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## Esociform Phylogeny

J. ANDRÉS LÓPEZ, WEI-JEN CHEN, AND GUILLERMO ORTÍ

Despite numerous studies aimed at resolving relationships among basal euteleost lineages, many aspects of their phylogeny remain the subject of debate. The Esociformes have proven particularly difficult to place, and although a hypothesis of relationships within this group first proposed by Nelson has been generally accepted, a recent hypothesis based on evidence from mitochondrial DNA sequences is not congruent with it. We have assembled an expanded dataset of DNA sequences from the mitochondrial and nuclear genomes to test existing hypotheses of esociform inter- and intraordinal relationships. This dataset includes representatives from all extant esociform lineages and a wide diversity of potential outgroups (51 taxa in total). We also conducted a review of the morphological information that supports currently held hypotheses of esociform inter- and intraordinal relationships. This review revealed potential problems with character state coding and interpretation of character states. However, the molecular evidence, particularly the nuclear sequences, produced unambiguous support for a sister-group relationship between esociforms and salmonoids and also offer similarly strong corroboration of the hypothesis of esociform intraordinal relationships based on mitochondrial sequences and for the monophyly of the subgenera *Esox* and *Kenoza* of *Esox*. In addition to the conclusions regarding esociform relationships, the molecular evidence we present offers support for the monophyly of the Osmeridae, for a sister-group relationship between the Retropinnidae and the Osmeroidei (Osmeridae + Salangidae + Plecoglossidae) and for a close relationship of Stomiiformes and Osmeriformes.

THE basal euteleost lineages include those fish groups that Greenwood et al. (1966) placed in the Protacanthopterygii, which was erected to collect the most primitive members of their Division III of teleost fishes, which broadly corresponds to the Euteleostei without the inclusion of Clupeomorpha. In the Protacanthopterygii, Greenwood et al. (1966) grouped salmoniforms, gonorhynchiforms, myctophoids (Aulopiformes and Myctophiformes), cetamimoids, giganturids, ateleopodids, and, tentatively, the Ostariophysii. Despite recurrent study of the phylogenetic relationships of the protacanthopterygian lineages, a robust hypothesis remains elusive and alternative hypotheses abound (e.g. Rosen, 1974; Fink, 1984; Johnson and Patterson, 1996). One consequence of the diversity of opinion on this subject is that protacanthopterygian membership has been modified extensively since its inception, at one point being reduced to the Salmoniformes (e.g., Rosen, 1974) and, thus, becoming a redundant taxonomic concept. Rosen (1974) con-

sidered the Salmoniformes to include esociforms (with *Lepidogalaxias*), argentinoids, alepocephaloids, salmonoids, osmeroids, and galaxioids.

Although ichthyologists have not yet arrived at a generally accepted reconstruction of basal euteleost relationships, there are some common features among the hypotheses advanced to date. One of these common features is the difficulty in placing the basal euteleost lineage of the Esociformes (pikes, pickerels, and mudminnows). So far, searches for suites of derived character states that may identify the esociform sister group have yielded scarce evidence and produced weakly supported hypotheses. It seems that the gross morphology of esociforms is largely characterized by autapomorphy and euteleost plesiomorphy. Esociformes have been proposed to be (1) sister taxon of all other euteleosts (Fink and Weitzman, 1982), (2) sister taxon of all other protacanthopterygians (with *Lepidogalaxias* included among esociforms; Rosen, 1974), (3) sister taxon of the salmonoids

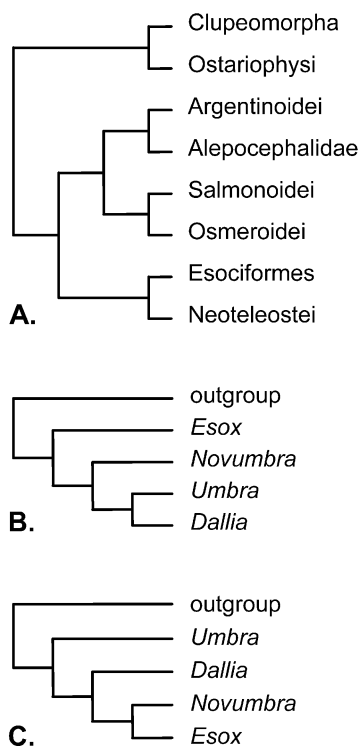


Fig. 1. Hypotheses of esociform relationships: (A) Johnson and Patterson, 1996; (B) Nelson, 1972, which was not derived from a strictly cladistic study; (C) López et al., 2000.

(Fink, 1984; Williams, 1987) and (4) sister taxon of the neoteleosts (Parenti, 1986; Johnson and Patterson, 1996).

Johnson and Patterson's (1996) study is the most recent, extensive, and comprehensive cladistic analysis of basal euteleosts relationships available. Their study offers the following conclusions regarding esociform relationships: (1) tentatively, a sister-group relationship between Esociformes and Neoteleostei (Fig 1A); and (2) agreement with the hypothesis of relationships among esociform genera of Nelson (1972; Fig 1B). In this last point, Johnson and Patterson (1996) deferred to prior studies by Nelson (1972) and Wilson and Veilleux (1982) and accepted their findings. Although, Nelson's (1972) analytical approach was not formally cladistic, the conclusions he supported formed the basis for a generally accepted set of evolutionary relationships among esociform taxa. In this contribution, we refer to Nelson's (1972) proposal for esociform classification as Nelson's hypothesis of relationships.

López et al. (2000) analyzed DNA sequences of two mitochondrial genes and proposed an arrangement of esociform genera incongruent

with Nelson's (1972) hypothesis and suggest a close relationship between Esociformes and Salmoniformes. According to the molecular hypothesis, *Umbra* is the most basal esociform genus, and *Novumbra* and *Esox* are sister genera (Fig. 1C). The monophyly of the family Umbriidae was strongly rejected by tree comparison tests. Unfortunately, the taxonomic sampling of that study did not include all species of *Esox* and had a very limited set of alternative outgroup taxa (López et al., 2000:429). Furthermore, all sequence data were derived from the mitochondrial genome only, and the strongest evidence in support of their conclusions came from the 16S sequences, which show levels of divergence among esociforms large enough to suggest potential problems with the alignment of some extremely variable loop regions.

A number of recent molecular studies support a close relationship between esociforms and salmonoids. Most of these were based on incomplete taxonomic samples that did not allow firm conclusions regarding esociform relationships (see summary in Zaragueta-Bagils et al., 2002). The most extensive molecular study of protacanthopterygian relationships is that of Ishiguro et al. (2003), using whole mitochondrial genome sequences. Their analysis supports (82% bootstrap) a sister-group relationship between the two esociforms (*Dallia pectoralis* and *Esox lucius*) in their study and the salmonoids.

Other evidence seemingly in conflict with Nelson's (1972) hypothesis of esociform relationships comes from a number of studies of the karyotypes and other cytological features. In the most recent of these studies, Crossman and Ráb (2001) surmised that the karyological characteristics of esociforms support a sister-group relationship between *Dallia* and *Novumbra* and an uncertain placement of *Esox* in relation to these two lineages. Unfortunately, the methodology employed so far in the study of esociform karyology results in observations that are difficult to frame in the context of a cladistic analysis, with the requisite assertions of homology of chromosome elements. Thus, although intriguing, the karyological information currently available are not adequate to reassess esociform relationships.

Paleontological evidence provides some indication of the age of the esociform lineage and the history of its morphological diversity. If the fragmentary fossils described by Wilson et al. (1992) are correctly assigned to the esociforms, they show that this lineage originated as early as the late Cretaceous. More definite evidence of esociforms comes from nearly complete and well-preserved fossil skeletons from the Paleo-

cene of Alberta and Saskatchewan, which can be assigned confidently to *Esox* (Wilson, 1980) and from material of similar quality from the Eocene of Wyoming that may represent species of the subgenus *Kenoza* of *Esox* (Grande, 1999). If López et al.'s (2000) hypothesis of esociform intrarelationships is correct, this fossil evidence would imply that all four lineages leading to extant esociform genera had originated no later than the Paleocene because in that hypothesis the origin of the lineages leading to *Novumbra* and *Esox* is younger than those of the two other esociform genera. Some putative early esociform fossils exhibit characteristics that have been interpreted as transitional between the "umbrid" and the esocid type (Sytchevskaya, 1976). However, Grande's (1999) summary of information on the fossils of *Esox* shows that at this point the paleontological evidence is insufficient to confidently infer the characteristics of the esociform ancestors and to guide our inference of relationships among esociform lineages.

As part of ongoing efforts to better understand the phylogeny of actinopterygians and the evolution of esociforms, we have assembled a new dataset consisting of DNA sequences from both the nuclear and mitochondrial genomes from most of the extant species of Esociformes and a wide sample of euteleosts to test previously proposed hypotheses of esociform intra- and interordinal relationships.

#### MATERIALS AND METHODS

We collected DNA sequences from representatives of the four extant esociform genera and a wide selection of possible outgroups. The taxonomic sample included at least one and more often several members of all the lineages that have been proposed as possible sister groups to the esociforms (e.g., four salmonoids, eight neoteleosts, 13 protacanthopterygians; see Material Examined). The sample also included secondary or more distant outgroups (*Scaphirhynchus*, *Amia*, two osteoglossiforms, three elopomorphs, and two clupeiforms) for the purpose of rooting the euteleost portion of the tree. One important species missing from this taxonomic sample is *Lepidogalaxias salamandroides*. The only tissue sample available from an individual of this species did not yield amplifiable DNA. Despite this important omission, this taxonomic sample is sufficient to test the most widely accepted hypotheses of esociform relationships because it includes several representatives of all the groups that have been allied to esociforms in those hypotheses.

We sequenced a fragment from the 3' end of

the single-copy recombination activation gene-1 (RAG1) from the nuclear genome and two fragments from the 3' halves of the 12S and 16S ribosomal RNA genes from the mitochondrial genome. We used PCR, gel electrophoresis, PCR-product purification, dye-deoxy chain termination, and automated sequence determination protocols to obtain the DNA sequences. For 12S, we used primers L1091 and H1478 (Kocher et al., 1989) and for 16S, 16Sar-L, and 16Sbr-H (S. Palumbi, unpubl.). These primers amplify fragments of the 12S and 16S mitochondrial rRNA genes corresponding to positions 1508 through 1896 and 3009 through 3588, respectively, of the *Oncorhynchus mykiss* mitochondrial genome (GenBank accession NC001717; Zardoya et al., 1995). For RAG1, we used primers designed to amplify the more slowly evolving 3' half of the gene (approximately 1400bp). The primer names and sequences are RAG1F1—CTG AGC TGC AGT CAG TAC CAT AAG ATG T; RAG1R1—CTG AGT CCT TGT GAG CTT CCA TRA AYT T; RAG1R2—TGA GCC TCC ATG AAC TTC TGA AGR TAY TT; and RAG1R3—GTC TTG TGS AGG TAG TTG GT. The primer set RAG1F1—RAG1R1 targets the region spanning between nucleotide positions 2215 and 3772 of the *O. mykiss* RAG1 sequence accessioned in GenBank (U15663). The primer RAG1R2 is an alternative reverse primer that we used when RAG1R1 produced unsatisfactory results and the primer RAG1R3 is an internal primer that we used to obtain the full DNA sequence of the fragment amplified by the external primers.

Because of the diversity spanned by our taxonomic sample, temperature and cycling conditions for PCR were optimized as required by different target genes and template species. In general, the amplification of mitochondrial genes required the least optimization and in most cases amplification was successful using 30 to 34 cycles with an annealing temperatures between 48 C and 55 C. The amplification conditions for RAG1 required more target specific conditions, but, in general, the number of cycles was between 32 and 40, and the range of annealing temperatures was between 52 C and 56 C. PCR conditions for specific taxa may be obtained from the authors.

We aligned the RAG1 sequences manually using the amino acid translation to guide the placement of the 10 amino acid insertions/deletions and the two introns (only found in *Argentina* and *Bathylagus*). We used the program Clustal X (Thompson et al., 1997) to align the 12S and 16S sequences and manually edited the resulting alignments guided by the secondary

structure of these molecules as proposed by Wang and Lee (2002) and Waters et al. (2000), respectively. To edit the alignment in regions of high variability, we used conserved stem regions as anchoring points. We discarded regions where the amount of length variation was very high and the resulting alignment would likely contain invalid assertions of homology (Swofford et al., 1996).

We compared base composition among sequences using the Chi-square test implemented in PAUP\* version 4.0b10 (D. Swofford, unpubl.) to determine the potential for artifacts in phylogenetic reconstruction that may result from convergence in base composition bias. To obtain optimal maximum parsimony (MP) and minimum evolution (ME) trees from each dataset, we conducted 1000 replicate heuristic searches with random taxon addition and random starting trees with tree-bisection-reconnection (TBR) branch swapping and saving the optimal tree from each replicate. We determined the bootstrap indices of support based on MP and ME criteria. In these bootstrap analyses, we conducted a full heuristic search for the optimal tree of 1000 bootstrap pseudoreplicates. Each heuristic search invoked TBR branch swapping. In MP bootstrap, each heuristic search consisted of five replicate searches with random taxon addition sequence and random starting tree.

We also conducted a heuristic search for the optimal maximum likelihood (ML) tree of each dataset to determine whether this analysis produced conflicting results (10 replicates with random starting trees, random taxon addition and TBR branch swapping). To select the substitution model for ME and ML, we used the routine devised by Posada and Crandall (1998) as implemented in MODELTEST version 3.06. ML searches were also conducted with the program Treefinder (G. Jobb, unpubl.) that implements a fast, deterministic, genetic tree search algorithm. Treefinder searches used default conditions under the General Time Reversible model with invariant sites and among site rate heterogeneity (GTR+I+ $\Gamma$ ). This program also was used for a bootstrap analysis under ML with 1000 replications. Bayesian analyses were conducted with the program MrBayes version 3.0 (Huelsenbeck and Ronquist, 2001) using the GTR+I+ $\Gamma$  model selected by MODELTEST for DNA sequences. Translated protein sequences for RAG-1 were analyzed under the Poisson model. For simultaneous analyses of RAG-1 and mtDNA data, we implemented a composite model approach, specifying one model for each data partition (i.e., nuclear DNA-mtDNA or nuclear protein sequence-mtDNA). Bayesian anal-

yses used 4 chains, ran for 300000 generations with a sample frequency = 200, and a burn-in value of 500 (i.e., a total of 1000 trees were sampled for each analysis to compute the consensus tree and posterior probabilities). The burn-in value was determined by examining a plot of overall model likelihood against generation of the chain to find the point where likelihood values stabilized. Results from more than one run were compared to provide additional confirmation of convergence among likelihood values, tree topologies, and posterior distributions.

We compared the phylogenetic hypothesis supported by the sequences with those that have been previously proposed using both parsimony and likelihood based tests (Kishino and Hasegawa, 1989; Shimodaira and Hasegawa, 1999) implemented in PAUP\* v.4.0b10. In addition, we visually inspected the aligned RAG1 amino acid translation in search of substitutions that could be interpreted as synapomorphies of the clades supported by alternative hypotheses of esociform phylogeny.

## RESULTS

*RAG1 sequences.*—The alignment of the RAG1 sequences used in this study consists of a fragment coding for 475 amino acid sites from the carboxyl half of the molecule (*O. mykiss*, U15663; Hansen and Kaattari, 1995). This fragment is located downstream of the intron RAG1b according to the nomenclature of Venkatesh et al. (1999). The DNA sequence alignment includes 1351 nucleotide sites after we eliminated sites with missing or ambiguous data. The sites eliminated include gaps created in the alignment by insertions/deletions in the amino acid sequence (10 residues) and two previously undescribed RAG1 introns that we discovered in the sequences of *Argentina sialis* and *Bathylagus ochotensis*. One of the two introns discovered in this study is shared by *B. ochotensis* and *A. sialis*, the only two argentiniforms in our taxonomic sample. This shared intron is located in the codon corresponding to residue 963 on the *O. mykiss* RAG1 protein. This intron is 338 bp long in *B. ochotensis* and 125 bp long in *A. sialis*. The other intron is unique to *B. ochotensis* among the taxa in our sample. It is of undetermined length, and it interrupts the codon corresponding to residue 899 of the *O. mykiss* RAG1 protein. The distribution of amino acid insertions/deletions in our taxonomic sample was not phylogenetically informative as concerns esociform relationships so we discarded the sites involved.

When all sites in the RAG1 sequence are con-



sidered, the null hypothesis of base composition stationarity among the species in this study is rejected using the Chi-square test implemented in PAUP\* ( $P < 0.0001$ ). Examination of base composition of the RAG1 sequences by codon position categories shows that the source of the heterogeneity is the third codon position sites. The null hypothesis of base composition stationarity at third codon position sites is strongly rejected ( $P < 0.0001$ ). Among the taxa represented in this study, the combined proportion of Gs and Cs (GC content), varies widely and continuously in a range from 0.46–0.95. *Ophichthus gomesii*, *Gonostoma bathyphilum*, and the two galaxiids in our sample (*Brachygalaxias bullocki* and *Galaxias fasciatus*) have GC content higher than 0.90 at third codon position sites. The range of GC content of osmeriforms (0.71–0.94) overlaps that of salmonoids (0.77–0.79), and the range of esociforms (0.65–0.74) overlaps with osmeriforms but not with salmonoids. The Chi-square test fails to reject the null for first and second codon position sites ( $P = 1.0$ ). At these sites, the magnitude of the range of GC content spanned by the taxa in this study is much reduced in comparison to that observed at third codon positions (0.11, 0.06, and 0.49 at first, second, and third codon position sites, respectively). Because salmonoids and esociforms do not show shared distinct patterns of base composition, it is unlikely that the sister-group relationship between salmonoids and esociforms supported by these sequences is an artifact of convergent base composition.

A plot of observed uncorrected transitions against transversions shows a deviation from a homogeneous relationship between transitions and transversions for RAG1 sequences. This deviation may be the result of changes in the substitution process and/or substitution saturation. The points in the plot become greatly dispersed at values above 6% transversions and 10% transitions. The extensive dispersion of points indicates heterogeneity in the substitution process among the taxa represented in the study. This observation is in agreement with the implications of the base composition heterogeneity and variability in GC content at third codon position sites described above.

We found six shortest trees under parsimony (5266 steps, CI 0.283, RI 0.532). The strict consensus of these trees is in general agreement with the MP bootstrap majority rule consensus tree (Fig. 2), and, where it concerns esociform relationships, the topologies of both trees are identical. The MP bootstrap analysis strongly supports the monophyly of the esociforms, the sister-group relationship of salmonoids and esociforms and the esociform intraordinal relationships proposed by López et al. (2000). Also, the taxa in this study representing the argentinoids (two species), eurypterigians (eight species), clupeiforms (two species), galaxiids (two species), ostariophysans (seven species), osmeroids (five species), retropinnids (two species), salmonoids (four species) and, stomiiforms (two species) form well-supported clades. Support for the Euteleostei (without Ostariophysa) is marginal (63%), and relationships among outgroup taxa are not well resolved, presumably because of poor taxonomic sampling. For example, the order Osteoglossiformes, represented by *Hiodon alosoides* and *Osteoglossum bicirrhosum*, receives weak to no support. Monophyly of elopomorph taxa *Albula vulpes*, *Megalops atlanticus* and, *Ophichthus gomesii* is not supported.

The base substitution model that best fits the data (GTR+I+ $\Gamma$ ) is the general time reversible with among site rate variation and invariant sites with the following parameter values: base frequencies (A 0.2253, C 0.2886, G 0.2848, T 0.2014), substitution rates ( $r_{AC}$  1.4203,  $r_{AG}$  3.4727,  $r_{AT}$  1.6624,  $r_{CG}$  0.8849,  $r_{CT}$  4.7203), gamma distribution shape parameter ( $\alpha$ ) 1.1595, and proportion of invariant sites ( $p_{inv}$ ) 0.384. Using this model for Bayesian analysis resulted in the tree shown in Figure 2. The same relationships found in the MP analysis are strongly supported, plus the grouping of stomiiform taxa (*Vinciguerria* and *Gonostoma*) and the galaxiids with the Osmeridae + Retropinnidae clade and the argentinoids as a sister group to the Eurypterygii clade. ML analysis (with Treefinder) had almost identical results, except that the position of argentinoids shifted to sister taxon of the salmonoid-esociform clade but with very low bootstrap support (53%). ME analyses of RAG-1 DNA sequences (under the GTR+I+ $\Gamma$  model distances) supports the same set of relationships for the euteleost taxa, with the exception of the position of argentinoids that are placed with the Osmeridae + Retropinnidae + Stomiiformes clade together with galaxiids (62% bootstrap support).

Parsimony and likelihood tree comparison tests indicate that alternative hypotheses of esociform and basal euteleost phylogeny represent significantly poorer tree topologies for the RAG1 sequences (Table 1). A visual inspection of the amino acid translation of these sequences revealed residues that may represent synapomorphies of (1) the salmonoid + esociform clade (valine to threonine at 777, proline to cysteine or arginine at 906, and threonine to valine at 958 of the *O. mykiss* translated sequence with GenBank accession no. AAA80281); (2) the

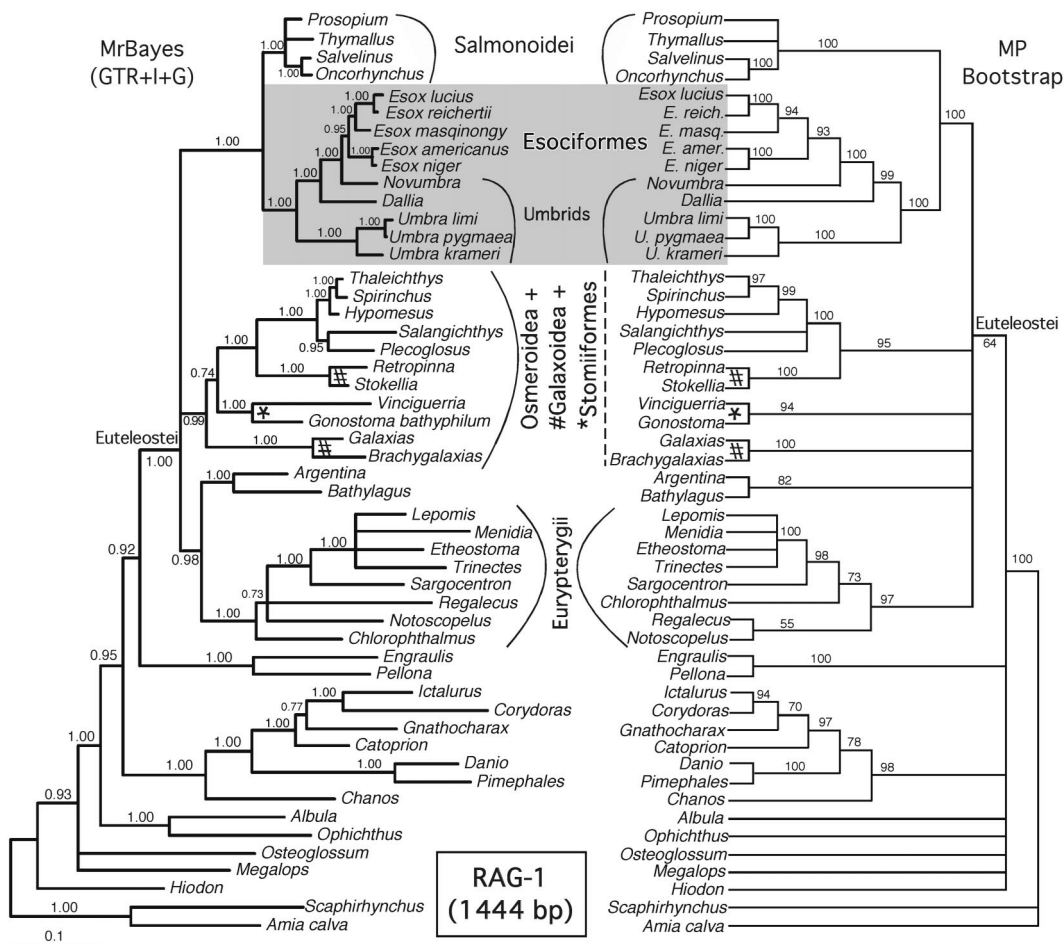


Fig. 2. Bayesian (left) and maximum parsimony (right) analyses of RAG-1 sequences. The majority-rule consensus phylogram obtained with Bayesian analyses shows posterior probability values at each node and was based on a GTR+I+G model (see text for more information). The parsimony bootstrap consensus tree was obtained from 1000 pseudoreplicates and only shows bootstrap support values > 60 (nodes with values under 60 were collapsed).

clade composed of *Dallia* + *Esox* + *Novumbra* (valine to isoleucine at 850, glutamine to asparagine or aspartic acid at 852, AAN codon to glutamine at 874, and glutamine to lysine at 923); and (3) the clade composed of *Esox* + *Novumbra* (threonine to asparagine 641). We could not detect any amino acid substitutions that could be best interpreted as shared derived residues of an osmeriform + salmonoid clade, the family Umbridae or the subfamily Umbrinae.

*Mitochondrial ribosomal DNA sequences.*—The alignment of the 12S sequences includes between 302 and 312 nucleotides corresponding to sites 1551 through 1856 on the *O. mykiss* mitochondrial genome (NC001717; Zardoya et al., 1995). This region includes stems 27 through

40 of the secondary structure model presented by Wang and Lee (2002). Seven of the loops included in the sequenced region show extreme variability; thus, we considered the homology of the sites implicit in the alignment suspect and report results of analyses with and without these hypervariable regions.

The alignment of 16S sequences includes between 358 and 399 nucleotides corresponding to sites 3102 through 3479 of the *O. mykiss* mitochondrial genome (NC001717; Zardoya et al., 1995). This region is a portion of the 3' half of the 16S rRNA gene and includes the sites between loops E27 and G16 as presented in Waters et al. (2000). Three loops included in the sequenced region show extreme variability so we gave them the same treatment described

TABLE 1. P-VALUES OF PARSIMONY AND LIKELIHOOD TREE COMPARISON TESTS.

Hypothesis	RAG1						mt rRNA					
	Parsimony			Likelihood			Parsimony			Likelihood		
	W.S. <sup>a</sup>	K-H <sup>b</sup>	S-H <sup>c</sup>	K-H <sup>d</sup>	S-H <sup>e</sup>	K-H	W.S.	K-H	S-H	K-H	S-H	K-H
Esociformes – 1972 <sup>e</sup>	<0.001	<0.001	<0.001	<0.001	0.052	0.033	<0.001	0.007	0.171	<0.001	<0.001	<0.001
Osmeroids + Salmonoids <sup>f</sup>	<0.001	<0.001	<0.001	<0.001	0.007	0.018	0.007	0.007	0.006	<0.001	<0.001	<0.001
Lower euteleosts – 1996 <sup>g</sup>	<0.001	<0.001	<0.001	<0.001	0.053	0.028	0.001	0.007	0.006	<0.001	<0.001	<0.001
Osmeroids – 1996 <sup>h</sup>	<0.001	<0.001	<0.001	0.001	0.004	0.002	0.001	0.007	0.007	<0.001	<0.001	<0.001

<sup>a</sup> Winning-sites test (nonparametric) as implemented in PAUP<sup>®</sup>v.4.0b10.

<sup>b</sup> Parsimony analog of test devised by Kishino and Hasegawa (1989) as implemented in PAUP<sup>®</sup>v.4.0b10.

<sup>c</sup> Likelihood test for a posteriori comparisons devised by Shimodaira and Hasegawa (1999) as implemented in PAUP<sup>®</sup>v.4.0b10.

<sup>d</sup> Likelihood test for a priori comparisons devised by Kishino and Hasegawa (1989) as implemented in PAUP<sup>®</sup>v.4.0b10.

<sup>e</sup> López et al.'s (2000) hypothesis of esociform relationships compared with Nelson's (1972) hypothesis.

<sup>f</sup> Sister group relationship between esociforms and salmonoids compared with sister-group relationship between osmeroids and salmonoids.

<sup>g</sup> Optimal tree compared with Johnson and Patterson's (1996)fig. 23) hypothesis of lower euteleostean relationships.

<sup>h</sup> Saruwatari's (2000)fig. 3) hypothesis of osmeroid relationships compared with Johnson and Patterson's (1996)fig. 19) hypothesis.

above for the 12S hypervariable regions. For the 12S, 16S and combined mitochondrial ribosomal DNA (mt rRNA) sets of sequences, the Chi-square test of base composition fails to reject the null hypothesis of stationarity.

Because the rRNA sequences are relatively short and given that they share many biological characteristics (e.g., genome location, transcribed but not translated components of the ribosome, function), we combined these two genes in all phylogenetic analyses. There are 65 shortest trees for the mt rRNA sequences (1290 steps, CI 0.325, RI 0.516). The MP bootstrap majority rule consensus trees of the mt rRNA sequences with and without considering the hypervariable regions are largely unresolved. Clades such as esociforms, salmonoids, and osmeroids receive moderate to strong support as indicated by bootstrap values. The osmeroid clade supported by parsimony (98% bootstrap) includes the osmerids, *Plecoglossus*, *Salangichthys*, and the retropinnids. The ostariophysans are not resolved as a monophyletic group and all higher relationships, with the exception of the Euteleostei without Ostariophysi (50–67%) remain unresolved.

The tree based on ME analysis using corrected distances supports similar results. There is little resolution of higher-level relationships, ostariophysan monophyly is not supported and strong bootstrap support is largely restricted to trivial groupings with the exception of the osmeroid clade described above, which is strongly supported (97%). When hypervariable sites are considered, esociforms and salmonoids form a clade with weak support (63%), but when these sites are removed, the support for this group is weaker (56%). The Euteleostei without Ostariophysi is weakly supported (67%) when all sites, including those from hypervariable regions are considered. The base substitution model that best fits the data is the GTR+I+Γ with the following parameter values: base frequencies (A 0.3186, C 0.2407, G 0.2038, T 0.2369), substitution rates ( $r_{AC}$  3.1199,  $r_{AG}$  10.2036,  $r_{AT}$  2.6920,  $r_{CG}$  1.0442,  $r_{CT}$  18.6542),  $\alpha = 0.6116$ , and  $p_{inv} = 0.3377$ .

Under ML, esociforms and salmonoids are sister groups both when all mt rRNA sites are considered and when the sites in the hypervariable regions are removed. Another grouping found in both of these ML trees is the one containing all the eurypterygian taxa included in this study. Generally, the inferred length of terminal branches relative to internal ones is large, but, not surprisingly, short terminal branches are inferred among osmeroids, salmonoids and esociforms, the groups that were most densely



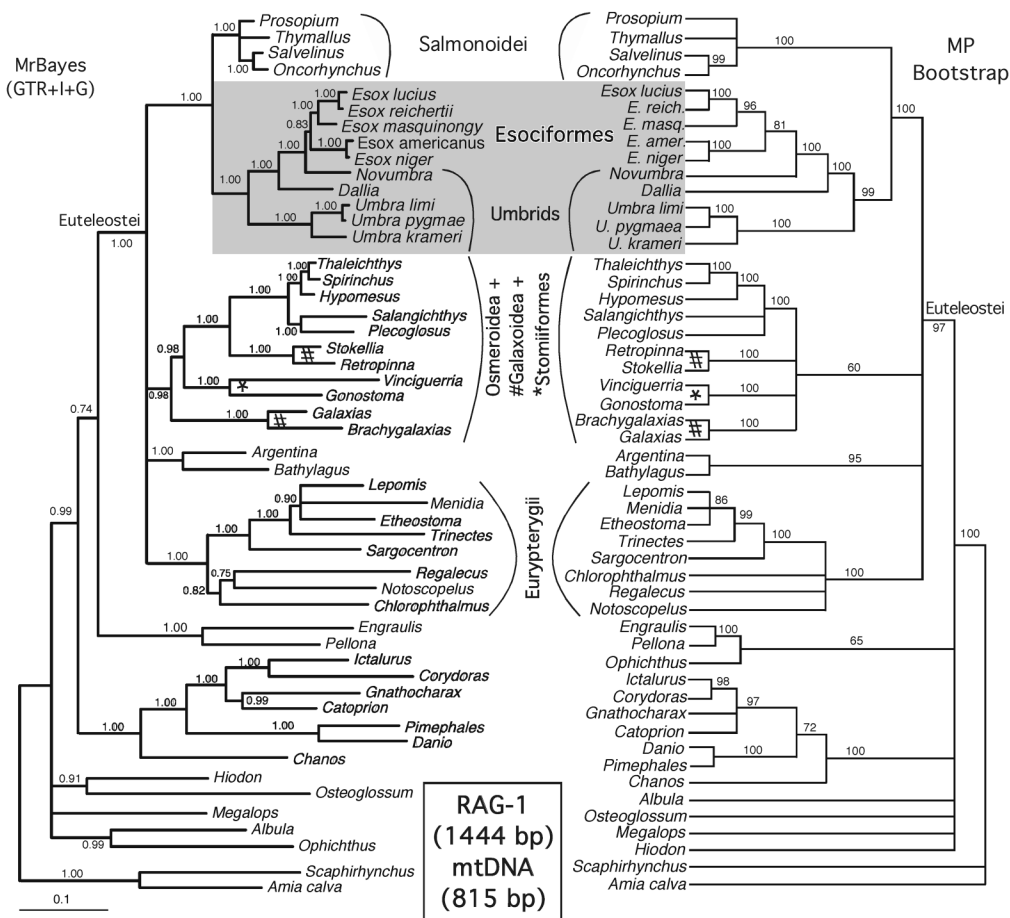


Fig. 3. Bayesian (left) and maximum parsimony (right) analyses of the combined molecular data set (RAG-1 + 12S and 16S). The majority-rule consensus phylogram obtained with Bayesian analyses shows posterior probability values at each node and was based on a GTR+I+G model fitted for each data partition (nuclear and mtDNA, see text for more information). The parsimony bootstrap consensus tree was obtained from 1000 pseudoreplicates and only shows bootstrap support values > 60 (nodes with values under 60 were collapsed).

sampled in this study. Generally, tree topology comparison tests suggest that alternative hypotheses of esociform and lower euteleost phylogeny are inconsistent with these sequences. However, the Shimodaira-Hasegawa test fails to reject a sister-group relationship between osmeroids and salmonoids based on the evidence provided by these sequences (Table 1).

**Combined DNA sequences.**—A total of 2259 bp combining the nuclear and mtDNA partitions was analyzed to obtain a general hypothesis of relationships. There is one MP tree of the combined RAG1 and mt rRNA dataset (6606 steps, CI 0.289, RI 0.524). The bootstrap majority rule consensus tree from the combined sequences show very strong support for the following groups (Fig. 3): Euteleostei minus Ostariophysii

(97%), Ostariophysii (100%), Eurypterygii (100%), and Esociformes + Salmonoids (100%). Osmeroidea + Retropinnidae + Stomiiformes only receives marginal bootstrap support (60%). Within Esociformes, the results of this analysis are in agreement with the hypothesis proposed by López et al. (2000). Within *Esox*, this analysis supports the monophyly of the subgenera *Esox* and *Kenosa* as defined by Nelson (1972). Results of Bayesian analyses are highly congruent with MP (compare both trees shown in Fig. 3) but provide somewhat more resolution, particularly within the Osmeroidea + Galaxoidea + Stomiiformes clade. This result is robust to the particular treatment of data partitions; thus the same clades presented in Figure 3 also are obtained using independent GTR+I+Γ models for mtDNA and RAG-1 or us-

ing protein sequences for RAG-1 analyzed with a Poisson model and DNA sequences for the mtDNA sequences with a GTR+I+ $\Gamma$  model F3.

The results of ME analyses of the combined dataset are in general agreement with those based on parsimony. The clades described above also are strongly supported by analyses based on corrected genetic distances. In addition, a clade composed of argentinoids, osmeroids, and stomiiforms is strongly supported (91%) and a clade composed of osmeroids and stomiiforms receives marginal support (63%). The base substitution model that best fits the data is the GTR+I+ $\Gamma$  with the following parameter values: base frequencies (A 0.2503, C 0.285, G 0.2683, T 0.1964), substitution rates ( $r_{AC}$  1.3218,  $r_{AG}$  3.5285,  $r_{AT}$  1.622,  $r_{CG}$  0.8839,  $r_{CT}$  5.528),  $\alpha$  0.9834, and  $p_{inv}$  0.381.

Finally, the ML tree based on the combined dataset contains the salmoniform + esociform, the osmerid + salangid + plecoglossid + retro-pinnid and the euteleost minus ostariophysan clades found in other analysis described above. It differs from parsimony and distance analyses of the same data in the placement of argentinoids and galaxiids as sequential sister groups of eurypterygians. The results of tree comparison tests based on the combined dataset mirror those based on RAG1 sequences alone (Table 1).

## DISCUSSION

*Esociforms among basal euteleosts.*—All analyses of the new molecular data show strong support for a sister group relationship between salmonoids and Esociformes. The esociform–salmonoid sister group relationship has been previously proposed by Fink (1984) and Williams (1987), who described characters of cheek musculature in support of this proposal but deemed the evidence too weak to be conclusive. Previous molecular studies offer corroborative evidence for the esociform + salmonoid clade. The nuclear rRNA sequences reported by Lê et al. (1989) and the growth hormone sequences analyzed by Bernardi et al. (1993) offer support for this group. Zaragüeta-Bagils et al. (2002) summarized evidence from six datasets of DNA sequences from teleosts to uncover phylogenetic relationships that could be accepted with confidence based on agreement between different sources of evidence. Although none of the datasets they examined includes a significant representation of protacanthopterygian taxa, in all cases where both esociforms and salmonoids were represented, these two lineages were placed as sister groups with relatively strong

measures of support. A more recent study examined the validity of the Protacanthopterygii concept using whole mitochondrial genome DNA sequences (Ishiguro et al., 2003) from a diverse sample of teleost taxa. The two esociforms (*Esox lucius* and *Dallia pectoralis*) included in that study were supported as the sister group of the salmonoids. We consider that this general consensus among the molecular evidence makes a strong case for the placement of salmonoids and esociforms as sister groups.

The base composition of the sequences in our study showed deviation from stationarity. This characteristic of the data has the potential to create artificial groupings of taxa based on convergence rather than descent. A closer examination of the patterns of base composition of the sequences in our dataset revealed that salmonoids and esociforms did not share common base composition characteristics. Therefore, the strong and consistent support for the salmonoid + esociform clade cannot be attributed to convergence. Even when RAG-1 protein sequences instead of DNA sequences are analyzed in the combined (Bayesian) analyses the salmonoid + esociform clade also is obtained. Further, although the ranges of base composition of the RAG1 sequences of salmonoids and osmeroids overlap the grouping of these two clades is strongly rejected.

*Esociform intrarelationships.*—Results from all analyses are in complete agreement and offer strong support for the hypothesis of esociform intergeneric relationships proposed by López et al. (2000). The bootstrap analyses in general show well-supported clades among the esociforms. The only node that does not receive high support (< 90%) is the one that diagnoses the monophyly of *Esox* to the exclusion of *Novumbra* (Figs. 2, 3).

Genetic distances between the most divergent species of *Esox* and between those species and *Novumbra hubbsi* are of similar magnitudes. Assuming that *Novumbra* is the sister group of a monophyletic *Esox*, this observation may be explained by (1) substitution saturation in the sequences we examined, (2) a markedly different rate of molecular evolution in species of *Esox* compared to that of *Novumbra*, (3) relative proximity in the time of origin for the lineages of *Novumbra* and the subgenera *Esox* and *Kenoza*, or (4) least likely, the genus *Esox* is not monophyletic. Substitution saturation is an unlikely explanation because the levels of divergence among these taxa are much lower than those observed in other comparisons and they are found well within range of linear change in sub-

stitution plots. Relative rate tests with *Dallia* as the reference outgroup point to a faster rate of substitution in the *Novumbra* lineage relative to lineages of *Esox*. Therefore, although we cannot rule out a similar time of origin for all these lineages, the differences in the rate of evolution evident in the sequences may explain the inconsistent support for *Esox* monophyly. Within the Esocidae, our data strongly support the division of the species of *Esox* into the subgenera *Kenoza* for the pickerels and *Esox* for the pikes and the muskellunge, and within the subgenus *Esox*, *Esox lucius*, and *Esox reichertii* are supported as sister species as proposed by Nelson (1972).

Given the consistent and strong support for esociform inter- and intraordinal relationships and because our study includes a thorough sample of extant esociform diversity, a broad sample of potential sister lineages, and sequence data from different genes and genomes (i.e., nuclear and mitochondrial), a revised classification of these taxa seems warranted. The classification proposed by López et al. (2000) may be accepted, however, the ranks given to the different groups in that classification may need revision once consensus on the placement of the salmonoid + esociform clade develops.

*Review of evidence supporting previously published hypotheses of esociform phylogeny.*—Because the set of esociform relationships supported by the molecular data is incongruent with currently accepted hypotheses of esociform phylogeny and because we find strong and consistent support in our data for the hypothesis of esociform relationships proposed here, we examined the most important bodies of evidence that support current hypotheses on esociform classification with the goal of determining possible reasons for the incongruence. This review focused on three relevant studies: (1) the study of lower euteleost relationships of Johnson and Patterson (1996) because it is the basis for a strongly supported sister-group relationships between salmonoids and osmeroids, which is incongruent with our findings; (2) Nelson's (1972) study of the cephalic sensory system because it is the foundation for the first proposal esociform intrarelationships that later found corroboration in the work of Wilson and Veilleux (1982) and was more recently accepted, although not explicitly examined, by Johnson and Patterson (1996). Wilson and Veilleux's (1982) osteology-based study of umbrid relationships constitutes the third and final focus of this review.

The conclusions of Johnson and Patterson (1996; summarized here in Fig. 1A) conflict with our results in two important aspects: the

placement of esociforms as the sister group of the neoteleosts and of salmonoids as the sister group of the osmeroids. As was noted by those authors (1996:315) the evidence supporting the sister-group relationship between esociforms and neoteleosts is not particularly strong. The following issues surrounding the evidence in support of the esociform + neoteleost clade are worth highlighting: (1) two of the four characters that support this grouping (presence or absence of uroneural 3 and cellular versus acellular skeleton) show some degree of homoplasy among basal euteleosts (Johnson and Patterson, 1996:287, 298); (2) as Fink (1981) pointed out, unique characteristics of the tooth attachment type in esociforms may make it distinct from the so-called type 4, which is one of the characters that implies affinity between neoteleosts and esociforms in Johnson and Patterson's data matrix; and (3) it should be noted that given the coding assigned to the presence of scales on the cheek and operculum (Johnson and Patterson, 1996:331), the trait is synapomorphic for the esociform + neoteleost clade through the parsimonious reconstruction of character states and because of this its status as synapomorphic for esociforms + neoteleosts is dependent on the treatment given to forward and reverse changes (see Johnson and Patterson, 1996:fig. 23).

The issues we have highlighted regarding these four characters do not change the fact that Johnson and Patterson's phylogenetic conclusions are those best supported by their character state matrix. However, in our view, they gain significance and are important to note in the context of the evidence from DNA sequences from multiple genes presented here and elsewhere (Zaragueta-Bagils et al., 2002; Ishiguro et al., 2003) that rejects the esociform + neoteleost sister-group relationship.

The sister-group relationship of osmeroids and salmonoids is one of the more strongly supported results of Johnson and Patterson's (1996) study. It is supported by 11 putative synapomorphies. In four of these characters (nos. 4, 7, 18 and 37 of appendix 4; Johnson and Patterson, 1996:332), the apomorphic character state is present in at least one esociform; therefore under an alternative coding of character states for the esociforms, these four characters would not exclude esociforms from a group containing salmonoids and osmeroids. This is not to say that the coding of these characters used by Johnson and Patterson (1996) is demonstrably incorrect but rather that there is an equally justifiable alternative coding that would exclude these four characters from the set of

evidence supporting a salmonoid + osmeroid group that does not include esociforms. Two other characters for which coding alternatives could affect the strength of the evidence excluding esociform from the salmonoid + osmeroid group are the ossification of epipleurals (no. 21) caused by the variation in this trait exhibited by osmeroids (as discussed by Johnson and Patterson, 1996:279) and the position of uroneural 2, which is absent in all esociforms, so its placement relative to uroneural 1 cannot be determined and given a demonstrably correct coding (no. 29). Another character that supports the salmonoid + osmeroid clade is the differential retention of upper pharyngeal toothplates among basal euteleost lineages (no. 16). Johnson and Patterson (1996) determined that the single toothplate retained in esociforms is upper pharyngeal 4 based on the relative position of this structure during its development, whereas in osmeroids and salmonoids, the retained toothplate is upper pharyngeal 5. Because in the three groups that concern us here, one of the two serial elements is missing, the homology determination must be based on the subtle differences in position during development of the retained pharyngeal plate relative to the underlying pharyngobranchial and epi-branchial elements of the fourth arch (see Johnson and Patterson, 1996:fig. 10). Although this in itself does not invalidate Johnson and Patterson's (1996) assessment of homology, it does bring to it a measure of uncertainty that we only highlight here given the context of the apparent strength of the molecular-based evidence in rejecting the salmonoid + osmeroid sister-group relationship.

The presence or absence of nuptial tubercles is another character that is offered in support of the salmonoid + osmeroid clade; however, the reconstruction of this character as a synapomorphy of that clade depends on the coding selected by Johnson and Patterson in their appendix 4 (1996:331–332). In the data matrix presented in that appendix, osmeroids and salmonoids are coded as having nuptial tubercles, but in the description of this character presented earlier in the text (Johnson and Patterson, 1996:297), it is stated that the primitive state for the salmonoids is ambiguous. Hence, this is another case where a reasonable argument could be made for an alternative coding under which the character would not constitute evidence supporting the salmonoid + osmeroid clade.

Finally, salmonoids and osmeroids are coded as having separate dermethmoid and supraethmoid based on the condition observed in cor-

egonids and osmerids. Under this coding, this condition is a synapomorphy of salmonoids + osmeroids; however, no explicit argument is given for considering that condition primitive for each of the two groups and for justifying the coding scheme applied to this character (Johnson and Patterson, 1996:254). Again, we point this out not because we know of a demonstrably better alternative coding of this character, but because there are equally defensible alternative codings that would result in this character not forming part of the evidence of the salmonoid + osmeroid clade, and we have presented molecular evidence that strongly rejects that clade.

The remaining two character states that support the salmonoid + osmeroid clade are the keel-like rostricaudal expansions of the last few neural and haemal spines and an anadromous life history. Our examination of the treatment of these two characters did not reveal any potential explanations for the incongruence between the molecular and morphological evidence.

Nelson (1972) proposed an arrangement of esociform genera (Fig. 1B) based on an extensive survey of the cephalic sensory system among esociforms and primitive teleosts. Although that study did not apply an explicitly cladistic methodology, Nelson's discussion of the evidence in support of his classification states that he emphasized derived character states in formulating his classification; therefore, his results can be interpreted as a hypothesis of phylogeny of esociforms. Nelson (1972) identified a reductive trend in the cephalic sensory system among esociforms involving the number and size of canal elements and the number of pores associated with those elements and devised a classification of Recent esociforms based on what he considered derived character states associated with this trend. Nelson's classification, and by extension the putative synapomorphies it implies, are incongruent with the molecular data presented here and in López et al. (2000). Our review of Nelson's evidence corroborated the accuracy of his observations; therefore, under the assumption that the molecular evidence reflects the history of the group, the incongruent results suggest that Nelson's determination of the derived states is incorrect or that homoplasy has rendered these character states misinformative. In this regard, we note that, although several characters support Nelson's classification, all of these refer to different aspects of the putative reductive trend in pore numbers and canal element continuity and, therefore, may not be independent. If Nelson's characters are in fact correlated, then reconstructing these traits on

the molecular-based hypothesis may represent a single homoplasy rather than many and hence have a small parsimony cost. As an aside, although some may also level the criticism of non-independence to the use of DNA sequences from one or a few genes, it is important to note that most known genes include a large proportion of sites whose variation has neutral or nearly neutral effect on the phenotype.

Nelson's (1972) proposed classification of esociform genera was later corroborated by evidence presented by Wilson and Veilleux (1982). The monophyly of the family Umbridae and its sister-group relationship to the Esocidae were explicitly stated assumptions of that study. These assumptions are significant in the context of the present findings for two reasons: (1) they led to the assertion that the character states observed in *Esox* were the primitive condition and any variants observed among umbrid genera represented derived states of those traits; and (2) synapomorphies in support for the monophyly of the umbrids were not presented. Because of this last omission, our review of the evidence presented by Wilson and Veilleux (1982) focuses on the list of 13 "shared derived characters" that support the monophyly of Umbrinae (*Dallia* + *Umbra*; Wilson and Veilleux (1982:appendix, Set I, p. 350). Two of these characters (no. 3: the presence of a spine on the sphenotic, and no. 12: uroneural not extending to preural 1) are miscoded according to the information provided earlier in the text (Wilson and Veilleux, 1982:331: "The apex of each [sphenotic] in *Dallia* and *Umbra* bears a small laterally-directed process from which the levator arcus palatini originates, as in *Esox*." And, p. 344: "[In *Novumbra*, t]he uroneurals, as in *Dallia* but not *Esox* and *Umbra*, do not reach anteriorly beyond the first ural centrum."). The coding of the presence or absence of a knob on the proethmoid (no. 1 from Set I, p. 350) is problematic for two reasons: the plesiomorphic condition cannot be determined because the shape of the proethmoids of all esociforms is different from that observed in other basal euteleosts with proethmoids and the description of the character (p. 326) does not specify to what extent the condition described for *Umbra* and *Dallia* (the putative apomorphy) differs from that reported for *Esox*, which also shows an anterior thickening of the proethmoid.

Two other putative synapomorphies (no. 4: supramaxilla reduced and, no. 6: ectopterygoid reduced) are coded as reduced in *Dallia* and *Umbra* but the structures to which those characters refer are absent in *Umbra* (no. 4; Wilson and Veilleux, 1982:fig. 6) and *Dallia* (no. 6; Wil-

son and Veilleux, 1982:fig. 7), which raises the question of whether a missing structure may be equated to a reduced structure in the context of character state coding; furthermore we did not observe significantly reduced ectopterygoids (no. 6) in *Umbra limi* and *Umbra pygmaea* (UMMZ 137450, UMMZ 164967). Four of the characters offered in support of the Umbrinae (nos. 8–11) refer to four different aspects of the anatomy of the caudal skeleton. Three of them (nos. 9–11) show within genus variation and are inconsistently coded when compared to illustrations presented by Rosen (1974:figs. 20, 21, 22, and 23) and Wilson and Veilleux (1982:figs. 13 and 14). For example, character no. 9 (no gap between second and third hypurals) is not consistent with Rosen's (1974) illustrations of the caudal skeletons of *Umbra pygmaea* (fig. 22D) and *Dallia pectoralis* (fig. 23D, E). Of the four characters of the caudal skeleton that are offered in support of the Umbrinae, the reduced difference in size between hypural 1 and hypurals 2 and 3 in *Dallia* and *Umbra* compared to *Esox* and *Novumbra* (no. 8) is the only one that is consistently illustrated and described.

Finally, for character no. 13 (fewer than four pectoral radials), *Umbra* is stated to have fewer than four radials, but we have observed four in all the specimens we checked (UMMZ 185076, UMMZ 137450, UMMZ 164967). Some specimens have the two ventralmost radials fused at their ends, but in all cases, the presence of four distinct elements is clear. Even if we ignore this observation, we consider it a strained argument to propose the highly modified unossified radial plate of *Dallia* and its rather ordinary homologue in *Umbra* as evidence of a shared character state transformation. Our review of Wilson and Veilleux's (1982) characters revealed that only four of the 13 putative synapomorphies of the Umbrinae are free of problems in the justification of the coding scheme. Two of these (nos. 2 and 5) were first presented by Nelson (1972) and were discussed above. The other two characters are (no. 7) the loss of the basihyal toothplate, a reductive trait, and (no. 8) the smaller difference in size between hypural 1 and hypurals 2 and 3 of *Dallia* and *Umbra* than the condition observed in *Esox* and *Novumbra*. We did not detect any possible reasons for the conflict between these characters and the molecular evidence.

As stated earlier, the goal of this review was to determine whether there were aspects of the evidence supporting those hypotheses of esociform relationships that are incongruent with our present results that suggest possible explanations for the incongruence. Accordingly, we



have highlighted a number of potentially problematic issues regarding that evidence. Considering that the issues highlighted affect a significant proportion of the morphological evidence and given the present context of consistent and strong support for the hypothesis of esociform relationships proposed here based on molecular data, it seems there is a strong case for the reevaluation of current ideas of the relationships of this group.

*Other phylogenetic considerations.*—Although our taxonomic sampling in this study was designed to address esociform relationships among proacanthopterygians, some of the results concerning other aspects of the phylogeny of basal euteleosts are worth highlighting. McDowall, tentatively, (1969) and later Rosen (1974) suggested a close relationship between Southern smelts (retropinnids) and Northern (osmerids) smelts. All of our analyses agree in placing the retropinnids as the sister group of the osmerid + salangid + plecoglossid clade to the exclusion of the two galaxiid species in our sample (Figs. 2, 3). Clearly, our galaxiid sample is taxonomically deficient and any hypothesis derived from these results must be further tested. However, the same hypothesis was obtained by a detailed analysis of mtDNA data based on a taxonomic sample better suited to address retropinnid affinities (Waters et al., 2002). One potential problem with the support given to the placement retropinnids by our sequence data is that the two galaxiids and the two retropinnids in our study show sharply contrasting patterns of base composition. The differences are more marked in the RAG1 sequences but they are also evident to a lesser extent in the mt rRNA data. In all cases, the sequence base composition of the two retropinnids is more similar to that of the five osmeroids than to that of the two galaxiids; therefore, it is possible that the retropinnid + osmeroid clade is an artifact of base composition similarity. Alternatively, similar base composition may be a shared derived trait and as such indicative of phylogenetic affinity. This seems to be the case, as suggested by analyses of the amino acid sequences of RAG-1 that also support the grouping of retropinnids and osmeroids.

Within the osmeroid clade, our results do not support the placement of the salangids among osmerids as proposed by Johnson and Patterson (1996). Again, our taxonomic sample is insufficient to produce a strong inference on this matter, but we consistently find very strong support for the monophyly of osmerids. Saruwatari et al. (2000) also reported strong support for

monophyletic Osmeridae and Salangidae from analyses based on 16S sequences from a taxonomic sample that included five osmerid and three salangid genera, as well as *Plecoglossus altivelis*. The comparison tests we conducted showed that Saruwatari et al.'s (2000) hypothesis of osmeroid relationships was consistent with our data, whereas that of Johnson and Patterson (1996) was not (Table 1). However, the four uncontradicted morphological synapomorphies placing salangids and the osmerid genus *Malotus* as sister groups in Johnson and Patterson's (1996:fig. 19; Appendix 1) analyses must be reconciled with this result. As our current understanding of the evolution of molecular and morphological traits is not sufficiently developed to confidently assert the relative value of putative molecular and morphological synapomorphies, at present the weight of the evidence seems to support osmerid monophyly.

Although our results concerning osmeroid, retropinnid and galaxiid relationships are in agreement with other molecular studies relevant to these questions, a strong conclusion will only be possible from a thorough critical examination of the evidence supporting existing hypotheses and the production of datasets from a sample of taxa designed to permit strong conclusions (i.e., complete or near complete representation of ingroup diversity and broad and well selected outgroup representatives). A most intriguing proposal arising from our analysis is the close relationship of osmeroids and the stomiiform taxa *Gonostoma* and *Vinciguerria*. Although taxonomic sampling also is limited for stomiiformes, strong indication of support for this grouping in all analyses suggests that it should be considered seriously.

Finally, although the DNA sequences we obtained for this study were informative about esociform relationships and promise to be informative about other relationships involving similar levels of divergence, there are deeper aspects of the basal euteleostean phylogeny for which we could not discern any relevant relationships. A denser taxonomic sample may show whether this lack of resolution is the result of poor sampling or the absence of phylogenetic information in the sequences. We suspect that to gain a better understanding of some of the more problematic aspects of euteleost evolution, it will be necessary to produce both, the appropriate taxonomic samples and, in some cases, to employ new sources of evidence such as the distribution of introns (Venkatesh et al., 1999), conserved insertion/deletions (Venkatesh et al., 2001) and mobile genetic elements (e.g., SINE's; Shedlock and Okada, 2000). For

example, the two novel RAG1 introns we found in the two argentinoids in this study may prove to have a phylogenetically informative distribution among members of this group. However, a critical flaw we have observed in many of the phylogenetic studies employing novel approaches is that in the rush to produce results, the quality of the taxonomic sampling has suffered, which makes the generality of the conclusions they support difficult to gauge (e.g., Venkatesh et al., 2001).

#### MATERIAL EXAMINED

Tissue samples for the molecular study are deposited in the personal collections of the authors and will be made available upon request. The following cleared-and-stained specimens were used to check some of the morphological characters used to support existing hypotheses of esociform relationships (see Discussion; Institution abbreviations follow Leviton et al., 1985). *Dallia pectoralis*, Bethel, AK, UMMZ 164848; Anchorage, AK, unaccessioned; *Esox americanus*, Livingston Co., MI, UMMZ 202358; Wilson Co., NC, unaccessioned; *Esox lucius*, Chippewa R., WI, FMNH 18090; Spirit Lake Hatchery, IA, unaccessioned; *Esox masquinongy*, Spirit Lake Hatchery, IA, unaccessioned; *Novumbra hubbsi*, Grays Harbor, WA, UMMZ 179398, UMMZ 187427; *Umbra krameri*, Lake Pantelimon, Romania, UMMZ 185076; *Umbra limi*, Mackinac, MI, UMMZ 137450; Jackson Co., IA, unaccessioned; *Umbra pygmaea*, Nansemond and Norfolk, VI, UMMZ 164967; Wilson Co., NC, unaccessioned.

Following are the GenBank accession numbers of the DNA sequences examined in this study (species names are followed by the GenBank accession number of the 12S, 16S and RAG1 sequences in that order). *Scaphirhynchus albus*, AY430247, AY430229, AY430198; *Amia calva*, AB042952, AB042952, AY430199; Osteoglossomorpha: *Hiodon alosoides*, AY430248, AY430230, AY430200; *Osteoglossum bicirrhosum*, AB043025, AB043025, AY430201; Elopomorpha: *Albula vulpes*, X99180, X99179, AY430202; *Megalops atlanticus*, X99178, X99177, AY430204; *Ophichthus gomesii*, AY430249, AY430231, AY430203; Clupeomorpha: *Engraulis japonicus*, AB040676, AB040676, AY430205; *Pellona flavipinnis*, AY430250, AY430232, AY430206; Ostariophysi: *Chanos chanos*, AY430251, AY430233, AY430207; *Danio rerio*, AC024175, AC024175, U71093; *Pimephales promelas*, AY430253, AY430235, AY430210; *Ictalurus nebulosus*, AY430252, AY430234, AY430209; *Corydoras sp.*, U15271, U15247, AY430208; *Gnathocharax steindachneri*, U33589, U33624, AY430211; *Catoptrion*

*mento*, AF283911, AF283932, AY430212; Osmeroidae: *Brachygalaxias bullocki*, AY430266, AF112328, AY430219; *Galaxias fasciatus*, AY430265, AF112333, AY430218; *Retropinna tasmanica*, AY430263, AF112342, AY430216; *Stokellia anisodon*, AY430264, AF454843, AY430217; *Salangichthys microdon*, AY430267, AY443566, AY380539; *Plecoglossus altivelis*, AY430261, AY443567, AY380536; *Thaleichthys pacificus*, AY430262, AY443568, AY380537; *Spirinchus thaleichthys*, AY430259, AY430239, AY430215; *Hypomesus olidus*, AY430260, AY443569, AY380538; Argentinoidae: *Argentina sialis*, AY430258, AY430238, AY430228; *Bathylagus ochotensis*, AY430257, AY443570, AY443564–5; Salmonoidae: *Prosopium williamsoni*, AY430254, AY430236, AY430213; *Thymallus thymallus*, AY430255, AY430237, AY430214; *Salvelinus malma*, AY430256, AF060445, AY380535; *Oncorhynchus mykiss*, L29771, L29771, U15663; Esociformes: *Esox masquinongy*, AY430274, AY443571, AY380543; *Esox reichertii*, AY430277, AY443572, AY380545; *Esox lucius*, AY430273, AF060446, AY380542; *Esox niger*, AY430276, AY443573, AY380544; *Esox americanus*, AY430275, AY443574, AY380541; *Novumbra hubbsi*, AY430272, AF060447, AY380546; *Dallia pectoralis*, AY430271, AF060448, AY380540; *Umbra krameri*, AY430269, AF060444, AY380547; *Umbra limi*, AY430268, AF060443, AY380548; *Umbra pygmaea*, AY430270, AF060442, AY380549; Neoteleostei: *Vinciguerra* sp., AY438704, AY443575, AY442363; *Gonostoma bathyphilum*, AY438705, AY443576, AY438703; *Notoscopelus kroeyeri*, AY430279, AJ277964, AY430221; *Chlorophthalmus* sp., AY430278, AY430241, AY430220; *Regalecus glesne*, AF049728, AF049738, AY430222; *Sargocentron punctatissimum*, AY430280, AY430242, AY430223; *Menidia menidia*, AY430281, AY430243, AY430225; *Lepomis macrochirus*, AY430284, AY430246, AY430227; *Etheostoma caeruleum*, AY430283, AY430245, AY430226; *Trinectes maculatus*, AY430282, AY430244, AY430224.

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