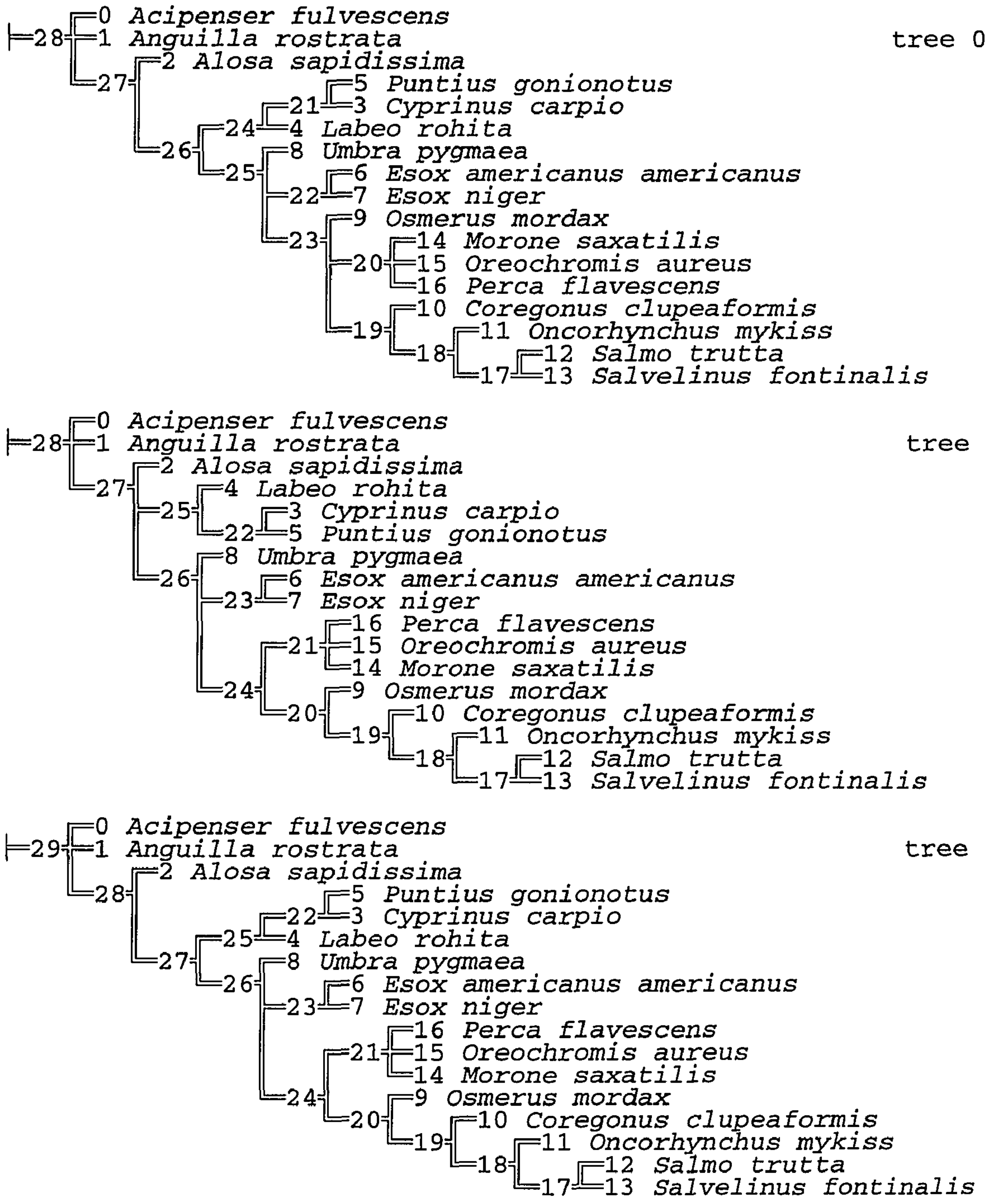


Figure 97. Equally parsimonious cladograms of the actinopterygians based on morphology analyzed with *mhennig, Hennig86.*

```
mhennig length 23 ci 95 ri 97 trees 3;
nelsen file 0 from mhennig 3 trees
tplot file 0 from nelsen 1 tree

    ╔═0 Acipenser fulvescens
╞═27╬═1 Anguilla rostrata
    ║   ╔═2 Alosa sapidissima
    ╚═26╣   ╔═4 Labeo rohita
        ╠═24╣   ╔═3 Cyprinus carpio
        ║   ╚═21╩═5 Puntius gonionotus
        ║   ╔═8 Umbra pygmaea
        ╚═25╣   ╔═6 Esox americanus americanus
            ╠═22╩═7 Esox niger
            ║   ╔═9 Osmerus mordax
            ╚═23╣   ╔═14 Morone saxatilis
                ╠═20╬═15 Oreochromis aureus
                ║   ╚═16 Perca flavescens
                ║   ╔═10 Coregonus clupeaformis
                ╚═19╣   ╔═11 Oncorhynchus mykiss
                    ╚═18╣   ╔═12 Salmo trutta
                        ╚═17╩═13 Salvelinus fontinalis
```

Figure 98. Nelsen consensus tree the actinopterygians based on morphology analyzed with *mhennig* followed by *nelsen*, *Hennig86*.

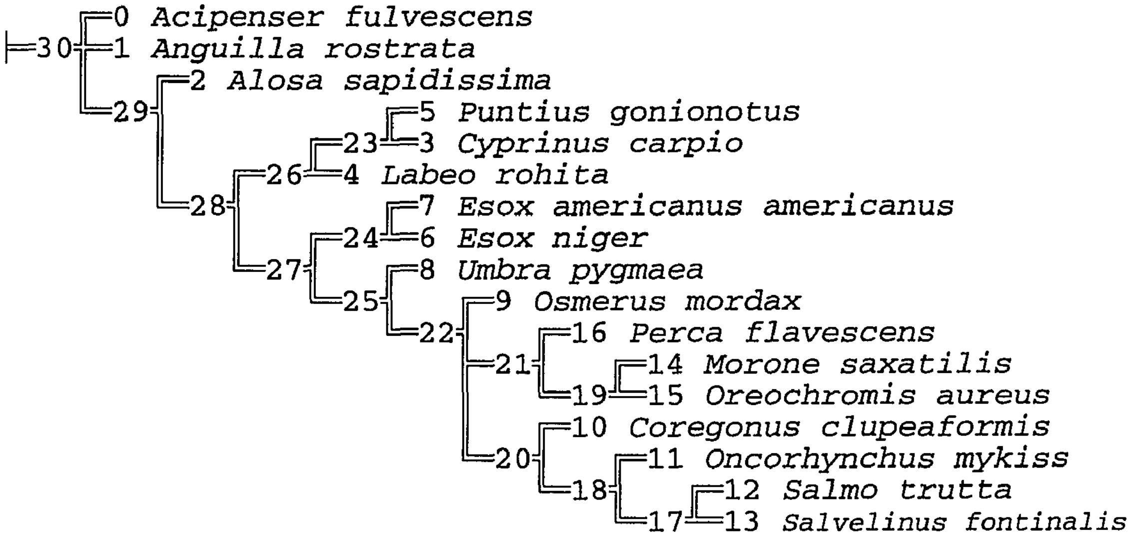
```
mhennig length 68 ci 60 ri 73 trees 4
tplot file 0 from mhennig 4 trees

tree 0
    ┌═0 Acipenser fulvescens
├═31┤═1 Anguilla rostrata
    │   ┌═2 Alosa sapidissima
    └═30┤           ┌═5 Puntius gonionotus
        │       ┌═24┴═3 Cyprinus carpio
        │   ┌═27┴═4 Labeo rohita
        └═29┤       ┌═7 Esox americanus americanus
            │   ┌═25┴═6 Esox niger
            └═28┤   ┌═8 Umbra pygmaea
                └═26┤           ┌═15 Oreochromis aureus
                    │       ┌═20┴═14 Morone saxatilis
                    │   ┌═22┴═16 Perca flavescens
                    └═23┤   ┌═9 Osmerus mordax
                        └═21┤       ┌═11 Oncorhynchus mykiss
                            │   ┌═17┴═10 Coregonus clupeaformis
                            └═19┤   ┌═12 Salmo trutta
                                └═18┴═13 Salvelinus fontinalis

tree 1
    ┌═0 Acipenser fulvescens
├═30┤═1 Anguilla rostrata
    │   ┌═2 Alosa sapidissima
    └═29┤           ┌═5 Puntius gonionotus
        │       ┌═23┴═3 Cyprinus carpio
        │   ┌═26┴═4 Labeo rohita
        └═28┤       ┌═7 Esox americanus americanus
            │   ┌═24┴═6 Esox niger
            └═27┤   ┌═8 Umbra pygmaea
                └═25┤   ┌═9 Osmerus mordax
                    └═22┤   ┌═10 Coregonus clupeaformis
                        ├═20┴═11 Oncorhynchus mykiss
                        │       ┌═13 Salvelinus fontinalis
                        │   ┌═18┴═12 Salmo trutta
                        └═21┤   ┌═16 Perca flavescens
                            └═19┤   ┌═14 Morone saxatilis
                                └═17┴═15 Oreochromis aureus
```

Figure 99. Equally parsimonious cladograms of the actinopterygians based on rDNA RE and morphological characters analyzed with *mhennig*, *Hennig86*.

tree 2



tree 3

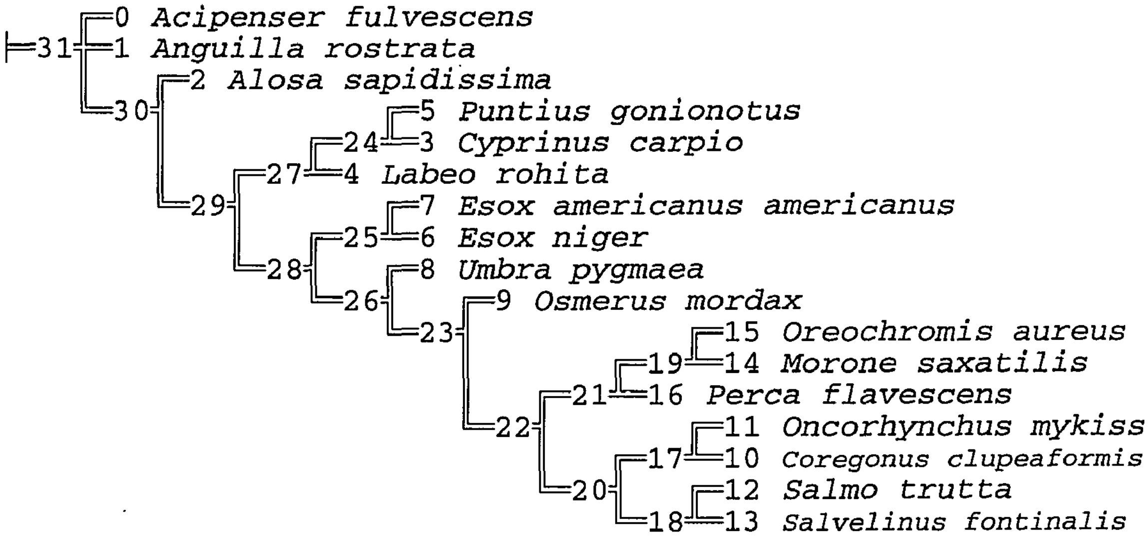


Figure 99. Continued.

```
mhennig length 68 ci 60 ri 73 trees 4
nelsen file 0 from mhennig 4 trees
tplot file 0 from nelsen 1 tree

    ╔═0 Acipenser fulvescens
╞═28╬═1 Anguilla rostrata
    ║   ╔═2 Alosa sapidissima
    ╚═27╣           ╔═5 Puntius gonionotus
        ║       ╔═21╩═3 Cyprinus carpio
        ║   ╔═24╩═4 Labeo rohita
        ╚═26╣       ╔═7 Esox americanus americanus
            ║   ╔═22╩═6 Esox niger
            ╚═25╣   ╔═8 Umbra pygmaea
                ╚═23╣   ╔═9 Osmerus mordax
                    ╚═20╬═10 Coregonus clupeaformis
                        ╠═11 Oncorhynchus mykiss
                        ║   ╔═12 Salmo trutta
                        ╠═18╩═13 Salvelinus fontinalis
                        ║   ╔═16 Perca flavescens
                        ╚═19╣   ╔═14 Morone saxatilis
                            ╚═17╩═15 Oreochromis aureus
```

Figure 100. Nelsen consensus tree of the actinopterygians based on rDNA RE and morphological characters analyzed with option *mhennig* followed by *nelsen*, *Hennig86*.

DISCUSSION

In the following sections, (i) the selection of specific rDNA regions for RE recognition site character matrix, (ii) rDNA RE recognition site homology, (iii) presence of RE recognition sites in the representative taxa, and (iv) the phylogeny analysis will be discussed.

V. Selection of rDNA region for RE recognition site character matrix:

Selection of rDNA regions for character information in order to study phylogeny varies with the diversity of the organisms. Since the experimental organisms in this study represent diverse groups of actinopterygians, an rDNA region containing 500 base pair long 5′ and 3′ ETS segments in addition to the 18S, 28S coding and ITS sequences from each of the rDNA maps, was selected for generating the rDNA RE site character matrix (Figure 8). Inclusion of ETS with the coding and ITS rDNA regions in determining phylogeny also is common for many other vertebrates (Baker *et al.*, 1991; Van Den Bussche, 1991; Phillips *et al.*, 1992).

The 5′ and 3′ ETS are known to contain rDNA transcription initiation and termination control sequences (Gerbi, 1985; Sollner-Webb and Mougey, 1991). Recently, Phillips *et al.* (1992) have described that the TR region of an eukaryotic rDNA contains about 1.3 Kb 5′ ETS and 0.3 Kb 3′ ETS flanking DNA sequences beyond the coding regions. Further, they summarized that the ETS in an rDNA evolves more slowly than the NTS, while rapidly in comparison to the coding regions (Phillips *et al.* 1992). Hence, the ETS regions may contain more conserved RE sites in relation to the NTS which may be important for determining the genealogy of distant euteleost groups.

The inclusion of rDNA ETS regions in determining euteleost phylogeny in the present study is supported by the following examples. Phillips *et al.* (1989) included RE sites present in both the 5′ and 3′ ETS regions in addition to the sites within the coding regions to reconstruct the phylogeny of six salmonid taxa from the following genera: *Oncorhynchus, Salmo,* and *Salvelinus.* Baker *et al.* (1991) included all the RE sites present within about 1 Kb 5′ ETS, in addition to many sites within the coding and ITS rDNA regions in order to determine the phylogeny of 20 chiropteran bats. Similarly, Van Den Bussche (1991) included RE site characters within about 2 Kb in the 5′ ETS, 0.1 Kb in the 3′ ETS in addition to the variable RE sites throughout the 18S, ITS, and 28S rDNA regions to determine the phylogeny of 42 phyllostomid bats.

Phillips *et al.* (1992), in their study, observed that all the evolutionarily conserved RE recognition sites are present within the coding regions of rDNA in seventeen salmonid species they studied. These taxa include four genera, *Hucho, Salvelinus, Salmo,* and *Oncorhynchus,* represented by one, six, two, and eight species, respectively (Phillips *et al.*, 1992). The RE used by Phillips *et al.* (1992) included *Bam*H I, *Eco*R I, *Hin*d III, *Pst* I, and *Pvu* II in addition to others. However, Phillips *et al.* (1992) reported the presence of several phylogenetically informative sites in the 5′ ETS in addition to a few sites in the 18S, ITS, and 28S rDNA regions among these closely related taxa. They indicated that the RE recognition sites in the 5′ ETS are more useful for the identification of species, thereby appearing to be more useful for determining the relationships between closely related taxa. However, in the present study representatives of phylogenetically distant euteleost groups were selected. Hence, the ETS as well as the coding regions of the rDNA are expected to contain phylogenetically important RE characters.

In summary, the studies cited above (Phillips *et al.*, 1989; 1992; Baker *et al.*, 1991; Van den Bussche, 1991) dealt with the phylogenies of closely related taxa, and hence, less diversity was expected to be found within the conserved coding rDNA sequences of those taxa. As a result they included (i) an extended area of 5′ ETS rDNA and (ii) more RE sites from the ETS regions in comparison to the coding regions for determining phylogeny (Phillips *et al.*, 1989; 1992; Baker *et al.*, 1991; Van den Bussche, 1991). Since the present study includes a diverse group of actinopterygians to determine the phylogeny of the euteleosts, the conserved coding regions of their rDNA are expected to possess diverse RE recognition sites when comparing between groups. Further, since the rate of evolution of the rDNA spacer regions increases with distance from the coding region, inclusion of longer ETS rDNA region may include homoplasy characters among the diverse fishes included in this study. For this reason, the RE recognition site characters present within about 500 bases of the 5′ and 3′ ETS rDNA, in addition to the coding and ITS rDNA regions, were included in generating the rDNA RE characters matrix in this study.

W. Ribosomal DNA RE recognition site homology:

Most of the RE sites within the maps of the selected rDNA regions did not require any adjustment for identifying homology. However, in a few, rare cases minor adjustments were needed for homology alignments. These adjustment of alignments may be considered similar to the alignment adjustment of amino acids and nucleotide sequences among homologous molecules at juxtapositions that are assumed to be positional homologues and applied for phylogenetic analysis (Swofford and Olsen, 1990).

In mapping the rDNA with the RE recognition sites in this study, the coding and ITS regions together were given a universal length of 6.8 Kb. This length is based on the previous observations in seventeen salmonid taxa reported by Popodi *et al.* (1985) and Phillips *et al.* (1989; 1992). In the absence of a specific and accurate length of this rDNA region for each specific taxa, the common length known for the salmonids was selected as a standard for rDNA mapping of all the fishes included in this study. However, it is known that the length of the ITS located between the 18S and 28S rDNAs varies among species even within actinopterygians (Phillips *et al.* 1989). The length of the ITS is reported to be 1.5 Kb in six salmonids, including *Salmo trutta, Salvelinus fontinalis,* and *Oncorhynchus mykiss,* (Phillips *et al.*, 1989), and 1.8, 1.6, 1.8, 1.9, and 4.5 Kb in the American eel

(*Anguilla rostrata*), goldfish (*Carassius auratus*), goosefish (*Lophius americanus*), toadfish (*Opsanus tau*), and in the spotted seatrout (*Cynoscion nebulosus*), respectively (Cortadas and Pavon, 1982: Tanhauser *et al.*, 1986). Since the lengths of the 18S and 28S rDNA are known to be relatively conserved among eukaryotes, the length variations of the ITS will affect the length of the TR. As a result, in some cases, although a RE site appears to be out-side the coding region in the map, this might be within the coding region if the accurate length of the ITS of that fish was known.

As stated above, the *Eco*R I site that appeared at about 650 bases upstream in the 5′ ETS of the *Puntius gonionotus* rDNA map, by character analysis, was found to be homologous to the *Eco*R I site within the 500 base 5′ ETS of the other taxa. Hence, it was considered to be a character present in the 5′ ETS region, and thus, was included in the character matrix.

Similarly, upon comparison, the *Eco*R I site that appeared at about 1.0 Kb upstream in the 5′ ETS of the *Oreochromis aureus* rDNA map was found to be homologous to the *Eco*R I site in the 5′ ETS rDNA of other species in this study. This homology suggests that possibly the length of the ITS in the white tilapia was longer than the standard size used in this map. A similar observation on the extended ITS length in another perciform (sea trout, *Cynoscion nebulosus*) has previously been reported by Tanhauser *et al.* (1986).

Similar to the above, in lake whitefish, *Coregonus clupeaformis*, the position of the *Bam*H I site in the 5′ ETS was found to be homologous to the *Bam*H I site in the adjacent area of the 18S rDNA. The alternate positions of a RE is considered as a single character, similarly, the alternate *Bam*H I sites in the 18S rDNA of *Salmo trutta* were considered as a single character.

Since the *Hin*d III site at the 3′ ETS rDNA of *Salvelinus fontinalis* is found to be variable, the homology of this site was reexamined. Upon removal of this site from the 3′ ETS to the adjacent NTS in the adjusted character matrix, the *Salvelinus fontinalis* appeared to represent the sister taxa to *Salmo trutta*. This observation is supported by morphological, behavioral, and mtDNA characteristics (McVeigh and Davidson, 1991; Phillips *et al.*, 1992; Stearley, 1992). Therefore, this site was excluded for further analyses.

It is known that the 28S rDNA contains several expansion segments, some of which are excised during processing of transcribed rRNA (for a review see Gerbi, 1985). It was further suggested that these expansion segments are relics of the DNA insertions in the nonfunctional regions known to be present in *Xenopus laevis* and *Xenopus borealis* rDNA and often get fully or partially deleted (for a review Gerbi, 1985). Upon analysis, it was determined that the *Hin*d III sites at the 3′ ETS rDNA of *Salvelinus fontinalis* and 5′NTS rDNA of *Oreochromis aureus* are homologous to the *Hin*d III sites in the adjacent area of the 3′NTS and 5′ ETS rDNA, respectively in the respective fishes. It is possible that due to a deletion of such an expansion segment(s), the length of the conserved area became reduced, and as a result, the position of the *Hin*d III site came closer to the 3′ ETS region in the common rDNA model used for constructing the *Salvelinus* *fontinalis* rDNA map. In the case of *Oreochromis aureus*, possibly due to unequal crossover or actions of transposons, the *Hin*d III sites might have been moved into the NTS rDNA. Further, since this species is widely used in aquaculture, it is possible that this sample fish represents a hybrid of other tilapia or cichlids. However, there is a single *Hin*d III site present within or close to the 5′ ETS rDNA regions of the other perciforms, *Morone saxatilis, Oreochromis aureus,* and *Perca flavescens* included in this study. Hence, this *Hin*d III site is considered to be homologous among all of these perciforms. Further, Tanhauser *et al.* (1986) have demonstrated that the length of the ITS is relatively longer in another perciform which may be true to some extent for the *Oreochromis aureus* which also is a perciform. Based on these assumptions, the above specific RE recognition characters are included in the character matrix.

Hence, it is possible, that all these acanthopterygians have slightly different ITS lengths than considered in the model rDNA unit based on salmonid rDNA data. Further, it also is possible for a RE recognition character to move to a distance due to DNA insertion that occurs during unequal crossing over, the actions of transposons, deletions or insertions of expansion segments in the rDNA as long as the functionality is not impaired.

The *Hin*d III site at the 3′ ETS area has been suggested to function as a transcription termination signal based on experimental observations in *Xenopus laevis* rDNA by Bakken *et al.* (1982). Hence, this site is expected to be conserved although this is not found to be true for all the fish taxa examined in this study.

Din *et al.* (1982) demonstrated that the presence of this *Hin*d III site was not absolutely essential in *Tetrahymena thermophilia* under the experimental conditions they used. Rather, poly T sites were found to perform the transcription termination signal in lieu of the *Hin*d III site (Din *et al.* 1982). From this information, it is understood that the *Hin*d III site at the 3′ ETS rDNA, where present, may function as a transcription termination signal which may be substituted by clusters of T's as shown by Din *et al.* (1982). Therefore, it was assumed that this *Hin*d III site at the 3′ ETS is not absolutely conserved. Hence, the *Hin*d III site found at the 3′ ETS of the *Salvelinus fontinalis* does not have to be homologous to the *Hin*d III site at the 3′ ETS of others.

X. Presence of RE recognition sites in the representative taxa:

X.1. *Bam*H I:

The rDNA area selected for the phylogenetic analysis was segmented into five regions, B^1, B^2, B^3, B^4 and B^5 (Figure 8), based on the available homologous *Bam*H I recognition sites. The *Bam*H I recognition site at the B^1 rDNA region was found to be present in the primitive actinopterygian (*Acipenser fulvescens*) and primitive teleost (*Anguilla rostrata*) and it reappeared in an esocoid (*Umbra pygmaea*) and two of the three acanthopterygian representatives (*Morone saxatilis*, and *Oreochromis aureus*). The reappearance of this *Bam*H I site in some derived taxa, as above, can be compared to the reappearance of dentigerous toothplate at the 4th basibranchial (a primitive feature) in esocoids while absent in all the euteleosts studied (Fink and Weitzman, 1982; Lauder and Liem, 1983; Fink, 1984; Rosen, 1985). Based on morphological characters, Stiassny (1992) suggested that within a lineage there may exist a vast reservoir of plesiomorphic characters which may be re-expressed at various levels. However, the reappearance of this character separated (1) the *Umbra* from the rest of the esocoids and (2) the *Perca* from the other two acanthopterygians (*Morone* and *Oreochromis*) included in this study. Further, this character brought the *Morone* and *Oreochromis* closer to each other and away from the *Perca*.

The *Bam*H I recognition site at the B^1 region also is found in several other vertebrates, like human (*Homo sapiens*), horse (*Equus caballus*), frog (*Xenopus laevis*), and perciform (*Cynoscion nebulosus*) while absent in cow (*Bos taurus*), pig (*Sus scofra*), sheep (*Ovis aries*) and alligator (*Alligator mississipiensis*) as reported by Tanhauser *et al.* (1986). Hence, this *Bam*H I site at B^1 rDNA region appears to be homoplastic.

The *Bam*H I recognition site at the B^2 rDNA region appears to be a synapomorphic to the protacanthopterygians since it was found to be common for all the esocoids, osmerid, and salmonids while absent among the rest of the taxa included in this study. It also may be possible that this character is a synapomorphy to neognaths and later lost by the acanthopterygians, as observed in this study. Popodi *et al.* (1985) and Phillips *et al.* (1989; 1992) also observed the presence of this homologous *Bam*H I site in the salmonids they studied, some of which also were included in the present study.

Tanhauser *et al.* (1986), on the other hand, found the presence of a homologous *Bam*H I site in (i) several placental mammals (human, *Homo sapiens;* cow, *Bos taurus;* horse, *Equus caballus;* pig, *Sus scofra;* sheep, *Ovis aries;* rat, *Rattus rattus*), a marsupial (opossum, *Didelphis marsupialis*), and (ii) a bird (chicken, *Gallus domesticus*). However, they could not find a homologous site in (i) a frog (*Xenopus laevis*), (ii) a reptile (*Alligator mississipiensis*), (iii) perciform (*Cynoscion nebulosus*), and (iv) an elasmobranch (*Carcharhinus* species, requiem shark). Since there is a structure/function relationship, this site, although conserved among protacanthopterygii, may not be essential for the rDNA functioning. Or it may be possible that another non-homologous *Bam*H I site substitutes its function, if needed. The *Bam*H I sites at the B^3 and B^4 rDNA regions in the present study appear to be partially synapomorphic traits. The *Bam*H I site at B^3 region was lost in *Cyprinus carpio, Puntius gonionotus, Esox,* and *Perca flavescens.* This character also has contributed to bring *Cyprinus* and *Puntius* closer together in support of their barbin lineage and separated them from the *Labeo,* representing the labein lineage. Further, this trait also has the potential to separate the esocid from the umbrid as well as from the rest of the protacanthopterygian lineage. In addition, it also contributed in separating *Perca* from the rest of the acanthopterygians used in this study.

The *Bam*H I site in the B^4 rDNA region in the present study is a symplesiomorphic character which is lost in two of the eight protacanthopterygians (*Umbra pygmaea; Oncorhynchus mykiss*). Loss of this site has contributed to the non-resolution of monophyly of the protacanthopterygians in this study.

The *Bam*H I sites in regions homologous to B^3 and B^4 in the present study are reported to be present in all the salmonids studied by Popodi *et al.* (1985) and Phillips *et al.* (1989; 1992). In the present study, the *Bam*H I site in the B^4 region was found to be absent in *Oncorhynchus mykiss* which may be related to the differences in the rDNA probes used. As a matter of fact, Popodi *et al.* (1985) and Phillips *et al.* (1989; 1992) used mouse/hamster rDNA probes alternately for hybridizing salmonid rDNA, while in this study only *Xenopus laevis* rDNA was used as the rDNA probe. Since a single probe was used throughout this entire study, this *Bam*H I site was included in the character matrix.

The *Bam*H I site at the B^5 region is only found in a primitive actinopterygian (*Acipenser fulvescens*) and in a salmonid (*Salvelinus fontinalis*). Although this character is lost throughout the teleostei, its reappearance in a salmonid can be an example of another atavism as recently explained and discussed by Stiassny (1992). This character also has the potential to affect the phylogeny resolution of the salmonids in this study.

The *Bam*H I recognition site at B^5 region also was found in an elasmobranch (*Carcharhinus* species, requiem shark) while absent in cow (*Bos taurus*), pig (*Sus scofra*), sheep (*Ovis aries*) and alligator (*Alligator mississipiensis*) as reported by Tanhauser *et al.* (1986). Hence, this *Bam*H I site at B^5 region appears to be rare and shows some degree of homoplasy.

X.2. *Eco*R I:

The rDNA selected for the phylogenetic analysis was again segmented into four regions, E^1, E^2, E^3 and E^4 (Figure 8), based on the occurrence of *Eco*R I recognition sites in the rDNA maps of the fishes in this study. The *Eco*R I recognition site at the E^1 region appears to be novel for *Puntius gonionotus* and *Oreochromis aureus* and contributes to separate them from their sister taxa in the phylogeny analysis.

The presence of the *Eco*R I recognition sites at E^2 was present in all the representative taxa used in this study, and thus, may be called synapomorphic. Similarly, the *Eco*R I site at the E^3 region was found to be present in all the experimental fishes except *Cyprinus carpio* and *Puntius gonionotus.* This has the potential to contribute in separating the barbin lineage (*Cyprinus* and *Puntius*) from the labein (*Labeo*) lineage.

The *Eco*R I sites at E^2 and E^3 regions are known to be present in almost all organisms studied, for example, in a loach (*Misgurnus fossilis*, Kuprijanova *et al.* 1982), salmonids (one *Hucho*, two *Salmo*, six *Salvelinus*, and eight *Oncorhynchus*, Popodi *et al.*, 1985; Phillips *et al.*, 1989; 1992), placental mammals (*Homo sapiens*, human; *Bos taurus*, cow; *Equus caballus*, horse; *Sus scofra*, pig; *Ovis aries*, sheep; *Rattus*, rat), a marsupial (*Didelphis marsupialis*, opossum), a bird (*Gallus domesticus*, chicken), a frog (*X. laevis*), a reptile (*Alligator mississipiensis*), a perciform (*Cynoscion nebulosus*), and an elasmobranch (*Carcharhinus* species, requiem shark) (Bakken *et al.*, 1982; Wilson *et al.*, 1984; Tanhauser *et al.*, 1986). Probably the presence of the *Eco*R I site at both the E^2 and the E^3 rDNA regions are functionally important, while the former is more so than the latter since the later is found to be absent in some cases in the present study. However, it is possible that the *Eco*R I site present at the non-homologous E^4 region in *Cyprinus carpio* and *Puntius gonionotus*, may substitute the function of the *Eco*R I site at the E^3 region since they are adjacently located.

The *Eco*R I site at the E^4 region was found in *Acipenser fulvescens*, *Alosa sapidissima*, *Cyprinus carpio*, *Puntius gonionotus*, *Salmo trutta*, and *Salvelinus fontinalis* (Table 26). This site has contributed in separating the barbins from the labein while bringing the clupein (*Alosa*) closer to barbins. Further, this site also has contributed in separating the *Salmo*-*Salvelinus* lineage from the rest of the salmonids.

The *Eco*R I site at E^4 rDNA region is not common in fishes and other vertebrates studied (Tanhauser *et al.*, 1986; Phillips *et al.*, 1989; 1992). However, Tanhauser *et al.* (1986) observed the presence of an *Eco*R I site at a position comparable to E4 region in an alligator. Hence, these sites appear to be specific for certain taxa.

X.3. *Hin*d III:

The selected rDNA region for the phylogenetic analysis was segmented into two regions (Figure 8), H^1 (including 5′ ETS and 18S), and H^2 (including 28S rDNA and 3′ ETS) based on homologous *Hin*d III recognition sites as found in the experimental fishes. However, *Hin*d III sites were found to be absent in the 3′ 18S, ITS and 5′ 28S rDNA areas. This is consistent within all the salmonids studied (Popodi *et al.*, 1985; Phillips *et al.*, 1989, Phillips and Pleyte, 1991; Phillips *et al.*, 1992). In murine rDNA, one *Hin*d III recognition site is present at the 5′ ITS downstream to 18S and another at the NTS (Christians and Hanawalt, 1994). A *Hin*d III recognition site is reported to be present at the end of the 3′ 18S in the mouse *Mus musculosus*, mole (*Scalopus aquaticus*), shrew (*Crocidura*), primates (*Homo sapiens* and *Lemur catta*), 18 Old World Fruit bat (Chiroptera, Baker *et al.*, 1991), and 42 New World Leaf-Nosed bat (Phyllostomidae, Van Den Bussche, 1991). Probably, this *Hin*d III site may be homologous to the *Hin*d III site present at the 5′ ITS downstream to 18S reported by Christians and Hanawalt (1994). In a sea urchin (*Paracentrotus lividus*), a *Hin*d III site is present in the 5′ ETS rDNA (Cantone *et al.*, 1993). All the above observations support the presence of a maximum of two *Hin*d III sites in the conserved rDNA regions. Further, the *Hin*d III sites are located outside the 18S and 28S rDNA coding regions or at their terminal regions. It can be seen that the number and locations of the *Hin*d III sites are limited, highly specific to rDNA locations, and present in derived forms of actinopterygians. Hence, consideration of these sites in generating the character matrix may be important in determining phylogeny of distantly related species.

The *Hind* III site at H^1 rDNA region (Figure 8; Table 26) was found in *Anguilla rostrata, Salmo trutta, Salvelinus fontinalis* and all the acanthopterygians included in this study. This site has contributed in producing the ostariophysans, the esocoids, *Coregonus-Oncorhynchus, Salmo-salvelinus* and the acanthopterygians as separate groups. Further, this has contributed to bringing *Osmerus* closer to the esocids and *Coregonus-Oncorhynchus* groups.

The *Hin*d III site at H^2 rDNA region (Figure 8; Table 26) was found in *Acipenser fulvescens, Alosa sapidissima, Osmerus mordax,* salmonids (except the *Salvelinus fontinalis*) and the acanthopterygian representatives while absent in others in this study. This site has contributed in separating the esocoids from the remaining protacanthopterygians and bringing them closer to the ostariophysans. Again the loss of this site independently in *Salvelinus,* while present in the rest of the salmonids, contributed to the anomaly observed in the phylogeny analysis. However, the *Hin*d III sites have supported the monophyly of acanthopterygians.

X.4. *Pst* I:

The rDNA area selected for generating the character matrix for the phylogenetic analysis was segmented into three regions, S^1, S^2, and S^3 (Figure 8) based on the homologous *Pst* I recognition sites. The *Pst* I site at S^1 was found to be an apomorphic character for *Oreochromis aureus* alone in this study (Table 26). This *Pst* I site was also found in some chiropteran and phyllostomid bats (Baker *et al.,* 1991; Van Den Bussche, 1991). In this study, this character contributed to separate *Oreochromis* from the rest of the fishes as well as from the other acanthopterygian representatives.

The presence of *Pst* I at S^2 has been found to be common for all the fishes except two of the outgroup taxa, *Acipenser fulvescens* and *Anguilla rostrata*, and thus, becomes a synapomorphic character for the Clupeocephala (Figure 1). This *Pst* I site was also found in (1) all the salmonids (*Hucho, Salmo, Salvelinus*, and *Oncorhynchus*), (2) placental mammals (*Homo sapiens*, human; *Bos taurus*, cow; *Equus caballus*, horse; *Sus scofra*, pig; *Ovis aries*, sheep; *Rattus rattus*, rat), a marsupial (*Didelphis marsupialis*, opossum), a bird (*Gallus domesticus*, chicken), a frog (*X. laevis*), reptile (*Alligator mississipiensis*) and an elasmobranch (*Carcharhinus* species, requiem shark), and (3) a mouse (*Mus musculosus*), a mole (*Scalopus aquaticus*), a shrew (*Crocidura*), primates (*Homo sapiens* and *Lemur catta*), and 18 Old World Fruit bats (Chiroptera), (4) 42 taxa of New World Leaf-Nosed bat (Phyllostomidae) as reported by (1) Phillips *et al.* (1989; 1992), (2) Tanhauser *et al.*, 1986, and (3) (Wilson *et al.*, 1984; Baker *et al.*, 1982), and (4) Van Den Bussche (1991), respectively. Hence, the *Pst* I site at the S^2 rDNA region appears to be highly conserved among these vertebrates with the two exceptions in the present study. However, in *Acipenser fulvescens*, there is a *Pst* I site in the NTS close to the 5′ ETS. If this *Pst* I site at the S^2 rDNA region is essential for function, then both *Acipenser fulvescens* and *Anguilla rostrata* must have analogous functional rDNA sequences. The above analogy justifies the maintenance of function in the absence of a functionally important homologous site. It may be possible that the *Pst* I site located in the NTS at the vicinity of 5′ ETS rDNA in *Acipenser fulvescens* may substitute that function. However, for *Anguilla rostrata*, a sequence other than the *Pst* I recognition site must perform that function, since no other *Pst* I site is available at nearby locations.

The other *Pst* I site at S^3 rDNA region was found in *Acipenser fulvescens, Anguilla rostrata,* and *Alosa sapidissima* and absent in the euteleosts with two exceptions (*Salvelinus fontinalis* and *Oreochromis aureus*). Although Phillips *et al.* (1989) reported a *Pst* I site to be present in the S^3 rDNA region in six salmonids, in a later report, Phillips *et al.* (1992) did not mention about its presence in any salmonid, which is confusing but may represent a correction of their earlier work. This *Pst* I site was also found to be present either in some chiropteran and phyllostomid bats (Baker *et al.*, 1991; Van Den Bussche, 1991). This character contributes to separate the *Salvelinus* from the other salmonids and the *Oreochromis* from the other acanthopterygians in this study.

X.5. *Pvu* II:

Based on the available homologous *Pvu* II recognition sites, the rDNA area selected for the phylogenetic analysis was segmented into six regions, V^1, V^2, V^3, V^4, V^5, and V^6 (Figure 8). Except for the *Pvu* II site at the V^5 region, all other *Pvu* II sites were variable among taxa. The *Pvu* II site at the V^5 region was common for all the salmonids, perciforms, *Osmerus mordax, Umbra pygmaea* and *Acipenser fulvescens,* and absent in the rest of the taxa used in this study. Phillips *et al.* (1989) also reported the *Pvu* II site at the V^5 region to be present for the six salmonids (*Salvelinus, Salmo,* and *Oncorhynchus*) they studied. However, later

Phillips *et al.* (1992) extended their study and found that this site is not present in 17 salmonids including one *Hucho,* two *Salmo,* six *Salvelinus,* and eight *Oncorhynchus.* However, they did not specify the taxa carrying the presence or absence of these sites. In the meantime, Baker *et al.* (1991) and Van Den Bussche (1991) found this *Pvu* II site to be universal for all the chiropteran and phyllostomid bats they studied. However, the other *Pvu* II sites at V^1, V^2, V^3, V^4, and V^6 regions also are found to be variable among salmonids (Phillips *et al.,* 1989, 1992), chiropteran bats (Baker *et al.,* 1991) and phyllostomid bats (Van Den Bussche, 1991).

Hence, it can be seen that the recognition sites for the *Pvu* II within the relatively conserved rDNA regions are not always conserved. While a few sites are conserved among many species, some others are conserved only within a few species; still others are specific for the taxa or the group they represent.

The *Pvu* II site at V^5 showed some degree of homoplasy, while the *Pvu* II sites at V^1, V^2, and V^6 showed a large degree of homoplasy (Table 26). While some of these will support phylogeny resolution in some cases, in others they create anomaly. The *Pvu* II site at V^2 contribute to bringing *Coregonus clupeaformis* and *Oncorhynchus mykiss* closer to each other than the other salmonids. Similarly, the *Pvu* II site at V^3 has the potential to separate the *Puntius gonionotus* from the other barbin and bring closer to the labein, which may have created the barbin labein anomaly. The presence of the *Pvu* II site at V^5 separates the esocids from the rest of the protacanthopterygians and puts them closer to ostariophysans and the other early teleosts in this study.

Y. Phylogeny analysis:

The primary goal of this thesis was to use the rDNA RE characters to analyze euteleost phylogeny (Figure 95). Since, the study covers a wide range of actinopterygians along with the outgroups, the relationships beyond the euteleosts were needed to be discussed. The phylogeny resolution obtained by rDNA was compared with the classical relationships of the actinopterygians vis-à-vis euteleosts as currently viewed based on morphology (Figure 96). Further, based on the available morphological traits, probable actinopterygian relationships were generated using parsimony analysis using the same analytical tool, *Hennig86*, and discussed (Figures 97 and 98). The above relationships also were compared with the rDNA phylogeny. Finally, the euteleost phylogeny obtained by combining the rDNA RE and morphological character matrices were analyzed in comparison to the phylogeny obtained with rDNA or morphology alone.

In the following sections the classical relationships of the actinopterygians based on morphology, rDNA RE characters alone and in combinations will be discussed in sequence. Further, both the most parsimonious trees and the consensus trees will be discussed with respect to their resolution, as appropriate. As stated by Wiley *et al.* (1991), there is a misnomer that the consensus tree provides the best estimate of the phylogeny of a group. Wiley *et al.* (1991) think that the consensus tree avoids solution of the difficult problems associated with equally parsimonious trees. Further, they suggested that a consensus tree should not be presented as a phylogeny unless it is identical to any one of the most parsimonious trees (Wiley *et al.*, 1991). The following excerpt from "The Compleat Cladist" (Wiley *et al.*, 1991) is added as additional information:

> "*As such, consensus trees must be used very carefully. It would be tempting, for example, to decide that a consensus tree, containing all the information from two different but equally parsimonious trees, gives you the best estimate of the phylogenetic relationships among groups. However, this is not true. ...... The use of consensus trees of all types has increased in the last few years .... what appears to be a simple solution of the difficult problems associated with choosing among several equally parsimonious trees. However, we feel that consensus trees solve no such problems and their use in this manner has the effect of avoiding the difficult problems associated with equally parsimonious trees. .... A consensus tree should not be presented as a phylogeny unless it is topologically identical with one or more of the most parsimonious trees.* Wiley et al., 1991: 80; 89-90.

Hence, a comparison between the most parsimonious trees and the consensus tree will be made and the areas of agreements as well as disagreements will be discussed. In addition, the steps needed to resolve the disagreements in future studies will be discussed.

Y.1. Classical relationships of the actinopterygians included in this study:

A phylogenetic cladogram of the actinopterygians in this study is constructed based on known morphological relationships (Figure 96). The relationships of *Acipenser fulvescens, Anguilla rostrata, Alosa sapidissima* and Euteleostei are based on Lauder and Liem (1983). These relationships match well with Nelson (1984; 1989; 1994). The interrelationships of the euteleosts, Ostariophysi, Protacanthopterygii, and Acanthopterygii are based on Lauder and

Liem (1983) which also match well with Nelson (1989) and Nelson (1994).

The interrelationships of the ostariophysan cyprinids are based on Chen *et al.* (1984), Howes (1991) and Cavender and Coburn (1992). The three ostariophysans included in this study belong to the subfamily Cyprininae based on the medial rostral process of supraethmoid, elongated lacrymal, anterior and posterior barbels, maxillaris nerve innervating anterior barbel through maxillary foramen, and occasionally serrated and enlarged dorsal and anal fin ray (Howes, 1991). The Cyprininae specimens belongs to two lineages: barbin and labein. The *Cyprinus* and *Puntius* belong to the barbin lineage characterized by the presence of foraminated dilatator fossa, and a lateral ethmoid articular facet, while the *Labeo* belongs to labein lineage characterized by vomero-palatine organ, double-foraminated dilatator fossa and hypertrophied supraneural bones usually contacting the cranium anteriorly (Howes, 1991).

The interrelationships of the protacanthopterygians (esocoids, osmeroids, and salmonids) are as in Nelson (1994). Based on the presence of dentigerous toothplates on the fourth basibranchial and lack of an adipose fin, Fink and Weitzman (1982) and Fink (1984) proposed esocoids to be primitive euteleosts, which also was supported by Lauder and Liem (1983). Later, Rosen (1985), based on several other morphological characters, e.g., acellular bone in the lower jaw, out growth of first uroneural, neural arches, and fused toothplates, proposed esocoids not to be euteleosts. However, Lauder and Wainwright (1992) based on the presence of posterior intermandibularis and interhyoideus, in addition to other characters as in Lauder and Liem (1983), proposed Esocae (Esocoidei) to belong to the Euteleostei as a sister group to the Osmerae-Salmonoidae. Elsewhere, Williams, in his Ph.D. thesis (1987), supported the esocoids to be a sister group to salmonids based on the suspensorium and its muscles (Nelson, 1994). Because of these conflicts, Nelson (1994) proposed to retain the esocoids as the primitive sister group to other protacanthopterygians, as before, and as shown in Figure 96.

The interrelationships of the esocoids, that the Esocidae and Umbridae are sister groups, are based on information available from Wilson and Veilleux (1982), Wilson (1984) and Wilson *et al.* (1992). The sister group relationships of the two terminal esocid taxa are supported by Wilson (1992). Wilson and Veilleux (1982) identified 45 osteological characters that separate umbrids from the esocids. The number of branchiostegals on the epihyal exceeds the numbers on ceratohyal, elongated and rectangular opercles, and the number of vertebrae exceed 42 in esocids (Wilson, 1984). On the other hand, in umbrids, the number of branchiostegals on the ceratohyal exceeds those on the epihyal. They have approximately triangular opercles and the number of vertebrae are less than 42 (Wilson and Veilleux, 1982; Wilson 1984). However, a Palaeocene esocoid, *Esox tiemani*, possessed characters that are intermediate between the modern esocids and umbrids (Wilson, 1984). This supports the sister group relationships of esocids and umbrids.

Although there are disagreements, the sister group relationships of the osmerid to salmonid, as included in the hypothetical cladogram (Figure 96), are supported by Fink and Weitzman (1982), Lauder and Liem (1983), Fink (1984), Begle (1991a) and reorganized by Nelson (1994).

The interrelationships of the salmonids are supported by several morphological characters (Phillips *et al.*, 1992; Stearley, 1992; Wilson and Williams, 1992). This group is characterized by a deep posterior myodome, peg-socket arrangement of the caudal haemal arch elements, reduced autopalatine, and toothless mesopterygoid (Wilson and Williams, 1992). The Salmoninae, represented by the trouts only in this study, possess a tripartite occipital condyle, reduced parietal, and narrow posterior infraorbitals in addition to other synapomorphic characters (Wilson and Williams, 1992).

The sister group relationships of the salmonids and Neoteleostei (represented by the acanthopterygians in this cladogram) are well supported by Lauder and Liem (1983) and Nelson (1994). Both the groups are brought together by (1) the presence of cartilage nodules between the ethmoid and premaxillae, and (2) the articulation of the basioccipital and exoccipital with the first vertebra (Lauder and Liem, 1983). However, due to the presence of the retractor dorsalis as a synapomorphic character among the acanthopterygians, they are separated from the salmonids.

The cichlids (represented by *Oreochromis aureus*) belong to Labroidei, which are highly derived perciforms (acanthopterygians) as compared to the Percoidei (*Morone saxatilis* and *Perca flavescens*) by the presence of (1) four parts of the transversus dorsalis, (2) specialized microbranchiospine on the gill arches, (3) extensively capped anterior second epibranchial with cartilage and (4) expanded fourth epibranchial head in addition to other characters (Lauder and Liem, 1983). Hence, the percoids and the labroids are separated as sister taxa. However, since the interrelationships of the percoid terminal taxa, *Morone saxatilis* and *Perca flavescens*, are not known, they have been put as sister taxa to each other in the hypothetical phylogeny (Figure 96).

Y.2. Phylogeny of the actinopterygians based on morphology with parsimony analysis:

In analyzing the phylogeny of the actinopterygians based on morphology using *Hennig86*, option *mhennig*, generated three trees, and option *mhennig* followed by command *nelsen* generated a Nelsen consensus tree (Figures 98 and 99). The option *mhennig* constructs several trees, each by a single pass, adding the terminals in several different sequences retaining the shortest trees using parsimony analysis. These trees demonstrate some of the possible relationships among the taxa under study, and thus, may differ due to character homoplasy and convergence. However, not more than one tree can represent the true phylogeny. The command *nelsen* calculated a Nelsen consensus tree from the trees generated by the option *mhennig*.

The consensus tree may not be the perfect solution to the problem to resolve the differences between the earlier trees. As mentioned above, a consensus tree is not a solution to this problem (Wiley *et al.*, 1991). Further, there are many techniques of consensus tree construction like Nelsen consensus, Adams Consensus, Strict Consensus and Majority Consensus, each coming with a different result for the same data matrix (Wiley *et al.*, 1991; Stiassny and Moore, 1992). However, careful comparison and examination of these trees with well established relationships are helpful in selecting the most appropriate phylogenetic tree based on the character matrix.

(1) *Acipenser* has been selected as the outgroup by default, as standard in this procedure. Since there was another outgroup *Anguilla*, based on the character matrix, a root was placed making the outgroup paraphyletic (as suggested in the user's guide of *Hennig86*). The *Anguilla* is a teleost based on the presence of uroneurals, a synapomorphic character for the teleosts. The above resolution was observed both in the most parsimonious and consensus trees (Figures 97 and 98). Although the *Alosa* was another outgroup, the parsimony program does not have to necessarily consider that as an outgroup.

(2) All trees, both most parsimonious and consensus, supported the monophyly of Clupeocephala, a hypothetical ancestor of the Clupeomorpha and the Euteleostei (Figure 1; Nelson, 1989). This monophyly is based on the synapomorphy of the retroarticular bone that is excluded from the quadromandibular joint.

(3) Two of the three equally most parsimonious trees generated by *mhennig* supports the monophyly of Euteleostei (tree 0, node 27; tree 2 node 27; Figure 97). The morphological character matrix contained information in support of the

euteleostean monophyly as shown by the presence of nuptial tubercle, and membranous outgrowth of the 1st uroneural. However, the consensus tree did not support the monophyly of Euteleostei. Because of the absence of a nuptial tubercles in esocids, unavailable information on the acanthopterygians, as well as a lack of knowledge on the presence of membranous outgrowth of the 1st uroneural in the representative ostariophysans the consensus tree fails to resolve this relationship.

(4) All three most parsimonious trees support the monophyly of Ostariophysi represented by the cyprinines (tree 0, node 24; tree 2 node 25; tree 3, node 25; Figure 97). Two out of three trees support ostariophysi as the sister group of Neoteleostei. The monophyly of the Ostariophysi is supported by the kenethmoid bone. The barbin and labein lineages are separated by the presence of a single or double foraminated dilatator fossa, lateral ethmoid articular facet and vomeropalatine organ. Although the consensus tree supports the monophyly of Cyprininae, it also placed Cyprininae as the sister group to both Clupeiform (*Alosa*) and Neognathi (Figure 98) which is not supported by the morphological characters, and hence, lacks resolution (Nelson, 1989).

(5) The monophyly of Neognathi is supported by either the most parsimonious trees and the consensus trees (Node 27, Figure 97; node 25, Figure 98). However, the monophyly of the protacanthopterygians is not supported. The topology of the esocoids (*Esox* and *Umbra*) also remained identical in either case (Figures 97 and 98). The relationship of the esocoids with other euteleosts

remains unresolved since there is no synapomorphic character in the morphology matrix for the esocoids in support of their monophyly. The esocids and the *Umbra* differ from each other, as well as from the osmerid-salmonids-percoids, in opercular structures as included in the matrix (Table 29). As a result, the *Umbra* is shown to represent a sister group to the esocids, and osmerid-salmonids-percoids (Figures 97, and 98).

(6) In two of the three most parsimonious trees, the monophyly of osmerid-salmonids is supported (tree 1, node 20; tree 2 node 20; Figure 97). This monophyly is probably related to the presence of (i) nuptial tubercles, and (ii) cartilaginous nodules between the ethmoid and the premaxillae. Further, this group is shown to be a sister group to acanthopterygii (tree 1, node 21; tree 2 node 21; Figure 97). However, this relationship remained unresolved in the consensus tree (Nodes 9, 19, and 20; Figure 98) since there was no synapomorphy in support of either the osmerid-salmonid, osmerid-acanthopterygians, or salmonid-acanthopterygians. The consensus tree placed the osmerid as a sister group of acanthopterygians and salmonids (Figure 98).

(7) In all the equally parsimonious trees and the consensus tree, the salmonids are grouped as monophyletic. This monophyly was also supported by Stearley (1992). The Coregonine, represented by *Coregonus* is presented as the most primitive salmonid. The presence of dentary kype in both *Salmo* and *Salvelinus* males makes them sister taxa. As a result the consensus tree is in agreement with the relationships of the salmonids as in the most parsimonious

trees (Figures 97 and 98).

(8) All the most parsimonious trees and the Nelsen consensus tree support the monophyly of the acanthopterygians included in this study (Figures 97 and 98). This monophyly was supported by their synapomorphic character to possess spiny and soft ray dorsal fins included in the matrix. Although the presence of a four part transverse dorsalis was an apomorphy for the *Oreochromis*, this single character could not separate the *Oreochromis* from the other acanthopterygians.

(9) The consensus tree (Figure 98) was found not to be identical to any one of the most parsimonious trees generated by *mhennig* (Figure 97). Hence, the consensus tree can not be taken as a phylogeny (Wiley *et al.*, 1991). Additional character information will be required to resolve their phylogeny. However, the relationships that are considered resolved in the above discussion, will be compared with the rDNA phylogeny and rDNA-morphology combined phylogeny in the following sections.

Y.3. rDNA phylogeny of the actinopterygians in this study:

Y.3.1. rDNA tree:

In the analyses of the rDNA RE character matrix using *Hennig86*, each of the options *hennig* and *mhennig* generated a single tree, and hence, each of these trees is a consensus tree. Among the above options: (i) *hennig* constructs a tree by a single pass through the data, and (ii) *mhennig* constructs several trees, each by a single pass, adding the terminals in several different sequences retaining the shortest trees.

The tree based on rDNA RE characters generated by *Hennig86,* option *hennig* gave a better resolution of the euteleostean relationships in comparison to any other tree and is presented in Figure 95. The tree length was 43, consistency index (*ci*) 44, and retention index (*ri*) 55. On the other hand, the *Hennig86,* options *mhennig* generated a tree with a length of 41, *ci* 46, and *ri* 59. Since there was character homoplasy in the rDNA, the option *mhennig* could not resolve the phylogeny. However, the tree generated by option *hennig* has a greater resolution and is more consistent and logical with the known morphological phylogeny while the other option generated highly ambiguous relationships. Further, the tree generated by *mhennig* is (i) inconsistent with the already established relationship of the euteleosts reported by Lauder and Liem (1983) and Nelson (1989), and (ii) does not resolve the relationships any further. Since the presence of uroneurals in teleosts (Lauder and Liem, 1983), exclusion of retroarticular bone from the quadromandibular joint in clupeocephalids (Lauder and Liem, 1983), kenethmoid bone in ostariophysans (Fink and Fink, 1981), cartilage nodules between the ethmoid and premaxillae in the neognathi (Lauder and Liem, 1983) are well established, these relationships are taken as indicators of distinguishing between these two trees. Hence, the rDNA phylogenetic tree (Figure 95) based on option *hennig* was accepted and discussed below.

Y.3.2. Outgroups:

Since no outgroup was assigned in this analysis, the parsimony program used lake sturgeon, *Acipenser fulvescens*, as the outgroup by default. It was suggested in the user's guide of *Hennig86* that if more than one outgroup is present, a root be placed making the outgroup paraphyletic. It appears that the analytical program accepted *Anguilla* as another outgroup based on the character matrix. Although the *Alosa* was another outgroup, the program did not have to consider that as an outgroup since it was not assigned.

This analysis showed that *Anguilla* and Clupeocephala (the hypothetical ancestor of the rest of the derived actinopterygian fishes together, Nelson, 1989; Figure 1) had a common rDNA ancestor in agreement with the hypothetical Elopocephala of Nelson (1989). This relationship also is in agreement with the previously described relationships based on (1) growth hormone amino acid sequence analysis (Bernardi *et al.*, 1993) and (2) morphology as in the hypothetical tree (Figure 96). Their monophyly based on morphology was substantiated by Lauder and Liem (1983) based on the presence of (1) two uroneurals extended over the second ural centrum anteriorly and (2) development of epipleural intermuscular bones throughout the abdomen and the anterior caudal regions. Further, the present rDNA relationships support two clupeocephalic lineages: (1) Clupeomorpha, and (2) Euteleostei as shown in Figure 95, which is in agreement with the morphological relationships (Figure 96).

The single clupeomorph representative, American shad (*Alosa sapidissima*), and the euteleosts, clearly show a common rDNA ancestor which supports the monophyly of the clupeocephalids. This is supported by a single synapomorphic rDNA RE character, *Pst* I site at S^2. This observation also is in agreement with the previously known relationships based on morphology (Lauder and Liem, 1983; Nelson, 1989; Nelson, 1994). The morphological characters that support the monophyly of this lineage are (1) exclusion of retroarticular bone from the surface of the quadratomandibular joint, (2) fused toothplates with endoskeletal gill arch elements, (3) absence or reduction of neural arch on the 1st ural centrum and (4) co-ossification of the articular bone with the angular, as summarized by Lauder and Liem (1983).

The above rDNA phylogeny provides additional support to the Euteleostei outgroups. *Acipenser fulvescens* is selected by the analytical program as an outgroup by default. However, both *Anguilla rostrata* and *Alosa sapidissima* are determined by the rDNA analysis to be the distant and closest outgroups of Euteleostei in this study, respectively. This observation is consistent with their relationships known from morphological character analyses (Figure 96; tree 0 and tree 2, Figure 97). It also is supported in part by Bernardi *et al.* (1993) who demonstrated that *Anguilla japonica,* a representative Elopomorpha, is an outgroup of Euteleostei based on growth hormone amino acid sequence. Further, Le *et al.* (1989) demonstrated the Clupeomorpha represented by *Clupea harengus,* to be an outgroup of Euteleostei based on a 300 base pair 28S rDNA sequence analysis. Hence, the relationships of the Euteleostei outgroups selected in this study are well supported by the present study, 28S rDNA sequence analysis (Le *et al.*, 1989; 1993), growth hormone amino acid sequence analysis (Bernardi *et al.*, 1993), and morphology (Figure 96; tree 0 and tree 2, Figure 97).

Y.3.3. Euteleostei:

The monophyly of Euteleostei is supported by the rDNA phylogeny in the present study (Figure 95; Node 27). The absence of a *Pst* I site at S^3 could be a synapomorphic character for the euteleosts while that site has reappeared in *Salvelinus fontinalis* and *Oreochromis aureus* as homoplasy. The ostariophysan samples included in this study represent the primitive euteleostei which is in agreement with the morphological relationships (Figure 96; tree 0 and tree 2, Figure 97). The monophyly of Euteleostei also is supported by Le *et al.* (1989) based on a 300 base pair 28S rDNA sequence. The growth hormone amino acid sequence analysis also supports this relationship (Bernardi *et al.*, 1993). However, Le *et al.* (1993) later could not resolve the monophyly of Euteleostei based on a 500 base pair 28S rDNA sequence taken from different regions. This lack of resolution may be due to the differences in the (1) rDNA sequences selected for the analysis or (2) the selection of the representative species or (3) both.

The monophyly of Euteleostei based on morphology is questioned since it is only supported by the following characters: the presence of (1) adipose fin, (2) nuptial tubercles and (3) membranous outgrowth of the 1st uroneural, most of which are lost in the derived forms (Lauder and Liem, 1983; Fink 1984; Rosen, 1985). The present observation of the rDNA monophyly of euteleostei may be further tested by adding additional taxa. In selecting the taxa, preference may be given to those groups that were not included in the present study. Since gonorynchiforms represent early ostariophysans, and the siluriforms being scaleless ostariophysans with modified armor, they may be included in order to reexamine the euteleostean monophyly based on rDNA RE character analysis. Again, incorporation of representatives from some selected neoteleosts other than the acanthopterygians will be important to test the validity of the rDNA supporting the monophyly of the euteleosts. As an example, samples from a limited but well established taxa covering from the lower to higher neoteleosts including stomiiforms, aulopiforms, myctophiforms and paracanthopterygians may be selected.

Y.3.3.1. Ostariophysi:

The ostariophysan samples included in this study belongs to Cyprininae. In the rDNA phylogeny, the Euteleostei (Node 27; Figure 95) gave rise to two clades: (1) barbin, and (2) labein-Neognathi. Barbin and labein are the two ostariophysan clades represented in this study by (1) *Cyprinus carpio* and *Puntius* and (2) *Labeo*, respectively.

The barbin and labein clades together represent subfamily Cyprininae. The rDNA phylogeny supports monophyly of the barbin and paraphyly of Cyprininae. The monophyly of Cyprininae is not supported by rDNA phylogeny which may be related to the limited number of taxa or the sample of *Labeo* selected in this study. Absence of the *Bam*H I site at B^3 region and *Eco*R I site at the rDNA E^3 region, and the presence of *Eco*R I site at E^4 region in *Cyprinus* and *Puntius* separate them from the *Labeo.* Presence of *Eco*R I site at the rDNA E^3 region in *Labeo* and the neognaths brought it closer to the neognaths. Another problematic feature is the absence of *Eco*R I site at the rDNA E^4 region in *Labeo* similar to most of the neognaths and has contributed in placing labein as the sister group of neognaths. However, had the *Eco*R I site at the rDNA E^3 region been absent and present at E^3 region in *Labeo,* it would have generated the monophyly of the cyprinines as well as the ostariophysan in this study. Since the sample of *Labeo* used in this study was sampled from an aquacultural stock, this may not represent a wild population. Although no specific published literature is available on the natural hybridization potentials of *Labeo* with other cyprinid species, in the past under experimental conditions, the FRI (Fisheries Research Institute), Bangladesh, produced hybrids between *Labeo rohita* X *Catla catla* and *Labeo rohita* X *Cirrhinus mrigalla* (Rahman, personal communication), and the present sample of *Labeo* was collected from FRI. Further, there are reports on the incidence of natural hybridization between cyprinid species (Economides and Sinis, 1988; Mir *et al.,* 1988). Hybrids between female *Ctenopharyngodon idella* X male *Hypopthalmichthys nobilis* (Beck *et al.,* 1983) and *Alburnus alburnus* X *Rutilus rubilio* (Berrebi *et al.,* 1989) are some examples. Since the sample of *Labeo rohita* used in this study comes from FRI, there has a potential for it to have genetic a composition different from its wild siblings. Or it may represent a interspecies hybrid generated some generations before since morphologically it is identifiable to be a *Labeo rohita*. Hence, a different sample of *Labeo rohita* or some other species of *Labeo* from the wild may be examined in order to resolve this ambiguity. This new sample may provide information on the rDNA characters important to answer the question of monophyly of cyprinine, vis-à-vis cyrpinids/ostariophysans.

Recent reports on the phylogeny of Cyprinidae based on morphological characters question the monophyly of this taxon since the morphological characters that qualify Cyprinidae also are shared by other cypriniforms (Howes, 1991). Cavender and Coburn (1992) pointed out that the Cyprinidae are defined by the lack of barbels and reduction in the pharyngeal tooth rows. However, (1) the reduction of tooth occurred several times independently within the family, and (2) barbels occur in about half of the North American cyrpinids, both of these facts puts the cyprinid monophyly into question. Based on this state of information, Cavender and Coburn (1992) proposed the cyprinids to be polyphyletic. Further, a strict consensus tree generated based on morphological data from Chen *et al.* (1984) supports a polyphyletic origin of Cyprininae from the outgroup Rasborinae (Cavender and Coburn, 1992). Hence, the rDNA phylogeny is in agreement with Howes (1991) and Cavender and Coburn (1992) by not supporting the monophyly of the cyprinids.

The separation of barbin (*Cyprinus-Barbus sensu lato, Puntius*) lineage from the labein (*Labeo*) lineage by the rDNA phylogeny is supported well by morphology based phylogeny (Cavender and Coburn 1992; Chen *et al.*, 1984; Howes, 1991). The growth hormone protein sequence analysis also separates the Cyprininae barbin lineage form the Leuciscinae (*Hypopthalmichthys*) lineage (Bernardi *et al.*, 1993). Both Chen *et al.* (1984) and Cavender and Coburn (1992) demonstrated the presence of two monophyletic lineages in the Cyprinidae and one such lineage is called the Cyprininae. The three cyprinid specimens selected in this study belong to the Cyprininae which is characterized by the presence of (1) medial rostral process of supraethmoid, (2) elongated lacrymal, (3) anterior and posterior barbels, (4) maxillaris nerve innervating anterior barbel through maxillary foramen and (5) occasionally serrated and enlarged dorsal and anal fin ray (Howes, 1991). However, the rDNA phylogeny in this study does not support the monophyly of the Cyprininae but rather supports a paraphyly. As discussed above, it may be related to the sample of *Labeo rohita* (collected from the FRI, a research station involved in aquaculture, and hence, may not represent a wild population) used in this study (Table 3).

Of the Cyprininae specimens used in this study, *Cyprinus* and *Puntius*, belong to the barbin lineage of the subfamily Cyprininae, family Cyprinidae (Howes, 1991). This lineage is morphologically characterized by the presence of foraminated dilatator fossa and lateral ethmoid articular facet. The monophyly of the barbin lineage is supported by the rDNA phylogeny as mentioned above. On the other hand, *Labeo* is different from the barbin lineage and belongs to the labein lineage (Howes, 1991). This lineage is characterized by a vomero-palatine organ, double-foraminated dilatator fossa and hypertrophied supraneural bones usually contacting the cranium anteriorly (Howes, 1991). Although the external appearance of *Labeo*, by its body size, scale size and color, appears close to *Cyprinus*, however, absence of the *Bam*H I site at the B^3 region and the *Eco*R I site at the rDNA E^3 region, and the presence of the *Eco*R I site at the E^4 region in *Cyprinus* and their neurocranial anatomy, separates *Labeo* and relates *Cyprinus* to *Puntius*.

There are reports on the presence of many morphological character differences between the subfamilies of Cyprinidae without resolving their interrelationships due to homoplasy (Howes, 1991; Cavender and Coburn, 1992). In order to resolve the relationships of the Cyprininae vis-à-vis, Cyprinidae and Ostariophysi, additional representative taxa from the labein lineage and other cyprinid subfamilies should be examined. Cavender and Coburn (1992) collected information from eleven cyprinid taxa, and upon analysis of their characters, came to the resolution as mentioned before. Additional rDNA RE character data also may improve the present implied relationships by extending the rDNA RE character matrix.

Y.3.3.2. *Protacanthopterygii:*

A hypothetical ancestor, Neognathi, encompasses the Protacanthopterygii and the Neoteleostei samples included in this study (Figure 1; Nelson, 1989). The rDNA phylogeny supports the monophyly of the neognaths (Figure 95, Node 24). Based on this rDNA phylogeny, the neognath rDNA appears to be derived from the labein lineage of the Cyprininae, Cyprinidae. Hence it appears that the labein lineage is close to Neognathi. However, there is no information available based on morphological characters or morphological phylogeny either in contrast or in favor of this lineage. Further, as mentioned before, since the sample of *Labeo* comes from an aquacultural stock, this may not represent a true lineage. Hence, this relationship remains as an open question to be confirmed in future studies as discussed before.

The *Bam*H I recognition site at the B^2 rDNA region is a synapomorphic character for the protacanthopterygians since it was found to be common for all the esocoids, osmerid and salmonids while absent among the rest of the taxa included in this study. However, due to character homoplasy at other sites of the rDNA, the parsimony analyses did not support the monophyly of protacanthopterygians.

Esocoidei:

The rDNA phylogeny does not support the monophyly of Esocoidei but rather their paraphyly. The Esocoidei is represented by two esocids and one umbrid in this study. Based on recent evidence, it is thought that the esocoids radiated in freshwater before the separation of Eurasia and North America as opposed to their origin in Eurasia followed by dispersion to North America, which has relevance to the evolution of the esocids and umbrids (Wilson *et al.*, 1992). However, the oldest fossil found in North America belongs to Esocidae and predates any umbrid fossil. Based on many skeletal characters, Wilson and Veilleux (1982) conclusively reported the monophyly of the Umbridae while the monophyly of the Esocoidei has never been demonstrated. Hence, the present rDNA phylogeny suggesting the paraphyly of the Esocoidei is in congruence with the present knowledge of their relationships.

The rDNA RE characters separated the umbrid from the esocid by the presence of *Bam*H I sites at B^1 and B^3, and *Pvu* II sites at V^1 and V^5 regions in the rDNA of the *Umbra pygmaea,* while absent in the esocids (Table 26). In addition, the presence of the *Bam*H I site at B^3, and the *Pvu* II site at V^5 regions in the rDNA of the *Umbra pygmaea,* and also the osmerid, salmonids, and acanthopterygians (except the *Bam*H I site at B^3 rDNA in *Perca flavescens*) brought the umbrid closer to osmerid-salmonid-acanthopterygians. Further, there was no rDNA RE character observed apomorphic for the esocoids in this study. If the esocoids are monophyletic, use of additional RE has the potential to reveal the apomorphic characters present in their rDNA.

Esocids:

The Neognathi gave rise to three lineages in the rDNA phylogeny as shown by Nodes 6, 7, and 23, in Figure 95. The two *Esox* clades were expected to form a separate group representing terminal taxa. It should be noted that although there was no difference in the RE recognition sites in the *Esox americanus americanus* and *Esox niger* rDNA region examined in this study, there was no apomorphic rDNA RE character available to support the esocids to form a group (Figures 39-48; Table 26). Due to the identical rDNA RE characters and lack of rDNA apomorphy in the conserved regions of the two esocid clades, each was chosen to be a sister of the rest of the neognaths in this analysis (Figure 95).

In order to resolve this problem, an additional RE has to be selected that will be able to find an apomorphic rDNA RE character of these two esocids. Since they represent two species, there is a potential to find RE site differences if not within the highly conserved regions, perhaps within the relatively less conserved ETS regions.

The similarity of the rDNA RE sites of the two esocids demonstrated clearly the presence of homologous characters between the two species. This observation supports the ability of the RE to find homologous characters of the rDNA across species lines.

The present phylogeny based on rDNA suggests that the *Esox* rDNA is derived from the ostariophysan (labein) lineage. Hence, they belong to the euteleost clades according to their rDNA ancestry. Further, the present rDNA phylogeny supports the *Esox* as a primitive protacanthopterygian which also is supported by morphological traits (Rosen, 1974). Molecular phylogeny of bony fishes based on growth hormone protein sequence also supports the *Esox* to represent a primitive protacanthopterygian clade (Bernardi *et al.*, 1993). Hence, the esocids remain related to rest of the protacanthopterygian lineage according to the rDNA character B^2, and with the morphology based tree (Figures 96-98).

Rosen (1974) demonstrated that the esocoids represent the most primitive protacanthopterygian based on hypobranchial anatomy. Later, several investigators, based on the presence of dentigerous toothplates on the fourth basibranchial, articular bone and retroarticular in the lower jaw, outgrowth of the 1st uroneural, and fused tooth plates in the esocids, suggested the esocids (esocoids) to be the sister group of clupeomorphs (Fink and Weitzman, 1982; Lauder and Liem, 1983; Fink, 1984; Rosen, 1985). However, a Southern hemispheric taxa, *Lepidogalaxias*, considered to be an esocoid by Rosen (1974) while possessing dentigerous toothplates on the fourth basibranchial, also possesses retractor dorsalis (RD), a synapomorphic character known to be present in the neoteleosts (Lauder and Liem, 1983, Fink, 1984). Following the presence of the RD in *Lepidogalaxias*, Fink (1984) proposed the southern hemispheric esocoids to form the sister group of neoteleosts. However, it is known today that the RD has developed independently also in *Amia*, *Lepisosteus*, and *Pantodon*, an osteoglossomorph (Lauder and Wainwright, 1992). Further, Parenti (1986) proposed the Esocae (that includes the Esocidae) as the most primitive euteleost clade based on cellular and acellular bone types. On the other hand, in a recent review, Wilson and Williams (1992) stated that the phylogenetic position of the esocids is uncertain. They also referred to several authors and summarized that, although a great deal of meristic and morphometric information including the sensory canal and pitlines adaptation are available for esocids and esocoids, they could not be polarized (Wilson and Williams, 1992). In the meantime, Williams (in his Ph.D. Thesis, 1987, University of Alberta) based on his observations of the suspensorium and its muscles, supported esocoids to be a sister group to salmonids (Nelson, 1994). On the other hand, Lauder and Wainwright (1992), based on the presence of intermandibularis and interhyoideus in addition to other characters, supported that esocoids belong to the Euteleostei as a sister group to the Osmerae-Salmonidae. Further, Nelson (1994) considers the esociforms to be the early protacanthopterygian based on Rosen (1974) and others as discussed before. The growth hormone protein sequence analysis also supports the *Esox* to be the primitive Protacanthopterygii as compared with multiple species of *Oncorhynchus* and *Salmo* (Bernardi *et al.*, 1993). Hence, the present rDNA phylogeny suggesting *Esox* as the primitive protacanthopterygii is supported by most conclusions from morphology based phylogeny and growth hormone protein sequence.

In summary, the position of *Esox* in the rDNA phylogeny supports the esocids to be a primitive Protacanthopterygii. In order to further confirm the position of esocids, an additional RE has to be selected that will be able to identify an apomorphic rDNA RE character of these two esocids. Since the two samples represent two species, there is a potential to find RE site differences, if not within the highly conserved regions, may be within the relatively less conserved ETS regions.

<u>Umbrids:</u>

The umbrids are represented by *Umbra pygmaea* in this study. An rDNA ancestor of *Umbra pygmaea* (Node 23, Fig 95) gave rise to two lineages (1) the *Umbra pygmaea* and (2) rest of the protacanthopterygians included in this study (Figure 95, Nodes 8 and 22). This observations also supports that *Umbra* is more derived than *Esox* in terms of rDNA sequences. Since umbrids are represented by a single taxon its monophyly cannot be conclusive. The rDNA RE characters separated umbrid from esocid by the presence of the *Bam*H I sites at B^1 and B^3 and the *Pvu* II sites at V^1 and V^5 regions in the rDNA of *Umbra pygmaea* while absent in esocids (Table 26). In addition, the presence of the *Bam*H I site at B^3, and *Pvu* II site at V^5 regions in the rDNA of *Umbra pygmaea* and also the osmerid, salmonids and acanthopterygians (except *Bam*H I site at B^3 rDNA in *Perca flavescens*) brought the umbrid closer to osmerid-salmonid-acanthopterygians. Further, there was no rDNA RE character observed apomorphic to esocoids in this study. If esocoids are monophyletic, use of additional RE has the potential to reveal the apomorphic characters present in their rDNA. Testing a hypothetical model with an additional apomorphic rDNA RE character for esocoids confirms the esocids and the umbrid into a monophyletic group. Hence, additional RE should be applied to reveal if an apomorphic character is present among esocoids that will include *Umbra pygmaea* in order to examine their monophyly. In order to determine the monophyly of umbrids based on rDNA, additional umbrid taxa (e.g. *Novumbra, Dallia,* and *Umbra*) as well as additional RE will be needed since the RE used in the current study did not differentiate the conserved rDNA regions between two species of *Esox* which are closely related.

Lauder and Liem (1983), Wilson (1984) and Wilson *et al.* (1992) proposed that both *Esox* and *Umbra* belongs to two sister groups. However, in the present study, the umbrid is more derived than the esocid in terms of its rDNA phylogeny. The North American fossil records support the earlier occurrence of *Esox* than *Umbrids* in the Late Cretaceous and Oligocene, respectively (Wilson and Williams, 1992). However, this cannot be considered as full proof supporting the derived nature of umbrids (Wilson and Williams, 1992). On the other hand, as reviewed by Wilson and Williams (1992), the number of vertebrae in the Umbridae ranges within 34-41 while the Esocidae has up to 56-67. If the gain in number of vertebrae is an example of a derived character, then umbrids are primitive while esocids are derived. Further, a Palaeocene fossil esocoid, *Esox tiemani*, possessed characters that are intermediate between recent esocids and umbrids (Wilson, 1984). This fossil supports the sister group relationships between esocids and umbrids. However, the above relationship does not deny that umbrids are more derived than esocids as found by the rDNA analysis in the present study. Further, the rest of the protacanthopterygians and the neoteleosts rDNA appear to be derived from umbrid lineage (Figure 95; Node 22).

In summary, (1) fossil records suggests an earlier appearance of esocids than umbrids (2) morphological evidence supports that umbrids and esocids are sister groups, and (3) molecular rDNA phylogeny suggests the umbrid rDNA is more derived than the esocid rDNA. In order to gain a deeper insight, additional esociform taxa should be included in future studies.

Osmerids:

Osmerids are represented by *Osmerus mordax* in this study. As shown in Figure 95, Node 22 gave rise to three rDNA lineages: (1) Osmerine (*Osmerus mordax;* Node 9), (2) *Coregonus-Oncorhynchus* (Node 20), and (3) *Salvelinus-Salmo-*Neoteleostei (node 21). The rDNA RE characters that separated osmerid from umbrid are the differences in the *Bam*H I sites at B^1 and B^4, the *Hin*d III site at H^2 and the *Pvu* II site at V^1 regions. However, *Osmerus* had no apomorphic rDNA RE character to define the group alone but had characters common to one or more salmonids. Both *Coregonus clupeaformis* and *Oncorhynchus mykiss* differed from the *Osmerus* by acquiring a *Pvu* II sites at V^2. *Oncorhynchus mykiss* acquired two RE characters, a *Bam*H I site at B^4 and a *Pvu* II sites at V^1, not present in *Osmerus*. These rDNA RE character differences separated osmerids from the *Coregonus-Oncorhynchus* as sister groups. If another osmerid taxon is selected, and if it possesses identical RE characters as *Osmerus mordax*, it will generate a bush without resolution. In order to resolve the monophyly of the osmerids using rDNA, two steps would be required: (i) selection of additional osmerid taxa preferably from a different genus; (ii) additional RE will be needed to find an apomorphic RE site in their rDNA, if present. The choice of additional taxa may be from a closer genus, *Plecoglossus* and a relatively distant genus *Hypomesus* or *Mallotus* (Begle, 1991a). If apomorphic sites are not present in the highly conserved rDNA regions in these taxa, relatively less conserved regions like the ETS may be extended to 1-2 Kb for the presence of apomorphic sites, as extended for the chiropteran and phyllostomid bats (Baker *et al.*, 1991; Van Den Bussche, 1991). Although the relationship of osmerids needs to be examined further, there is some agreement with this rDNA phylogeny based on morphology as shown in Figure 96 and Begle (1991a). However, it is possible that addition of some osmerid taxa mentioned above, and additional RE has the potential to resolve the above relationship.

The single osmeroid representative, *Osmerus mordax*, showed a sister group relationship with (1) *Coregonus-Oncorhynchus*, and (2) *Salvelinus-Salmo*-Neoteleostei lineages based on the rDNA RE characters used in this study. Begle (1991a) hypothesized that the osmeroids and argentinoids together belong to a sister group with salmonid-neoteleost lineage based on several morphological character analyses. In this rDNA phylogeny, the monophyly of osmerid can not be concluded or denied. However, the representative osmerid showed a sister group relationships with salmonids (Node 20) and salmonid-neoteleost (Node 21) lineages. Thus, the present observation in rDNA phylogeny of the osmerid-salmonid and acanthopterygians (neoteleosts) derive support from Begle (1991a).

Rosen in 1973 included the Osmeroidea with Protacanthopterygii as a sister group of neoteleostei. Later Rosen (1974) demonstrated that osmeroids are the most derived of the Protacanthopterygii based on both caudal and hypobranchial anatomy. However, Fink and Weitzman (1982), based on several morphological features, that include (1) large teeth along the medial border of mesopterygoid, (2) absence of basisphenoid, (3) lack of orbitosphenoid, grouped osmeroids together with ostariophysans, argentinoids, and salmonids to form a sister group to neoteleosts. Lauder and Liem (1983), based on (1) loss of the dentigerous tooth plate on the fourth basibranchial and (2) fusion of the postal neural arches in the in the caudal fin with either the 1st ural vertebra or uroneural, separated the osmeroids from the salmonids. They also described salmonids to be further derived and to be closer to the neoteleost lineage (Lauder and Liem, 1983). Parenti (1986) again grouped osmeroids together with ostariophysans, argentinoids, and salmonids based on cellular and acellular bones. Begle (1991a), based on the presence of the occipital condyle formed by basioccipital and exoccipital in salmonids, separated salmonids from the osmeroids and proposed salmonids to be a sister group to neoteleosts. Thus, Begle (1991a) supported osmeroids to be more primitive than salmonids. Later, Begle (1992) included 24 additional characters to his previously used 84 characters (Begle, 1991a) and upon analysis, suggested the Osmerae (Osmeroidei and Argentinoidei) as the sister group of Neoteleostei. However, in the present study, the rDNA phylogeny supports osmerid vis-à-vis osmeroid to be a sister group to both (i) *Coregonus-Oncorhynchus*, and (ii) *salvelinus-Salmo*-Neoteleostei lineages.

Salmonids:

The rDNA phylogeny based on the present character matrix do not support the monophyly of the salmonids. Instead it supports separate origins of rDNA for *Coregonus-Oncorhynchus*, and *Salvelinus-Salmo* lineages as shown by Nodes 20 and 21, Figure 95. The rDNA RE characters at the *Eco*R I site at E^4, the *Hin*d III site at H^1 and the *Pvu* II site at V^2 regions separated the *Coregonus-Oncorhynchus* and *Salvelinus-Salmo* lineages from each other. Further, the rDNA RE characters at one locus, the *Eco*R I site at E^4, brings the *Coregonus-Oncorhynchus* lineage closer to the acanthopterygians (neoteleosts), while at two other loci, the *Hin*d III site at H^1 and the *Pvu* II site at V^2 regions, brought the *Salvelinus-Salmo* lineage closer to the acanthopterygians (neoteleosts) than each other. Since more rDNA loci supported the relationship of the *Salvelinus-Salmo* lineage to the acanthopterygians (neoteleosts), the parsimony analysis supported them together as a group (Node 21, Figure 95). On the other hand, there was no rDNA RE apomorphic character found for the salmonids in the present study. Additional RE has the potential to determine if there are apomorphic characters in the conserved rDNA and the relatively less conserved ETS rDNA in support of their monophyly.

Stearley (1992) described the monophyly of salmonids based on their (i) tetraploid origin, (ii) presence of parr marks, (iii) detachment of neural spine from the 2nd pleural centrum, (iv) toothless endopterygoid, (v) short supraorbital, and absence of (vi) basibranchial teeth and (vi) supraopercle. However, the presence of these characters in fishes other than salmonids have not been reported, and if present, may question their monophyly. Buth *et al.* (1991) summarized several reports on the existence of many tetraploid cyprinid species (including *Barbus, Tor, Pseudobarbus,* and *Spinibarbus*). Hence, the tetraploid origin does not support the monophyly of the Salmonidae. There are some morphological characters that support the sister group relationship between salmonids and neoteleosts. For example, (i) both the exoccipital and basioccipital articulates with the 1st vertebrae, and (ii) cartilage nodules attaches the anterior ethmoid to the premaxillae (Fink and Weitzman, 1982; Lauder and Liem, 1983; Begle, 1991a) in salmonids and neoteleosts, and hence, these characters do not support the monophyly of salmonids. The present study based on rDNA may be extended further to reexamine the monophyly of the Salmonidae. Use of additional RE has the potential to reveal more rDNA RE characters, and thus, reveal if there exists characters in the rDNA supporting their monophyly. In doing so, the extension of the relatively less conserved rDNA ETS up to 2 Kb may be informative to this question. Since there are reports on the existence of natural hybridization among some salmonids (Hurrell and Price, 1991; Jansson *et al.*, 1991; Phillips *et al.*, 1992), multiple samples of the same species from separate locations may reveal their common rDNA RE pattern. It also is possible that sequencing specific segments of 28S rDNA may be more informative on this particular case, which may be examined following Le *et al.*, 1989 and 1993. Examination of other conserved genes, for example the histone genes, has the potential to yield information on their phylogeny. Only actual examination of these genes can determine their usefulness on this specific case.

As already indicated above, the rDNA phylogeny supported separate rDNA lineages for *Coregonus-Oncorhynchus* and *Salvelinus-Salmo* lineages as shown by Nodes 20 and 21, Figure 95. The absence of rDNA RE characters at the *Eco*R I site at E^4, and the *Hin*d III site at H^1 and presence of the *Pvu* II site at V^2 regions separated the *Coregonus-Oncorhynchus* lineage from the *Salvelinus-Salmo* lineage. The recently reported euteleost phylogeny based on growth hormone amino acid sequence also supports the separation of *Salmo* from *Oncorhynchus* lineages (Bernardi *et al.*, 1993).

Allendorf and Thorgaard (1984) described that salmonoids have undergone extensive radiations since their polyplodization giving rise to three major tetraploid lines: (1) Coregonidae (whitefishes), (2) Salmonidae (trouts and salmons) and (3) Thymallidae (graylings). However, based on morphology and karyotype, Phillips and Pleyte (1991) hypothesized that the Coregonidae represent the primitive salmonid taxa while *Oncorhynchus* represents the most derived form. Further, they hypothesized that *Salvelinus* is closer to the Coregoninae than to *Oncorhynchus* and *Salmo* is closer to *Oncorhynchus*. Similar conclusions also have been made by Stearley (1992) based on several morphological and behavioral characters. Hence, the present observation of salmonid phylogeny based on rDNA is not in agreement with both Phillips and Pleyte (1991) and Stearley (1992).

Stearley (1992) separated the Coregoninae from the rest of the salmonids based on the presence of subterminal mouth, vestigial vomeral, premaxillary and palatine teeth and toothless maxilla. The *Salvelinus-Salmo-Oncorhynchus* lineages are supported by the presence of several synapomorphic characters such as low coronoid dentition, large eggs, orbitosphenoid structure, stout maxillary teeth in addition to others (Stearley, 1992). The spawning behavior of coregonines, characterized by the absence of territoriality and mid-water spawning by scattering eggs over the bottom, are considered symplesiomorphic, and therefore, this group was considered to be a primitive salmonid (Stearley, 1992).

In the present study based on RE characters of the rDNA, Coregoninae, represented by *Coregonus clupeaformis*, is found to belong to a primitive salmonid group with *Oncorhynchus*. *Oncorhynchus* is found to be the sister group of *Coregonus*, and hence, closer than *Salvelinus* based on rDNA RE characters. However, this relationship of *Oncorhynchus* is not supported by morphological relationships (Stearley, 1992; Figure 96). The Coregninae also has been suggested to be the primitive salmonid clade based on morphological analyses both in the presence or absence of characters from fossil taxa (Phillips and Pleyte, 1991; Stearley, 1992; Wilson and Williams, 1992), which is supported by the rDNA phylogeny in this study.

Morphology based phylogeny supports the *Salvelinus-Salmo* lineage to be derived from the Coregoninae lineage (Phillips and Pleyte, 1991; Stearley, 1992; Figure 96). The present phylogeny based on rDNA in this study supports their sister group relationships and also supports the monophyly of *Salvelinus* and *Salmo* terminal taxa.

McVeigh and Davidson (1991) have demonstrated that in salmonids the *Oncorhynchus* lineage forms a sister group with the *Salvelinus-Salmo* lineage based on mitochondrial cytochrome b DNA sequence. This relationship is in agreement with the present observation based on rDNA. Similarly, Bernardi *et al.* (1993), based on growth hormone protein sequence phylogeny, support the above relationship. However, the position of *Oncorhynchus mykiss* as a sister taxa to *Coregonus clupeaformis* is in disagreement with the phylogeny based on morphology and behavioral analyses (Phillips and Pleyte, 1991; Stearley, 1992). In contrast to the present observations, phylogeny based on morphology suggests that *Oncorhynchus* is the most derived salmonid (Stearley, 1992). However, the interrelationships of these taxa may be reconfirmed using additional samples of *Oncorhynchus mykiss* from a different source or a different species of *Oncorhynchus*, since there are reports on the occurrence of natural hybridization among trouts and salmons (Hurrel and Price, 1991; Jansson *et al.*, 1991). The rDNA phylogeny suggests *Coregonus-Oncorhynchus* to form a sister group relationship with the *Salvelinus-Salmo* lineage. As mentioned above, the *Salvelinus-Salmo* lineage separated from *Oncorhynchus* is supported by cytochrome b mitochondrial DNA analysis (McVeigh and Davidson, 1991).

In summary, the *Salvelinus-Salmo* lineage separate from *Oncorhynchus* is supported by mitochondrial DNA analyses and growth hormone sequence (McVeigh and Davidson, 1991; Bernardi *et al.*, 1993). Although Phillips *et al.* (1989) initially suggested that *Salmo trutta* is closer to *Oncorhynchus mykiss* than *Salvelinus fontinalis*, later, Phillips and Pleyte (1991) and Phillips *et al.* (1992) repositioned *Salmo trutta, Salvelinus fontinalis* and *Oncorhynchus mykiss* forming a bush at their origin based on rDNA RE recognition sites. These differences in their own results may be related to the RE sites included in their analysis, methods of analysis or sample differences. There are reports on the high rate of natural hybridization between *Salmo trutta* and *Salmo salar* in tributaries around South-West England and Sweden (Hurrel and Price, 1991; Jansson *et al.*, 1991). If hybridization has occurred in the *Salmo trutta* sample, analyzed by Phillips *et al.* (1989; 1991), then it is possible to have variations in the less conserved segments of the rDNA in terms of RE recognition characters between samples of *Salmo trutta* as observed by them.

Y.3.3.3. *Acanthopterygii:*

The Neoteleostei are represented in this study by three acanthopterygian terminal taxa. Node 19, Figure 95, of the rDNA phylogeny supports the monophyly of the perciforms as represented by the genera *Perca, Morone* and *Oreochromis.* The *Bam*H I site at B^2 region is possibly synapomorphic, however, it is homoplastic to the ostariophysans and the outgroups in this study. The monophyly of the Acanthopterygii also is supported by Rosen (1973) and Stiassny and Moore (1992). Further, this rDNA lineage is derived from the *Salvelinus-Salmo* lineage which represents some of the salmonids, and supported by Lauder and Liem (1983) and Begle (1991a) as shown in Figure 96. The rDNA phylogeny derived from the present study resulted in a better resolution of the euteleosts vis-à-vis actinopterygii than reported by Le *et al.* (1993) based on a 500 base long 28S rDNA sequence analysis.

Le *et al.* (1993) generated a phylogenetic tree for 38 gnathostomes including

21 actinopterygians using PAUP based on a 500 base long 28S rDNA sequence. However, one rDNA ancestor node gave rise to two lineages: one such lineage gave rise to (1) pleuronectiforms (*Limanda limanda*) and (2) salmoniforms (*Oncorhynchus mykiss; Salmo salar*) and the other lineage gave rise to (1) lophiforms (*Lophius piscatorius*) and (2) perciforms represented by (*Scomber scombrus*), *Perca flavescens*, and *Trigla lucerna* which was highly ambiguous. In order to resolve the ambiguity created by the position of the genus *Limanda*, Le *et al.* (1993) eliminated a total of seven taxa from their analysis including all otophysans, clupines and elegines considering them as fast evolving. When the rRNA sequences of the remaining 31 taxa were analyzed, the relationships of the acanthopterygians and the salmonids remained as in their previous analysis except *Lophius* that formed an outgroup to the above (Le *et al.*, 1993). Hence, the relationships of the representative acanthopterygian perciforms with salmoniforms revealed by the present rDNA analysis based on RE characters are greatly resolved in comparison to the findings of Le *et al.* (1993). The present observation also is in partial agreement with the morphological relationships (Figure 96).

The rDNA interrelationships of the acanthopterygian representatives are shown in Figure 95 which also has partial agreement with the morphological relationships as shown in Figure 96. The acanthopterygians included in this study belong to the Order Perciformes, Suborders (I) Percoidei, represented by two families (1) Percichthyidae (*Morone saxatilis*) and (2) Percidae (*Perca flavescens*) and (II) the Labroidei family and (3) Cichlidae (*Oreochromis aureus*) as in Nelson

(1994). The percichthyids are brackish-marine fish while both the percids and the cichlids are freshwater species. The percichthyids were previously included within the serranids with uncertain phylogeny (Nelson, 1984). The cichlids are known to contain several derived morphological characters and are now considered to belong to the Labroidei separate from the Percoidei. *Sensu stricto,* there is no well established phylogenetic relationships known between these three families. However, since the cichlids possess several derived anatomical features and absent in two other percoids (Lauder and Liem, 1983), the cichlids can be considered as the most derived form of the three as shown in Figure 96.

The rDNA phylogeny supports the percid, *Perca flavescens,* to be the most primitive form of the sample acanthopterygians and the *Morone-Oreochromis* lineage to be more derived. Although these relationships are not in absolute conflict with the morphological knowledge, a finding of *Perca-Morone* as primitive and *Oreochromis* as derived would be more compatible based on morphological and behavioral information (Lauder and Liem, 1983; Stiassny and Gerstner, 1992). Since *Oreochromis* represents one of the sister groups of the derived perciforms, it is conceivable that the rDNA phylogeny has great potential for resolving the phylogenetic relationships of these fishes. As understood from the present study and the current literature, selection of additional representative perciform taxa and inclusion of appropriate rDNA segments and additional RE will be required in future studies in order to answer the unresolved questions. Additional representatives from the following percids *Gymnocephalus, Stizostedion* and *Zingel,* may be important to examine the topology of the *Perca* in the rDNA phylogeny since they are known to be closely related (Wiley, 1992). Another sample from any of the following cichlid genera may re-establish the position of the cichlids, *Paratilapia, Cichlasoma,* and *Hemichromis,* since they are closely related (Stiassny and Gerstner, 1992). Samples from the following percichthyids *Perichthys* and *Percilia* would also be important to re-examine their actual relationships.

Y.4. Phylogeny based on RE and Morphological Characters:

The option *mhennig, Hennig86,* generated four equally parsimonious trees based on RE and morphological character transformation series (TS) (Figure 99). The command *nelsen* reanalyzed these trees and constructed a single Nelsen consensus tree (Figure 100). The consensus tree retained the monophyly of the Clupeocephala, Euteleostei, Neognathi, *Salmo-Salvelinus* lineage and Acanthopterygii as observed by *hennig* based on rDNA RE characters alone (Figures 95 and 100). One of the most parsimonious trees demonstrated the Acanthopterygii to form a sister group to the osmerid-salmonid lineage (Tree 0; Figure 99) while the Nelsen consensus failed to resolve the Osmerus-salmonid-acanthomorph relationship. In the following sections, a summary of the above discussion and analyses is presented.

(1) The outgroups, supported by the rDNA phylogeny, remained as outgroups.

(2) The Clupeocephala was established to be monophyletic in the most

parsimonious trees and the Nelsen consensus tree. This monophyly is supported by a single synapomorphic rDNA character, the *Pst* I site at S^2 and a morphological character, TS 3, the exclusion of the retroarticular bone from the quadromandibular joint.

(3) All the most parsimonious trees and the Nelsen consensus tree supported the monophyly of euteleostei. The absence of the *Pst* I site at the S^3 region in the rDNA is a partial synapomorphy for the euteleosts since the character reappeared in *Salvelinus fontinalis* and *Oreochromis aureus* as homoplasy. Similarly, the nuptial tubercle is a partial synapomorphy for the euteleosts and information on the presence of membranous outgrowth of the 1st uroneural is not available for the ostariophysans included in this study. However, the morphological and RE character matrices together could resolve this relationship to be monophyletic.

(4) All the most parsimonious trees and the Nelsen consensus tree supported the monophyly of the ostariophysan cyprinines and also separated the barbin and labein lineages. Although the rDNA RE characters alone separated the barbin and labein lineages it failed to demonstrate the monophyly of the cyprinines vis-à-vis ostariophysans. The absence of the *Bam*H I site at B^3 region and the *Eco*R I site at the E^3 region, and presence of the *Eco*R I site at the E^4 region in the rDNA in *Cyprinus* and *Puntius* separated them from the *Labeo.* Presence of the *Eco*R I site at the rDNA E^3 region in *Labeo* and the neognaths brought the *Labeo* closer to the neognaths. Another problematic feature is the

absence of the *Eco*R I site at the rDNA E^4 region in *Labeo* which is similar for most of the neognaths. Hence, this site has contributed in placing the labeins as the sister group of the neognaths. However, the presence of a kinethmoid bone in the ostariophysans has settled the monophyly of the cyprinines vis-à-vis ostariophysans irrespective of the presence of the problematic rDNA RE characters as mentioned above. Since there is a possibility that the labein sample used may not represent a wild population and could be a hybrid from an aquacultural stock, a different sample of *Labeo rohita* or some other species of *Labeo* from the wild may be examined in order to resolve the ambiguity observed in the phylogeny based on rDNA RE characters. The selection of another sample of *Labeo* may provide character information of the rDNA important to answer this question of monophyly of cyprinine based on rDNA.

(5) The monophyly of Neognathi is supported by all the most parsimonious trees and the Nelsen consensus tree (Figures 99 and 100). Although the option *hennig* demonstrated the monophyly of Neognathi based on rDNA RE characters alone, because of extensive homoplasy, the other options could not show the monophyly of Neognathi. However, the apomorphic morphological characters of the neognaths could resolve the monophyly of the Neognathi in spite of their rDNA RE character homoplasy. Hence, the combination of rDNA RE and morphological characters has the potential to resolve this ambiguous relationship.

(6) Neither the most parsimonious trees, nor the Nelsen consensus tree

could demonstrate protacanthopterygian monophyly (Figures 99 and 100). Although the rDNA RE characters demonstrated an apomorphic character for the protacanthopterygians with a *Bam*H I site at the B^2 region, due to homoplasy at other loci, the rDNA demonstrated the protacanthopterygians to be paraphyletic. There was no apomorphic morphological trait for the protacanthopterygii present in the matrix. Hence, the combined matrix suggested the protacanthopterygii to be a polyphylectic group. An additional apomorphic character of protacanthopterygians could establish their monophyly. Since the rDNA has demonstrated an apomorphic character for the protacanthopterygians, there is a potential for the existence of additional apomorphic rDNA characters for the protacanthopterygians. Since the present study included a limited number of RE, the ambiguity should be reinvestigated using additional RE.

(7) Esocoidei:

Neither the most parsimonious trees nor the Nelsen consensus tree could demonstrate the monophyly of the esocoids (Figures 99 and 100). Further, the rDNA RE characters or the morphological characters alone could not show their monophyly. There is no apomorphic morphological character known for the esocoids, and hence, there is none incorporated in the matrix. On the other hand, the opercle characters separated the esocids from the umbrid. The rDNA RE characters that separated the umbrid from the esocid are the presence of the *Bam*H I sites at B^1 and B^3, and the *Pvu* II sites at V^1 and V^5 regions in the rDNA of the *Umbra pygmaea* while absent in the esocids (Table 26). In addition, the presence of the *Bam*H I site at B^3, and the *Pvu* II site at V^5 regions in the rDNA of the *Umbra pygmaea* and the osmerid, salmonids, and acanthopterygians (except the *Bam*H I site at B^3 rDNA in *Perca flavescens*), brought the umbrid closer to osmerid-salmonid-acanthopterygians, as compared to esocids. Hence, it appears that the rDNA RE character synapomorphy strongly supports the umbrid-osmerid-salmonid-acanthomorph common ancestry.

When the rDNA RE and morphological characters were combined and analyzed, the option *mhennig* generated four trees all showing *Umbra* closer to the osmerid-salmonids-percoids lineage (Figure 99). The esocids and the umbrids are known to form a sister group to each other although their relationships with other teleosts are frequently debated (Wilson, 1984; Begle 1991a; Begle,1992; Nelson, 1994). In Nelsen consensus tree (Figure 100), the *Umbra* has been shown to be closer to the osmerid-salmonids-percoids than the esocids. This relationship appears to be influenced by the RE characters. Umbrids are known to contain more branchiostegal rays than esocids, which is a derived character. As an example, the primitive actinopterygian representative used in this study, *Acipenser*, possesses a single branchiostegal ray while some ostariophysans contain 3-4 and salmonids contain 7-20 (Nelson, 1984). However, there is no known linearity in the number of branchiostegal rays present in fishes, hence, it is homoplastic and can not be used to determine the global phylogeny based on their numbers. On the other hand, if an increase in the number of vertebrae present is an acquired character then esocids are more derived (Wilson, 1984), which is debatable.

(8) Osmerid-salmonid-acanthopterygian relationship:

The osmerid-salmonid-acanthopterygian relationship is not resolved fully by the rDNA RE and morphological characters alone or together. The Nelsen consensus tree (Figure 100) has generated a bush giving rise to Osmerid, coregonine, *Oncorhynchus*, *Salmo-Salvelinus* and acanthomorph lineages without resolution.

The rDNA RE characters that separated osmerids from umbrids are the differences in the *Bam*H I sites at the B^1 and B^4, the *Hin*d III site at the H^2 and the *Pvu* II site at the V^1 regions. However, *Osmerus* has no apomorphic rDNA RE character to define the group alone but the *Bam*H I site at B^1 is a common character with the salmonids. Further, the *Bam*H I site at B^4 and the *Hin*d III site at the H^2 brought the osmerid-salmonid-acanthopterygians closer. As a result the parsimony analysis of the rDNA RE characters supported osmerids to have a sister group relationship to the (1) *Coregonus-Oncorhynchus* and (2) *Salvelinus-Salmo*-Neoteleostei lineages (Figure 95).

The presence of an adipose fin cartilage separates the osmerids from the others in this study. However, this character also is present in some myctophiforms (Matsuoka and Iwai, 1983). Hence, this cartilage can not be a valid character. Begle (1991a) demonstrated that the presence of a cartilaginous vane in the 1st basibranchial is an apomorphic character for the osmerids. However, the status of this character in other euteleosts have not been thoroughly examined.

Hence, there is a problem with the position of the Osmeridae. There is no character apomorphy that supports either the osmerid-salmonid or osmerid-acanthopterygian lineages. The Nelsen consensus based on morphology alone supported osmerid, salmonid and acanthopterygian sister group relationships (Figure 99). One of the most parsimonious trees (Tree 2, Figure 97) supported the Osmeridae is a sister group to the Salmonidae while the osmerid-salmonid lineage is sister to the acanthopterygians. This relationship made esocids equidistant from the osmerid-salmonid and acanthopterygian lineages.

The rDNA RE and morphological characters together generated four equally parsimonious trees with different topology. One of them retained the relationship generated by the rDNA characters alone while some others only with modifications (Figure 99). The Nelsen consensus tree retained the *Salmo-Salvelinus* and acanthopterygian relationships as generated by the rDNA RE character alone (Figure 100). However, the respective positions of the osmerids, coregonines and *Oncorhynchus* remained unresolved.

On the other hand, the four equally parsimonious trees generated by option *mhennig* gave different topologies with some resolutions (Figure 99). However, because of character homoplasy both in the rDNA RE and morphological characters, relationships of the osmerid-salmonid-acanthopterygian could not be resolved. Since the present study included a limited number of RE, additional RE should provide additional information from the rDNA in support of their phylogeny.

(9) One of the most parsimonious trees (Tree 2, Figure 99) based on rDNA RE and morphological characters together, showed *Coregonus* to be the most primitive salmonid, while *Salmo-Salvelinus* was the most advanced. This relationship is identical to the Nelsen consensus based on morphology alone. The Nelsen consensus tree based on rDNA RE and morphological characters could not retain this relationship due to rDNA character homoplasy.

(10) The Nelsen consensus tree based on rDNA RE and morphological characters retained the relationship of the acanthopterygians shown by the rDNA RE characters alone. Further, the addition of morphological characters with the rDNA characters resolved this relationship strongly. In comparison to the other acanthopterygians, rDNA RE characters E^1, S^1, S^3 and V^4 supported the *Oreochromis* to be derived while S^1 and V^4 are autoapomorphies for this genus. Further the B^1 rDNA character along with others supported *Morone* to be closer to *Oreochromis*. As a result *Perca* has been shown to be the primitive acanthopterygian perciform. However, lack of synapomorphic characters supporting percoidei lineage failed to bring *Morone* and *Perca* together while the rDNA B^1 character contributed to separated them.

Z. Synthesis and Conclusions:

This study is the first of its kind to investigate the phylogeny of euteleost using rDNA RE characters. Hence, this investigation brings in new information on the rDNA of euteleosts in addition to a few other actinopterygians. While considerable numbers of groups of fishes in this study contained synapomorphic rDNA RE characters, there was evidence of homoplasy too. Homoplasy in rDNA RE characters also were seen in salmonids and bats (Baker *et al.*,1991; Van den Bussche, 1991; Phillips *et al.*, 1992). As observed in this study, the RE *Eco*R I site at E^2 rDNA is common for all actinopterygians while the *Bam*H I site at B^2, and the *Pst* I site at S^2 are synapomorphic for the protacanthopterygians and clupeocephalids, respectively. Since rDNA is a universal molecule, the conserved regions have the potential to possess in addition to phylogenetically informative RE sites, symplesiomorphic sites based on the functional importance of the sequence. Further, in the eukaryotic rDNA, there exists many expansion segments within the conserved regions which varies among species in length and sequence (Gerbi, 1985). These segments also might have contributed in the present study to identifying group specific RE sites. Potentials of these segments in storing synapomorphic information for closely related groups are relatively greater than other areas in the conserved rDNA regions. Since these segments are distributed widely in the conserved rDNA regions, RE characterization has a greater potential to extract more information from them. This is due to the fact that the RE will be able to characterize a wider region of the rDNA at a time than DNA sequencing since DNA segments of smaller lengths are chosen for sequencing. Hence, the use of additional RE have the potential to reveal more rDNA character information that will contribute in determining the euteleost phylogeny.

Here is another important factor that need to be considered is the selection of phylogenetically informative rDNA RE sites in the conserved regions. Recently, Phillips *et al.* (1992) selected an rDNA RE site that was present in at least two taxa and at the same time absent in two other taxa. In that sense, RE sites E^2, S^1 and V^4 included in the present study may not be informative. However, there is a large difference between the present study and that of Phillips *et al.* (1992). The present study encompasses diverse groups of actinopterygians while Phillips *et al.* (1992) included multiple species from a single genus covering salmons, trouts and their close relatives. Hence, an rDNA RE site observed in one sample species in this study may be present in several species as well as in multiple genera, in the group represented by the sample. In determining the phylogeny of the chiropteran (Baker *et al.*, 1991) and phyllostomid (Van den Bussche, 1991) bats, only the variable rDNA RE sites present in the conserved regions were included. However, the authors never indicated the criteria they followed to determine a variable site. Although there in no conflict between the above and the current study, those studies included a narrow group of bats, either chiropteran or phyllostomid. However, if needed, a length of up to 2 Kb 5′ ETS rDNA may be included to determine the phylogeny of the Euteleostei since ETS rDNA is known to contain regulatory sequences and are relatively conserved than the NTS rDNA (Phillips *et al.*, 1992). Further, Baker *et al.*, (1991), Van den Bussche, (1991) and Phillips *et al.* (1992) used more than 2

Kb 5′ ETS rDNA to determine the phylogeny of bats and salmonids.

Baker *et al.* (1991), Van den Bussche (1991) and Phillips *et al.* (1992) observed most of the variable sites to be present in the ETS and ITS rDNA regions in bats and salmonids while the present study shows the variable sites also to be present within the 18S and 28S rDNA. The presence of the variable sites within the conserved rDNA regions in this study may be related to the phylogenetic distance of the groups included. Hence, the conserved rDNA regions may play an important role in studying euteleost phylogeny.

Further, the present study also demonstrated the presence of identical RE characters in the rDNA regions included in this study between two closely related species of the genus *Esox*, although additional RE may reveal their species specific diversity. This observation demonstrated beyond doubt the ability of the RE to identify homologous sites in the euteleost rDNA. Hence, RE characterization of the rDNA has been found to be highly promising in determining homologous sites in the euteleost rDNA.

As stated above, the present study has demonstrated the presence of enough diversity even within the conserved regions including the 5′ and 3′ ETS (each of 500 base pair in length) rDNA among the euteleosts needed to determine their phylogeny. The rDNA mapping with RE is an efficient method to identify homologous characters present in the rDNA. Since the present study employed a limited number of RE, additional RE have the potential to detect additional rDNA characters in these taxa which will contribute to a higher degree of phylogeny resolution. Further, in the previous sections of discussion, the need for additional sampling was identified in specific cases. Some of these will be briefly cited below in this conclusion while the previous discussion may be consulted for the details.

(1) Based on the entire rDNA matrix *Anguilla* and *Alosa* were shown to be euteleostean outgroups, which is in agreement with the known morphological relationships (Nelson, 1989). The synapomorphy of the *Pst* I site at the S^2 rDNA region supported the monophyly of the Clupeocephala that re-establishes the hypothesis of the clupeocephalan monophyly based on morphology (Nelson, 1989).

(2) Although *hennig* supported the monophyly of euteleosts based on rDNA, there was no specific synapomorphic character for the euteleosts in support of that. Probably, the monophyly was achieved as a consensus. Additional RE may be able to detect additional rDNA characters synapomorphic to the euteleosts. The morphological characters alone were not sufficient to conclude the monophyly of the Euteleostei. However, the combination of the rDNA RE and morphological characters have demonstrated the monophyly of euteleosts. This is a positive indication for the integration of both molecular and morphological information to resolve phylogeny where information from an individual source is not resolving.

(3) Although the ostariophysans are known to be monophyletic, the cyprinids are debated either to be poly- or paraphyletic (Howes, 1991; Cavender

and Coburn, 1992). The ostariophysans included in this study belong to two separate lineages, barbin and labein, in the subfamily Cyprininae. Although Cavender and Coburn (1992) demonstrated the monophyly of the Cyprininae based on morphology, the relationship based on rDNA characters alone could not demonstrate their monophyly. This discrepancy is related to the rDNA E^3 and E^4 characters of *Labeo* which separated *Labeo* form the barbins and also supported *Labeo* to be close to the neognaths. This may be due to the fact that the sample of *Labeo* was collected from an aquacultural stock where interspecies breeding involving *Labeo* has been practiced in the past. Hence, there is a potential that the sample of *Labeo* used did not represent the wild stock of *Labeo*. This relationship should be reinvestigated by including a sample of *Labeo rohita* from the wild or another species of wild *Labeo* to answer the question of cyprinine monophyly based on rDNA. However, the combination of rDNA RE and morphological characters resolved the monophyly of the Cyprininae beyond doubt.

(4) The rDNA RE and morphological characters separately and in combination supported the monophyly of the Neognathi. Although there was no single synapomorphy in the rDNA RE characters supporting the neognath monophyly, several characters together probably contributed to this resolution. The morphological characters alone also demonstrated the monophyly of the neognaths supported by a specific synapomorphy (Figures 97 and 98) and the addition of rDNA RE characters to the morphological character matrix did not change this relationship. Hence, the Neognathi appears to be a monophyletic

group beyond doubt.

(5) The rDNA RE and morphological characters alone or together do not support the monophyly of the protacanthopterygians. Although the rDNA B^2 character appears to be a synapomorphic character for the protacanthopterygians, homoplasy of the other rDNA characters masked their resolution. The growth hormone amino acid sequence analysis involving *Esox, Salmo,* and *Oreochromis* supported the monophyly of the Protacanthopterygii. However, the fate of this topology after the inclusion of *Umbra* and *Osmerus* growth hormone sequence is yet to be seen (Bernardi *et al.,* 1993). Since an rDNA synapomorphy was found for the protacanthopterygians, there exists a possibility to find another protacanthopterygian synapomorphic character in the rDNA. Addition of another synapomorphic character for the protacanthopterygians and one for the esocoids to the present rDNA character matrix can resolve the monophyly of the protacanthopterygians. This relationship was confirmed by analyzing a simulated character matrix. Hence, experiments with additional RE may be able to find an additional rDNA synapomorphic character of the protacanthopterygians, if present.

(6) There is no synapomorphic character supporting the monophyly of the Esocoidei both in the rDNA RE and morphological characters. Hence, the rDNA RE and morphological characters singly or in combination did not generate a monophyletic tree for esocoids. Additional RE may be used to investigate further if esocoids possess any synapomorphic characters in their rDNA. Since the rDNA

has both highly conserved and highly variable domains, there is a great potential for the existence of a synapomorphic rDNA character among esocoids if they are monophyletic. Although the monophyly of esocoids was not shown by the present study with rDNA, the present study amply demonstrated them to belong to the Euteleostei as well as to the Neognathi.

(7) There was no rDNA RE synapomorphy found for the two esocids studied. Since the esocids are very close to each other, there is a greater chance to find rDNA synapomorphic characters among them. Further, since esocids retained a synapomorphic rDNA character along with other protacanthopterygians, esocid rDNA has the potential to retain their own synapomorphic characters. The few RE used in the present study may not be enough to find those synapomorphic characters. Hence, in future studies, additional RE should be used in order to find them.

(8) *Umbra* was found to possess at least one rDNA RE character (B^1) that separated it from the osmerid-salmonid lineage. Addition of another umbrid taxa with the same rDNA character may produce a monophyletic umbrid group. Hence, addition of rDNA RE characters of another umbrid taxa (e.g., *Novumbra* and *Dallia*) would be very important in establishing their phylogeny. Since the Umbridae contains only three genera, samples from genera other than *Umbra* would be appropriate.

(9) Although the osmerids are represented by a single representative, the potential for the monophyly of osmerids was tested by duplicating the rDNA

characters of the *Osmerus* for another osmerid in a simulated matrix. In that analysis, the osmerids appeared to represent a monophyletic group although their relationships with other euteleosts still remained to be an open question. If the monophyly of the protacanthopterygians that includes osmerids, and the monophyly of salmonids are established, then the osmerids will form a sister group to the salmonids which can be inferred from the present study. In order to demonstrate the monophyly of the osmerids, additional taxa from a closer genus, *Plecoglossus*, and another from a relatively distant genus, *Hypomesus* or *Mallotus*, may be included. Selection of these samples will provide enough scope to find diversity as well as similarity among the Osmeridae.

(10) Although the rDNA RE characters E^4 and V^2 show partial synapomorphy for the *Salmo-Salvelinus* and *Coregonus-Oncorhynchus* lineages, there exists no rDNA RE character synapomorphy for the salmonids. Since in this preliminary study only a limited number of RE were included, use of additional RE has a great potential to find out a synapomorphic rDNA character for the salmonids, needed to establish their monophyly. Further, the samples of *Salmo, Salvelinus,* and *Oncorhynchus* used in this study were obtained from an aquacultural stock (Table 3). Since there are reports on the natural hybridization among the salmonids (Phillips *et al.*, 1992), additional samples of these genera from different sources will be needed to confirm their present rDNA RE characters. This may resolve some of the problems encountered in salmonid phylogeny in the present study.

(11) Although *Perca* and *Morone* belong to Percoidei, there was no rDNA RE character synapomorphy in support of their monophyly. However, the *Bam*H I B^1 rDNA character supported *Morone* to be closer to *Oreochromis* although the latter belongs to Labroidei, a highly derived group. Since the present study only includes a few RE, additional RE will reveal more rDNA character information, and hence, has the potential to resolve the current unresolved relationships of the percoids. It was found out that two additional synapomorphic rDNA characters for *Perca* and *Morone* would be able to resolve the percoid and labroid relationships without morphological characters. Further, additional sample taxa related to each of the current sample also would be important in resolving their phylogeny and demonstrate monophyly of the each individual clade. The groups that are related to *Perca, Morone* and *Oreochromis* are the percids (*Gymnocephalus, Stizostedion,* and *Zingel*), perichthyids (*Perichthys,* and *Percilia*) and cichlids (*Paratilapia, Cichlasoma,* and *Hemichromis*), respectively, which may be used in future studies.

In summary, the present study demonstrates that the selected rDNA region contains enough RE site variability to study the phylogeny of the Euteleostei. Since the present study included a limited number of RE, inclusion of additional RE will be necessary in order to obtain additional RE characters to reveal synapomorphy for specific groups, if present. Further, in some cases additional taxa need to be included to extend the groups already sampled in order to resolve their phylogeny and identify meaningful relationships. As pointed out in the discussion, if necessary, the rDNA ETS may also be extended up to 2 Kb in generating the rDNA character matrix in order to identify synapomorphies for each group. Finally, both molecular and morphological data may be combined in order to resolve the phylogeny of an especially difficult group.

As in the words of Stiassny and Moore (1992) pertaining to acanthomorph relationships, "*.....considerably more data from many more taxa are necessary....*", this study also requires more taxa and more RE to increase their rDNA characters for conclusive information on euteleost phylogeny.

APPENDIX

APPENDIX 1
LIST OF CHARACTERS USED FOR GENERATING MORPHOLOGICAL MATRIX

Actinopterygii (Lauder and Liem, 1983)
1. Presence median fins supported internally by paired segmented dermal ray.

Teleostei (Lauder and Liem, 1983)
2. Presence of uroneurals.

Clupeocephala (Lauder and Liem, 1983)
3. Retroarticular bone excluded from the quadromandibular joint.

Euteleostei (Lauder and Liem, 1983)
4. Nuptial tubercle.
5. Presence of membranous outgrowth of the 1st uroneural.

Ostariophysi(Fink and Fink, 1981; Lauder and Liem, 1983; Howes, 1991; Nelson, 1994)
6. Kinethmoid bone.

Barbin lineage (Howes, 1991)

7. Foraminated dilatator fossa.
8. Lateral ethmoid articular facet.

Labein lineage (Howes, 1991)
9. Vomeropalatine organ.

Neognathi (Lauder and Liem, 1983)
10. Cartilage nodules between the ethmoid and premaxillae.
11. Specialized suspensorium muscle present (Begle, 1992; Nelson, 1994).

Protacanthopterygii

Esocoidei
12. Rudimentary neural arch while developed in salmonids and osmerids (Rose, 1974).

Esocid
13. Opercle rectangular (Wilson, 1984).

Umbrid
14. Opercle triangular (Wilson, 1984).

Osmeroidei, *sensu stricto*, Osmeridae
15. Adipose fin cartilage (Matsuoka and Iwai, 1983).

Salmonidae (Stearley, 1992)
16. Tetraploid karyotypic origin.
17. Parr marks present.

Coregoninae (Stearley, 1992)
17. Parr marks absent (R).

Salvelinus (Stearley, 1992)
18. Dentary with kype in breeding males.

Salmo (Stearley, 1992)
18. Extreme development of kype.

Oncorhynchus (Stearley, 1992)
19. Cutthroat mark present.

Neoteleostei/Acanthopterygii

Percoidei: (Nelson, 1984; Roberts, 1993)
20. Spiny and soft ray dorsal fins.

Labroidei/Cichlidae (Kaufman and Liem, 1982)

21. Transversus dorsalis divided into four parts.

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