

Phylogenetic relationships among North American *Alosa* species (Clupeidae)

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A phylogeny of the six North American species in the genus *Alosa*, with representatives of three Eurasian species, was generated using mtDNA sequences. This was accomplished by obtaining sequences for three North American species and additional geographical sampling of the other three species. The subgenus *Alosa*, including the formerly recognized subgenus *Caspialosa*, formed a strongly supported monophyletic group. *Alosa alabamae* was part of a polytomy with *Alosa sapidissima*, which was interpreted to support the recognition of *A. alabamae* as an incipient, yet distinct, species. The subgenus *Pomolobus* was not recovered as a monophyletic group. *Alosa chrysochloris* was basal to all other *Alosa*, although this position was only weakly supported. Previous work had indicated that *Alosa pseudoharengus* and *Alosa aestivalis* are not reciprocally monophyletic, but additional sampling in this study did not detect any further cases of shared haplotypes between the two species. The phylogeny supports previous hypotheses that the evolution of North American *Alosa* species in the Gulf of Mexico (*A. chrysochloris* and *A. alabamae*) was the result of two independent events. First, the ancestor of *A. chrysochloris* was isolated in the Gulf of Mexico, likely by the close of the Suwannee Straits, and this was followed later by dispersal of the ancestor of *A. alabamae* around the Florida peninsula into the Gulf of Mexico sometime during or after the Pleistocene.

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INTRODUCTION

Many members of the family Clupeidae support valuable fisheries across much of the world. Clupeids are predominately marine, although *c.* 29 species within the family are diadromous. Seasonal migrations of shad (subfamily Alosinae) have long been exploited by humans (McDowall, 2003). Populations of many migratory clupeids are now declining due to the same sorts of environmental perturbations and anthropogenic influences that have threatened other diadromous species such as salmonids (Nehlsen *et al.*, 1991) and sturgeons (Billard

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& Lecointre, 2000). Despite their economic importance and management concern, shads have generally been poorly studied (Waldman, 2003). One critical missing piece of information is knowledge of the basic taxonomic units within the genus *Alosa* and their phylogenetic relationships.

Seven subfamilies are recognized within the Clupeidae (Nelson, 2006). Of interest in this study is the genus *Alosa* Linck in the subfamily Alosinae, a group that contains marine, freshwater and diadromous species. Of the seven alosine genera, *Alosa* accounts for about half of the 31 species in the subfamily. At one time, species within *Alosa* were split into three genera: *Alosa*, *Caspialosa* Berg and *Pomolobus* Rafinesque. Regan (1917; in Bentzen *et al.*, 1993) considered *Pomolobus* to include four of the six species from North America [*Alosa aestivalis* (Mitchill), *Alosa chrysochloris* (Rafinesque), *Alosa mediocris* (Mitchill) and *Alosa pseudoharengus* (Wilson)], while *Caspialosa* was comprised of species from Eastern Europe (the Ponto-Caspian drainage systems). *Alosa* was reserved for the European species *Alosa alosa* (Linnaeus) and *Alosa fallax* (Lacepède) and the North American species *Alosa alabamae* Jordan and Evermann, and *Alosa sapidissima* (Wilson). Bailey *et al.* (1954) later united *Pomolobus* with *Alosa*. Similarly, the work of Svetovidov (1964) on osteological characters led him to group both *Pomolobus* and *Caspialosa* into *Alosa*, although he did recognize that the differences between *Alosa* and *Pomolobus* could support the recognition of *Pomolobus* as a distinct subgenus.

The taxonomic status of the genus *Alosa* now appears stable (although see Baglinière *et al.*, 2003 for some exceptions), but the number of species in the genus remains in a state of flux. Considerable polymorphism in the genus has resulted in the recognition of numerous sub-species (Baglinière *et al.*, 2003). For example, six sub-species of *A. fallax* (reviewed by Aprahamian *et al.*, 2003) and nine sub-species of *Alosa brashnikovi* (Borodin) (Waldman, 2003) have been described. Conversely, other workers have questioned the taxonomic distinctiveness of some species. Chapman *et al.* (1994) suggested that further investigation was warranted to determine if *A. alabamae* is distinct from its sister species *A. sapidissima*. Boisneau *et al.* (1992) doubted that *A. alosa* and *A. fallax* were distinct species given the frequency of hybridization between the two. However, recent work (Alexandrino *et al.*, 1996; Alexandrino *et al.*, 2006) has documented that the two species do represent distinct lineages based on differences in morphology (gill rakers) and molecular markers (allozymes and mtDNA).

Recent systematic work on the genus *Alosa* has relied on genetic variation in mtDNA. Bentzen *et al.* (1993) used whole mitochondrial genome restriction digests to examine the phylogenetic relationships among five species: two from Europe (*A. alosa* and *A. fallax*) and three from North America (*A. sapidissima*, *A. pseudoharengus* and *A. mediocris*). This work recognized distinct clades representing the subgenera *Alosa* and *Pomolobus* and indicated that there was evidence of hybridization between *A. alosa* and *A. fallax*. Chapman *et al.* (1994) compared restriction site variation in the entire mitochondrial genome and two specific mitochondrial genes [cytochrome *b* (*cyt b*) and NADH dehydrogenase subunit 1 (ND1)] in the North American sister species *A. sapidissima*/*A. alabamae* and *A. aestivalis*/*A. pseudoharengus*. *Alosa alabamae* was weakly differentiated from *A. sapidissima*, which led Chapman *et al.* (1994) to express doubts that *A. alabamae* warranted recognition as a distinct species. *Alosa*

aestivalis and *A. pseudoharengus* appeared to be polyphyletic based on their ND1 data, a result that Chapman *et al.* (1994) attributed to incomplete lineage sorting in a recently diverged pair of species. A follow-up study by Nolan *et al.* (2003) also found limited genetic differentiation between *A. alabamae* and *A. sapidissima*, although they detected two fixed restriction site differences. Nolan *et al.* (2003) appeared to interpret this as evidence favouring continued recognition of *A. alabamae* while recognizing that the two species have only recently diverged. Faria *et al.* (2006) have produced the most inclusive phylogeny to date, and the only one so far using mitochondrial sequence data. Their work focused mainly on the Eurasian *Alosa* species, but they did include three of the six North American *Alosa* in their phylogeny. *Alosa* and *Pomolobus* were again supported as being distinct clades, but *Caspialosa* species did not represent a unique group.

The Florida peninsula appears to have played a major role in the evolution of North American *Alosa* species, as for other marine organisms (examples reviewed in Avise, 2000). Both *A. alabamae* and *A. chrysochloris* are restricted to Gulf of Mexico drainages, and their presumed closest relatives, *A. sapidissima* and *A. mediocris*, respectively, occupy Atlantic drainages (Berry, 1964). The Suwannee Straits connected the Gulf of Mexico to the Atlantic Ocean and separated Florida from mainland North America during the Miocene (Riggs, 1984). This connection was eventually closed as sea levels dropped, but it may have reopened during the high sea stands of the Pliocene (reviewed by Bert, 1986). Swift *et al.* (1986) suggested that the close of the Suwannee Straits isolated the ancestor of *A. chrysochloris* in the Gulf of Mexico. However, it appeared unlikely that the ancestor of *A. alabamae* and *A. sapidissima* was present on both sides of the Florida peninsula prior to the Pleistocene, meaning that the ancestor would have had to later disperse around the peninsula (Swift *et al.*, 1986).

The goal of this study was to collect comparable sequence data to that reported by Faria *et al.* (2006) and build upon their impressive phylogeny by adding complete taxonomic sampling of North American *Alosa* species. As far as is known, this is the first attempt to apply DNA sequence data to questions about North American *Alosa* species raised by previous studies that used mtDNA restriction digests (Bentzen *et al.*, 1993; Chapman *et al.*, 1994; Nolan *et al.*, 2003). Specifically, the goals were to: (i) produce a complete North American *Alosa* phylogeny, (ii) determine the taxonomic distinctiveness of *A. sapidissima* and *A. alabamae*, (iii) characterize the extent of genetic differentiation between *A. pseudoharengus* and *A. aestivalis* through increased sampling, (iv) determine if *A. chrysochloris* and *A. mediocris* are sister species and (v) better understand the biogeography of North American *Alosa* species.

MATERIALS AND METHODS

Samples of North American *Alosa* species were obtained from numerous sources (Table I; Fig. 1). Fin clips were placed into a preservation buffer (Seutin *et al.*, 1991) and then stored at -20°C at the University of Southern Mississippi until DNA was extracted. Sequence data from Faria *et al.* (2006) were taken from GenBank for all available North American *Alosa* species and for selected Eurasian species (Table I).

TABLE I. List of taxa sampled, sample size and collection locations given in decimal degrees. GenBank accession numbers are provided for the *cyt b* and ND1 sequences. Accession numbers for the *cyt b* and ND 1 sequences are listed in order of their pairing in each unique composite haplotype. Sources of samples at a given location are identified by the following symbols: *Faria *et al.*, 2006, †Florida Fish and Wildlife Conservation Commission, ‡Personal collections, §Michael Bruening, ||North Carolina Co-operative Fish and Wildlife Research Unit, ¶Duke Power and #Georgia Department of Natural Resources

Taxa	Location	N	Latitude	Longitude	Cyt <i>b</i>	ND1
<i>A. aestivalis</i>	Miramichi River (Canada)*	4	47·100° N	65·217° W	DQ419777, DQ419779	DQ419804, DQ419805
	St Johns River (FL, U.S.A.)†	2	28·786° N	81·171° W	EF653228, EF653229	EF653236, EF653237
<i>A. alabamae</i>	Pascagoula River (MS, U.S.A.)‡	2	31·460° N	89·171° W	EF653230, EF653230	EF653238, EF653239
	Apalachicola River (FL, U.S.A.)‡	2	30·156° N	85·133° W	EF653230	EF653240
	Ouachita River (AR, U.S.A.)‡	2	33·800° N	93·177° W	EF653230	EF653240
	Gasconade River (MO, U.S.A.)‡	1	38·605° N	91·635° W	EF653230	EF653240
<i>A. alosa</i>	Lima River (Portugal)*	2	41·683° N	8·830° W	DQ419761, DQ419763	DQ419785, DQ419784
	Garonne River (France)*	1	44·133° N	0·733° E	DQ419760	DQ419782
	Dordogne River (France)*	1	44·833° N	0·450° E	DQ419762	DQ419785
<i>A. chrysochloris</i>	Pascagoula River (MS, U.S.A.)‡	3	31·349° N	89·306° W	EF653231, EF653231, EF653232	EF653241, EF653242, EF653243
	Ohio River (OH, U.S.A.)§	1	38·798° N	84·177° W	EF653232	EF653243
<i>A. fallax fallax</i>	Tywi River (England)*	2	51·750° N	4·383° W	DQ419764, DQ419765	DQ419786, DQ419786

TABLE I. Continued

Taxa	Location	N	Latitude	Longitude	Cyt <i>b</i>	ND1
<i>A. immaculata</i>	Danube Delta (Romania)*	1	44·867° N	29·617° E	DQ419769	DQ419796
	Danube Delta (Romania)*	2	44·183° N	28·800° E	DQ419770, DQ419771	DQ419794, DQ419795
<i>A. mediocris</i>	Roanoke River (NC, U.S.A.)	3	36·309° N	77·563° W	EF653233, EF653233	EF653244, EF653245
	Altamaha River (GA, U.S.A.)#	2	31·316° N	81·285° W	EF653233	EF653244
<i>A. pseudoharengus</i>	Miramichi River (Canada)*	2	47·100° N	65·217° W	DQ419777, DQ419778	DQ419804, DQ419806
	Lake Ontario (NY, U.S.A.)*	2	43·250° N	77·533° W	DQ419776	DQ419803
	Catawba River (NC, U.S.A.)¶	3	35·436° N	80·958° W	DQ419778	EF653246
<i>A. sapidissima</i>	Connecticut River (CT, U.S.A.)*	4	41·267° N	72·563° W	DQ419773, DQ419773, DQ419774, DQ419775	DQ419799, DQ419800, DQ419801, DQ419802
	Roanoke River (NC, U.S.A.)	2	36·309 °N	77·563° W	EF653234, EF653234	DQ419799, EF653247
	Altamaha River (GA, U.S.A.)#	5	31·316° N	81·285° W	DQ419775, DQ419775, DQ419775, EF653235, EF653234	EF653248, EF653249, EF653250, EF653251, EF653247
	St Johns River (FL, U.S.A.)†	2	28·786° N	81·171° W	DQ419774, DQ419774	EF653252, DQ419801

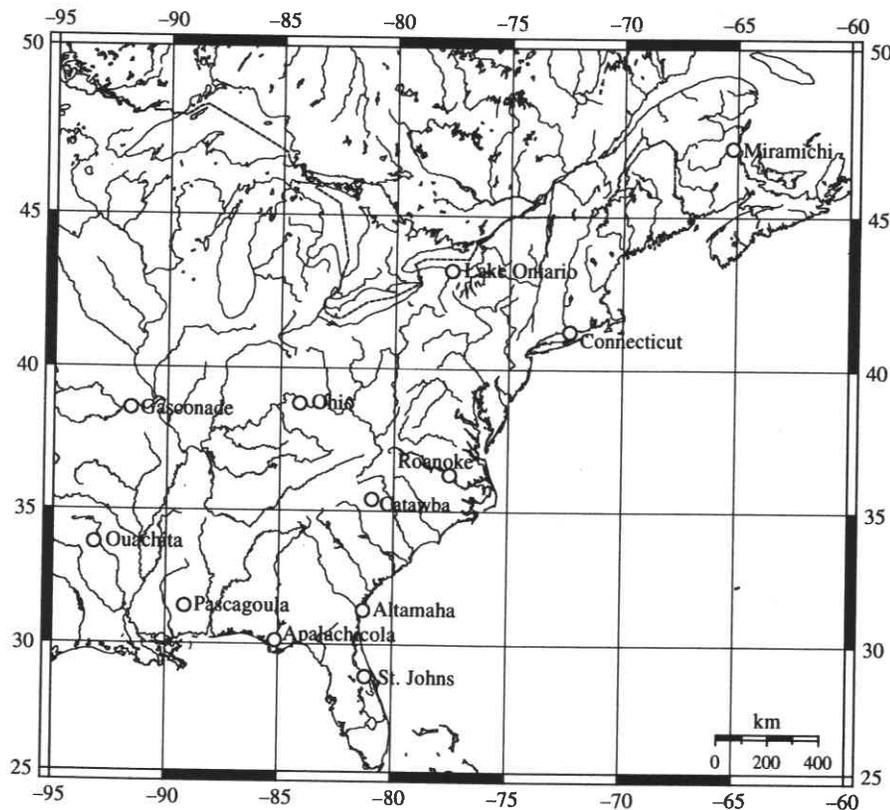


FIG. 1. North American collection sites for *Alosa* used in this study. Sites are labelled by drainage as identified in Table I. The base map was generated with the online map creation website (<http://www.aquarius.geomar.de/omc>).

Following Faria *et al.* (2006), one of the outgroup taxa was *Sardinops melanostictus* (Temminck & Schlegel) (subfamily Clupeinae), and *Dorosoma cepedianum* (Lesueur) (subfamily Dorosomatinae) was selected for the second outgroup. Both outgroup taxa have complete mitochondrial sequences available on GenBank (accession numbers AB032554 and DQ536426).

Total genomic DNA was extracted from fin tissue using the Qiagen DNeasy extraction kit (QIAGEN Inc., Valencia, CA, U.S.A.). The polymerase chain reaction (PCR) was used to amplify the complete mitochondrial ND1 and *cyt b* genes. The primers reported by Faria *et al.* (2006) were used for ND1. Primers matching conserved regions in the flanking tRNAs of *cyt b* were designed based on the sequences available on GenBank for the clupeiform fishes *S. melanostictus* and *D. cepedianum*. These primers, ShadcytbF1 (5'-CCACCGTTGTTATTCAACTATAGAAAC-3') and ShadcytbR1 (5'-AGAATRCTAGCTTTGGGAG-3'), allowed us to amplify the entire *cyt b* gene. An internal primer, ShadcytbR3 (5'-AGAAGGGCTARTACTCC-3'), was designed for sequencing across the region reported by Faria *et al.* (2006).

PCR amplifications were conducted in a total volume of 25 μ l using 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.01% gelatin, 2 mM MgCl₂, 200 μ M dNTPs, 0.5 units Taq polymerase, 0.3 μ M of each primer, c. 100 ng template DNA and water to the final volume. PCR cycling conditions consisted of an initial 1 min denaturing step at 95° C followed by 30 cycles for 1 min at 95° C, 1 min at 50° C and 1–3 min at 72° C. A final elongation step for 7 min at 72° C completed the cycle. PCR products were

cleaned using the ExoSAP-IT system (USB Co., Cleveland, OH, U.S.A.) and then used as the template in a cycle sequencing reaction with an ABI BigDye Terminator cycle sequencing kit (Foster City, CA, U.S.A.) using the primers described above. All sequencing reactions were sephadex cleaned (Princeton Separations, Adelphia, NJ, U.S.A.) prior to gel runs at the Iowa State University DNA Sequencing and Synthesis Facility. Sequence data were edited and aligned using Sequencher v. 4.1 (GeneCodes Co., Ann Arbor, MI, U.S.A.). Only the portion of *cyt b* matching that reported by Faria *et al.* (2006) was used in subsequent analyses.

PAUP* 4.0b10 (Swofford, 2002) was used to calculate pair-wise distances and the number of transitions and transversions. Saturation of nucleotide substitutions was assessed by plotting the number of transitions and transversions *v.* pair-wise uncorrected *p* distances. The degree of congruence in the phylogenetic signal of the *cyt b* and ND1 data sets was examined using the incongruence length difference test as implemented by PAUP* (Farris *et al.*, 1994). For all ingroup taxa, MEGA 3.1 (Kumar *et al.*, 2004) was used to calculate the average within-species uncorrected *p* distances and the net nucleotide divergence between species.

Phylogenetic relationships were inferred using maximum parsimony (MP), maximum likelihood (ML) and Bayesian analyses. All redundant sequences were excluded from these analyses to reduce the computational demand. The MP analysis was performed by PAUP* with a branch-and-bound search, and the initial upper bound was calculated by stepwise addition. GARLI v. 0.95 (Zwickl, 2006) was used to perform the ML analysis. A Bayesian inference of the phylogeny was performed using MrBayes v. 3.1 (Ronquist & Huelsenbeck, 2003). Tree space was explored starting with a random tree and employing two independent runs of four Markov chains of 1 000 000 generations, each sampled every 100 generations. Plots of log-likelihood scores *v.* generation time were examined to ensure that each run had reached stationarity, and the first 2500 trees were then discarded as burn-in. The most appropriate model of sequence evolution for the ML and Bayesian analyses was selected by ModelTest v. 3.5 (Posada & Crandall, 1998) as a GTR+G model with a gamma distribution shape parameter of 0.146. Phylogenetic support was assessed through bootstrapping (Felsenstein, 1985) with 1000 rounds of resampling for the MP and ML analyses. Bremer support values (Bremer, 1988) were calculated using MacClade 4 (Maddison & Maddison, 2000) to produce the command file for PAUP*. The majority-rule consensus of the 7500 trees saved by the Bayesian analysis was used to obtain the posterior probabilities of each clade.

The Templeton's two-tailed Wilcoxon signed-ranks test (Templeton, 1983) and the Shimodaira-Hasegawa test (SH, Shimodaira & Hasegawa, 1999) were used to compare the results of the phylogenetic analyses with prior hypotheses of relationships within *Alosa*. Two hypotheses were tested: monophyly of *A. sapidissima* and a sister relationship between *A. mediocris* and *A. chrysochloris*. The most parsimonious trees identified by the branch-and-bound search were compared with the trees generated by the constrained searches using the non-parametric two-tailed Wilcoxon signed-ranks test as implemented by PAUP*. The SH test compared the most likely tree from the constrained search with the most likely tree from the unconstrained search and the set of most parsimonious trees. The most parsimonious trees were included in case the ML search did not identify the true topology since the SH test assumes that the true tree is included. The SH test was conducted in PAUP* with 1000 bootstrap replicates using the resampling estimated log-likelihood (RELL) method.

RESULTS

New sequences for 30 individuals representing the six North American *Alosa* species were obtained as a part of this study (see Table I for GenBank accession numbers), including three species (*A. alabamae*, *A. chrysochloris* and *A. mediocris*) that were not sampled by Faria *et al.* (2006). In combination with 18 individuals selected from Faria *et al.* (2006), the data set was comprised of 35 unique haplotypes for the ingroup taxa. The final sequence alignment

included 1423 bases of which 448 bp were of *cyt b* and 975 bp of ND1. When considering just the ingroup taxa, 68 sites were variable in *cyt b* and 179 in ND1, of which 62 and 165 were parsimony informative, respectively. The incongruence length test found congruent phylogenetic signal ($P = 0.73$) in the two data sets, so both were combined in all phylogenetic analyses. All codon positions were weighted equally in the parsimony analysis, since no evidence of saturation at third codon positions was detected in either gene.

Average within-species p distances for both genes combined (Table II) for North American taxa were all relatively small – ranging from 0.0003 (*A. mediocris*) to 0.0052 (*A. aestivalis*). Net nucleotide divergence between the ingroup taxa (Table II) ranged from 0.0042 (*A. alabamae*–*A. sapidissima*) to 0.0936 (*A. fallax*–*A. chrysochloris*). The *A. aestivalis* with an *A. pseudoharengus* haplotype reported by Faria *et al.* (2006) was excluded from these calculations. Considering the subgenera *Alosa* and *Pomolobus*, the average net nucleotide divergence among species within each subgenus was 0.018 and 0.042, respectively, and the net nucleotide divergence between the two was 0.046.

Even though *cyt b* was a partial sequence, there were nearly as many amino acid substitutions in *cyt b* (seven) as there were in ND1 (eight). In *cyt b*, five of the amino acid substitutions were synapomorphies for the subgenus *Alosa*, and one was a synapomorphy for Eurasian *Alosa*. Four of the amino acid substitutions in ND1 differentiated *A. chrysochloris* from other *Alosa*, but two of these differences were also shared with one of the outgroup taxa. Likewise, the one amino acid difference in ND1 distinguishing the subgenus *Alosa* from *Pomolobus* was not a true synapomorphy for either group. However, one amino acid substitution in this gene was a synapomorphy for *A. mediocris*.

The branch-and-bound search identified 48 equally parsimonious trees [$L = 795$, consistency index (CI) = 0.731, retention index (RI) = 0.869]. The ML ($-\ln L = 5465.84$) and Bayesian phylogenetic analyses recovered the same overall topology, and the ML tree was selected to represent the phylogeny (Fig. 2).

TABLE II. Net nucleotide divergence (below the diagonal) and standard error (above the diagonal) between ingroup species based on the combined mitochondrial *cyt b* and ND1 nucleotide sequences. Average within-species uncorrected p distances are reported in bold along the diagonal. Species are abbreviated as follows: Alab., *A. alabamae*; Sap., *A. sapidissima*; Alosa, *A. alosa*; Fallax, *A. fallax*; Imm., *A. immaculata*; Pseudo., *A. pseudoharengus*; Aest., *A. aestivalis*; Med., *A. mediocris* and Chryso., *A. chrysochloris*

	Alab.	Sap.	Alosa	Fallax	Imm.	Pseudo.	Aest.	Med.	Chryso.
Alab.	0.0004	0.0016	0.0050	0.0048	0.0046	0.0059	0.0060	0.0070	0.0075
Sap.	0.0042	0.0043	0.0046	0.0044	0.0041	0.0059	0.0060	0.0068	0.0073
Alosa	0.0329	0.0299	0.0052	0.0039	0.0034	0.0065	0.0066	0.0069	0.0078
Fallax	0.0316	0.0290	0.0220	0.0007	0.0028	0.0066	0.0065	0.0071	0.0082
Imm.	0.0283	0.0252	0.0156	0.0110	0.0052	0.0061	0.0062	0.0068	0.0077
Pseudo.	0.0683	0.0672	0.0685	0.0694	0.0669	0.0040	0.0029	0.0050	0.0070
Aest.	0.0668	0.0650	0.0698	0.0700	0.0672	0.0151	0.0052	0.0050	0.0064
Med.	0.0750	0.0710	0.0754	0.0752	0.0740	0.0420	0.0436	0.0003	0.0072
Chryso.	0.0906	0.0863	0.0928	0.0936	0.0908	0.0707	0.0659	0.0746	0.0015

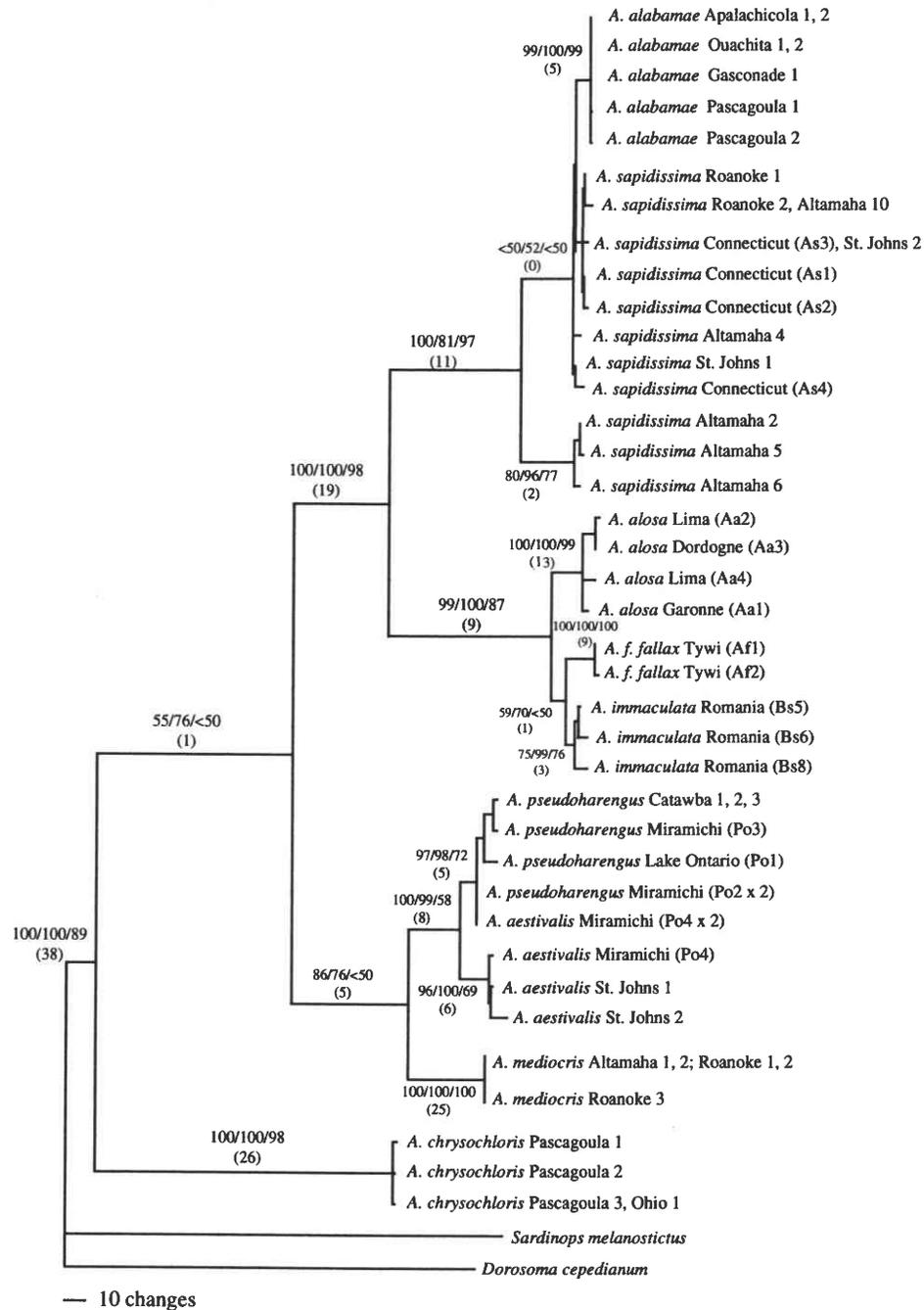


FIG. 2. The phylogram recovered from the maximum likelihood (ML) analysis of the combined mitochondrial *cyt b* and ND1 sequence data. Values provided for the more basal branches of the tree correspond to maximum parsimony bootstrap, Bayesian posterior probabilities and ML bootstrap for a given node, while the value in parentheses is the decay index. Individuals are identified by species and the river system from which they were collected. The sequences from Faria *et al.* (2006) have also been given the haplotype designation provided by the authors.

Alosa chrysochloris was recovered as the most basal *Alosa* species, but this relationship should be considered tentative given the low support values for the node leading to all remaining species (e.g. MP bootstrap = 55). Other species within the subgenus *Pomolobus* formed a moderately to poorly supported clade with *A. mediocris* basal to *A. aestivalis* and *A. pseudoharengus*. The subgenus *Alosa* was strongly supported by all measures of phylogenetic support. Within this clade, species fell into well-supported North American (*A. alabamae* and *A. sapidissima*) and Eurasian clades (*A. alosa*, *A. fallax* and *Alosa immaculata* Bennett). *Alosa alabamae* was recovered as a strongly supported monophyletic group that was part of what essentially was a polytomy with *A. sapidissima*.

The results of the phylogenetic hypothesis testing reflected the measures of phylogenetic support for the clades in question. When *A. sapidissima* was constrained to be monophyletic, the MP search recovered trees only one step longer ($L = 796$), and neither Templeton's ($P > 0.564$) nor the SH ($P > 0.425$) tests were significant. The position of *A. chrysochloris* as basal to the remainder of *Alosa* was only poorly supported by bootstrapping, and this topology was not significantly better than a constraint tree reflecting a sister relationship between *A. chrysochloris* and *A. mediocris* (Templeton's $P > 0.170$; SH $P > 0.408$).

DISCUSSION

As far as is known, this is the first molecular-based phylogeny including all North American *Alosa* species. Faria *et al.* (2006) extensively sampled Eurasian taxa, but they only included *A. aestivalis*, *A. pseudoharengus* and *A. sapidissima* from North America. In this study, the remaining three species (*A. alabamae*, *A. chrysochloris* and *A. mediocris*) have been added along with increased geographical sampling in the other three species, especially *A. sapidissima*. As found by Faria *et al.* (2006), the subgenus *Alosa* was a well-supported clade in the phylogeny. However, the subgenus *Pomolobus* was no longer a monophyletic group after the inclusion of *A. mediocris* and *A. chrysochloris*. Although *A. chrysochloris* was recovered as being basal to the remainder of *Alosa*, the phylogeny should realistically be presented as a polytomy of *A. chrysochloris* with the *A. pseudoharengus/A. aestivalis/A. mediocris* clade and the subgenus *Alosa*.

The inclusion of *A. alabamae*, the putative sister species to *A. sapidissima*, in the phylogenetic analyses did not alter the relationships within the subgenus *Alosa*. The *A. alabamae/A. sapidissima* clade was strongly supported with an average net nucleotide divergence of 0.029 between them and the Eurasian *Alosa* species. *Alosa alabamae* was recovered as a strongly supported monophyletic group that was part of a polytomy with *A. sapidissima*. Previous workers using mtDNA restriction site data had either refuted (Chapman *et al.*, 1994) or supported (Nolan *et al.*, 2003) the recognition of *A. alabamae* as a distinct, albeit a recently diverged, species. The sequence data presented here can also be interpreted as evidence of recent divergence of *A. alabamae* from *A. sapidissima*, and the lack of reciprocal monophyly between the two supports Swift *et al.*'s (1986) characterization of *A. alabamae* as an incipient species. However, to claim that *A. alabamae* and *A. sapidissima* do not represent separate species fails to recognize the fact that they are allopatric, separated by the Florida peninsula. The two taxa also exhibit important life-history differences; *A. alabamae*

is iteroparous whereas southern populations of *A. sapidissima* tend to be semelparous (Mettee & O'Neil, 2003). *Alosa sapidissima* was widely introduced in the Mississippi River drainage and across the Gulf of Mexico during the late 1800s, although these introductions were never deemed successful (Mettee & O'Neil, 2003). The present study does not address this issue since detecting the extent, if any, that these two species hybridized after the introductions would require much more intensive sampling and the use of nuclear and mtDNA markers (Alexandrino *et al.*, 2006). However, at least within the narrow scope of the sampling, no *A. sapidissima* haplotypes were detected in *A. alabamae*.

Previous workers (Chapman *et al.*, 1994; Faria *et al.*, 2006) reported that *A. aestivalis* and *A. pseudoharengus* were not reciprocally monophyletic. In the mitochondrial restriction site data of Chapman *et al.* (1994), the two species (both sampled from North Carolina, U.S.A.) were monophyletic when considering *cyt b*, but not ND1, which they considered to represent incomplete lineage sorting in the two species. Faria *et al.* (2006) detected two individuals of *A. aestivalis* from the Miramichi River (Canada) that shared a haplotype with *A. pseudoharengus* collected from the same location. They cited Chapman *et al.*'s (1994) explanation of incomplete lineage sorting, and they also suggested that this might represent hybridization since the sequence divergence between the two species was *c.* 2%. Additional sampling from one new location for each species failed to reveal any additional cases of shared haplotypes. Given the extent of sequence divergence between *A. aestivalis* and *A. pseudoharengus* compared with the relatively low level within each species, incomplete lineage sorting may not be the most likely explanation. As far as is known, potential hybridization between the two species has not been thoroughly tested with mtDNA or nuclear loci (*e.g.* microsatellites).

The hypothesized close relationship between *A. chrysochloris* and *A. mediocris* has apparently been based on biogeography and not formal taxonomic study (Berry, 1964; Swift *et al.*, 1986). The phylogeny presented here does not support this hypothesized relationship. However, the basal position of *A. chrysochloris* in the phylogeny was only weakly supported, and this topology was not significantly better than one in which *A. chrysochloris* was constrained to be sister to *A. mediocris*. Further work will be required before the exact relationship between *A. chrysochloris* and the remainder of the subgenus *Pomolobus* and the genus *Alosa* as a whole can be resolved.

The work presented here provides some insight into the biogeography of North American *Alosa* species. Ideally, the sequence divergence data could be used to date cladogenetic events, which would then be correlated with important geologic events. Bentzen *et al.* (1993) arrived at a rate of sequence divergence in *Alosa* mtDNA (0.22% per million years) that was much lower than those calculated for other teleost fishes, but recent work has raised doubts about the age of the fossils used to calibrate this rate (Zaragüeta, 2001; in Faria *et al.*, 2006). Despite this setback, the phylogeny and sequence divergence values can still be used to make biogeographic inferences. Faria *et al.* (2006) inferred that two biogeographic events led to the evolution of taxa on both sides of the Atlantic. Presumably, the first event involved an ancient divergence between the subgenus *Pomolobus* in North America and the subgenus

Alosa in Eurasia. A second more recent divergence then took place between Eurasian *Alosa* and the common ancestor of *A. sapidissima* and *A. alabamae*. Since *Alosa* are anadromous fishes capable of movement in the marine environment, the isolation of the two groups could have taken place well after the widening of the Atlantic due to sea-floor spreading. The similarly distributed *Acipenser sturio* Linnaeus (eastern Atlantic) and *Acipenser oxyrinchus* Mitchill (western Atlantic) may have a similar evolutionary history, perhaps only having diverged as recently as the Pliocene or Pleistocene (Choudhury & Dick, 1998).

For the North American *Alosa* species, Swift *et al.* (1986) proposed that the evolution of *A. chrysochloris* and *A. alabamae* in the Gulf of Mexico was the product of two independent biogeographic events: the close of the Suwannee Straits and dispersal around the Florida peninsula sometime during or after the Pleistocene. The phylogeny and sequence divergence data roughly corroborate this situation. Although the phylogeny does not show strong support for a sister relationship between *A. chrysochloris* and *A. mediocris*, the large amount of sequence divergence (0.0659–0.0746) between *A. chrysochloris* and the remainder of the *Pomolobus* clade suggests that the first divergence event between North American *Alosa* in the Gulf of Mexico and those in the Atlantic was an ancient one. The lack of reciprocal monophyly and limited sequence divergence (0.0042) between *A. alabamae* and *A. sapidissima* support the hypothesis that the ancestor of *A. alabamae* arrived in the Gulf of Mexico relatively recently in geological terms. A very similar evolutionary history has been inferred for two species of hermit crabs, both with populations in the Atlantic and Gulf of Mexico. Divergence of *Pagurus pollicaris* populations probably occurred sometime prior to the Pleistocene, while populations of *Pagurus longicarpus* likely diverged at some point during the Pleistocene (Young *et al.*, 2002).

The work presented here, in conjunction with that of Faria *et al.* (2006), provides a comprehensive molecular phylogeny of the genus *Alosa*. The complete taxonomic sampling of North American species has allowed us to address unresolved taxonomic issues in the group and to test several biogeographic hypotheses. Future work employing additional markers (other mtDNA genes and nuclear loci) may yet be able to provide a more resolved phylogeny for the genus.

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