Short communication

A molecular phylogenetic perspective on the evolutionary history of *Alosa* spp. (Clupeidae)

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1. Introduction

Clupeidae is one of the world’s most commercially important family of fishes. Within Clupeidae, the genus *Alosa* (subfamily Alosinae) has received research attention concerning its ecology and life-history (Baglinière, 2000 and references therein), as well as conservation issues due to recent declines in many of its species (Waldman, 2003). Despite their importance, little is known about the phylogenetic relationships within *Alosa*, resulting in systematic and taxonomic uncertainty, which may undermine the establishment of adequate conservation measures.

Drawing on the work of Economidis and Sinis (1986) and Whitehead (1985); Baglinière (2000) listed 16 species native to the northern hemisphere distributed through the western and eastern Atlantic coasts; Mediterranean, Black, and Caspian Seas; and Lake Volvi (Greece). Although most of these species are anadromous, there are two amphidromous, one marine and one strictly freshwater species. However, within some anadromous species there are populations that complete their entire life cycle in freshwater environments. This variation in life-history strategies together with the capacity to colonize new habitats makes species of *Alosa* interesting models to study speciation and adaptation. In addition, enormous intraspecific morphological variability has been described making the application of molecular techniques crucial to understand the phylogenetic relationships between species.

*Alosa* was initially classified into three genera: *Pomolobus*, composed of four North American species (now *A. aestivalis*, *A. pseudoharengus*, *A. mediocris*, and *A. chrysochloris*); *Caspialosa*, for Ponto-Caspian species; and *Alosa*, represented by *A. alosa* and *A. fallax* in Europe and *A. sapidissima* and *A. alabamia* in North America (Regan, 1917 in Bentzen et al., 1993). Based on morphological criteria, Svetovidov (1964) considered a single genus (*Alosa*) with two subgenera (*Alosa* and *Pomolobus*), rejecting the validity of *Caspialosa* as a distinct subgenus. However, the species of the Ponto-Caspian region are often considered a distinct biogeographic and morphological entity that merits subgeneric status (Baglinière, 2000; Bobori et al., 2001). The existence of the first two subgenera (*Alosa* and *Pomolobus*) is congruent with an mtDNA restriction length polymorphism analysis (RFLP) analysis reporting a 6.5% divergence between them (Bentzen et al., 1993), but this study did not include any Ponto-Caspian species. The distinction of *A. alosa* and *A. fallax* has also been questioned by some authors (Boisneau et al., 1992), but differences in morphology and allozyme genetic data support the existence of two divergent lineages that hybridize (Alexandrino et al., 1996; Bentzen et al., 1993). Uncertainty on the distinction of some North American species (*A. sapidissima* vs *A. alabamia*; *A. pseudoharengus* vs *A. aestivalis*) has also been presented (Chapman et al., 1994; Nolan et al., 2003), and no molecular information has been reported for the seven Ponto-Caspian species. There have also been numerous descriptions of subspecies (e.g., six for *A. fallax*; Aprahamian et al., 2003), but no effort has been invested to determine the degree of phenotypic plasticity or molecular differentiation of these putative taxonomic units.

Herein, we screen mtDNA sequence variation in cytochrome *b* (cyt *b*) and NADH dehydrogenase subunit one (ND1) in eight *Alosa* species providing the first sequence based perspective on the evolutionary relationships within
the genus. In particular, the study aimed to: (i) distinguish if *A. sapidissima* is closer to European or to other North American species; (ii) understand the phylogenetic position of the Ponto-Caspian relatively to the European and to the North American representatives of the genus; (iii) discern if *A. alosa* and *A. fallax* are sister taxa; and (iv) evaluate the genetic differentiation between different *A. fallax* subspecies.

2. Material and methods

*Alosa* specimens were collected by angling or nets between 1993 and 2004 from throughout most of the geographic range of the genus (see Table 1). At least two individuals were analyzed for each of eight putative species: *A. sapidissima*, *A. pseudoharengus*, and *A. aestivalis* from North America; *A. alosa*, *A. fallax*, *A. macedonica*, *A. caspia*, and *A. immaculata* (considered a priority name of *A. pontica*, Kottelat, 1997) from Eurasia. Within *A. fallax*, samples were taken from five putative subspecies (*A. f. fallax*, *A. f. rhodanensis*, *A. f. nilotica*, *A. f. lacastris*, and *A. f. killarnensis*). Two *Sardinella* sp. individuals (Clupeidae) collected in Turkey, and a Japanese sardine (*Sardinops melanostictus*, Clupeidae) for which both cyt *b* and ND1 sequences were available in GenBank (Accession No. NC002616), were used as outgroups.

Whole genomic DNA was extracted using a standard high-salt protocol (Sambrook et al., 1989). A 515 bp fragment of the cyt *b* and the complete ND1 (975 bp) were amplified by polymerase chain reaction (PCR). The cyt *b* fragment was amplified using *Alosa* specific primers: Alocytf1 (CCT TCT AAC ATT TCA GTC TGA TG) and Alocytr1 (AGG ATT GTG GCC CCT GCA ATT AC), both located at cyt *b*. ND1 was amplified using *Alosa* specific primers: Alond1f1 (GTA CGA AAG GAC CGG CT) and Alond1r2 (CGT GGT TCA CTC TAT W), located in the tRNA-Ile and tRNA-Gln regions. For *A. aestivalis*, *A. pseudoharengus*, and *Sardinella* sp., a different reverse primer for the ND1 gene was used (Alond1r2: CGT GGT TCA CTC TAT W). Amplification conditions are available upon request. PCR products were purified with ExoSap-IT (Amersham-Pharmacia Biotech) and sequenced following the ABI PRISM BigDye Terminator

### Table 1

<table>
<thead>
<tr>
<th>Sampled taxa</th>
<th>River/basin (country)</th>
<th>Sample code (N)</th>
<th>Latitude</th>
<th>Longitude</th>
<th>GenBank a.n. (cyt <em>b</em>)</th>
<th>GenBank a.n. (ND1)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. aestivalis</em></td>
<td>Miramichi/Atlantic (Canada)</td>
<td>A aesti.Can (4)</td>
<td>47°06’N</td>
<td>65°13’W</td>
<td>DQ419777</td>
<td>DQ419804</td>
</tr>
<tr>
<td><em>A. pseudoharengus</em></td>
<td>Miramichi/Atlantic (Canada)</td>
<td>A pseud.Can (2)</td>
<td>47°06’N</td>
<td>65°13’W</td>
<td>DQ419777</td>
<td>DQ419804</td>
</tr>
<tr>
<td><em>A. pseudoharengus</em></td>
<td>Lake Ontario (EU)</td>
<td>A pseud.Eua (2)</td>
<td>43°15’N</td>
<td>77°32’W</td>
<td>DQ419776</td>
<td>DQ419803</td>
</tr>
<tr>
<td><em>A. sapidissima</em></td>
<td>Connecticut/Atlantic (EU)</td>
<td>A. sapid.Eua (4)</td>
<td>41°16’N</td>
<td>72°20’W</td>
<td>DQ419773</td>
<td>DQ419799</td>
</tr>
<tr>
<td><em>A. alosa</em></td>
<td>Lima/Atlantic (Portugal)</td>
<td>A. alosa.Lim (2)</td>
<td>41°41’N</td>
<td>8°50’W</td>
<td>DQ419761</td>
<td>DQ419785</td>
</tr>
<tr>
<td><em>A. alosa</em></td>
<td>Garonne/Atlantic (France)</td>
<td>A. alosa.Gar (1)</td>
<td>44°08’N</td>
<td>0°44’E</td>
<td>DQ419760</td>
<td>DQ419782</td>
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<tr>
<td><em>A. alosa</em></td>
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<td>A. alosa.Dor (1)</td>
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<td>0°27’E</td>
<td>DQ419762</td>
<td>DQ419785</td>
</tr>
<tr>
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<td>Tywi/Atlantic (England)</td>
<td>A. fall.Tyw (2)</td>
<td>51°41’N</td>
<td>4°23’W</td>
<td>DQ419764</td>
<td>DQ419786</td>
</tr>
<tr>
<td><em>A. f. fallax</em></td>
<td>Tejo/Atlantic (Portugal)</td>
<td>A. fall.Tej (1)</td>
<td>38°41’N</td>
<td>9°15’W</td>
<td>DQ419764</td>
<td>DQ419787</td>
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<tr>
<td><em>A. f. fallax</em></td>
<td>Sebou/Atlantic (Morroco)</td>
<td>A. fall.Mar (2)</td>
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<td>6°40’W</td>
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<td>DQ419791</td>
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<tr>
<td><em>A. f. killarnensis</em></td>
<td>Lake Lcane/Atlantic (Ireland)</td>
<td>A. f. kill.Irl (1)</td>
<td>52°02’N</td>
<td>9°31’W</td>
<td>DQ419764</td>
<td>DQ419786</td>
</tr>
<tr>
<td><em>A. f. rhodanensis</em></td>
<td>Rhône/Mediterranean (France)</td>
<td>A. f. rhod.Rho (2)</td>
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<td>4°50’E</td>
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<td>DQ419789</td>
</tr>
<tr>
<td><em>A. f. rhodanensis</em></td>
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<td>A. f. rhod.Her (1)</td>
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<td>3°26’E</td>
<td>DQ419764</td>
<td>DQ419788</td>
</tr>
<tr>
<td><em>A. f. lacastris</em></td>
<td>Lake Garda/Mediterranean (Italy)</td>
<td>A. f. laca.Gar (1)</td>
<td>45°32’N</td>
<td>10°42’E</td>
<td>DQ419764</td>
<td>DQ419789</td>
</tr>
<tr>
<td><em>A. f. nilotica</em></td>
<td>Pinios/Mediterranean (Greece)</td>
<td>A. f. nilo.Gre (1)</td>
<td>39°51’N</td>
<td>22°31’E</td>
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<td>DQ419790</td>
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<tr>
<td><em>A. immaculata</em></td>
<td>Danube Delta/Black Sea (Romania)</td>
<td>A. immac.Rom (1)</td>
<td>44°52’N</td>
<td>29°37’E</td>
<td>DQ419769</td>
<td>DQ419796</td>
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<tr>
<td><em>A. immaculata</em></td>
<td>Danube Delta/Black Sea (Romania)</td>
<td>A. immac.Rom (2)</td>
<td>45°11’N</td>
<td>28°48’E</td>
<td>DQ419770</td>
<td>DQ419794</td>
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<tr>
<td><em>A. immaculata</em></td>
<td>Black Sea (Northeastern Turkey)</td>
<td>A. immac.Tur (3)</td>
<td>41°03’N</td>
<td>39°41’E</td>
<td>DQ419771</td>
<td>DQ419795</td>
</tr>
<tr>
<td><em>A. caspia</em></td>
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<td>A. caspi.Rom (2)</td>
<td>45°06’N</td>
<td>29°16’E</td>
<td>DQ419772</td>
<td>DQ419792</td>
</tr>
<tr>
<td><em>A. macedonica</em></td>
<td>Lake Volvi/Mediterranean (Greece)</td>
<td>A. maced.Gre (2)</td>
<td>40°40’N</td>
<td>23°27’E</td>
<td>DQ419772</td>
<td>DQ419796</td>
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<tr>
<td><em>Sardinella</em> sp.</td>
<td>Samandag/Mediterranean (Turkey)</td>
<td>Sardinella sp. (2)</td>
<td>34°04’N</td>
<td>35°56’E</td>
<td>DQ419780</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>DQ419781</td>
<td>DQ419808</td>
</tr>
</tbody>
</table>
Cycle Sequencing protocols, using Alcytbf1 and Alc
tybfr for cyt b, and the internal primers Alond1f2 (GTA AAT GCA GGA AGC CTA AG; located in the tRNA-
Leu) and Alond1r2 for the ND1. Sequencing products
were electrophoresed on an ABI PRISM 310 automated
sequencer (PE Applied Biosystems). Alignments were
done separately for each gene using BioEdit 5.0.9 (Hall,
1999).

Sequences were imported into PAUP* 4.0b10 (Swofford,
2002) to calculate observed pairwise sequence divergence
(uncorrected p distances) and the number of transitions and
transversions. To assess the degree of saturation in each
gene, we plotted: (i) the absolute number of transitions and
absolute number of transversions under the corrected
pairwise distances (p distances); and (ii) p distances against
ML distances. Base frequency homogeneity across taxa was
tests using a chi-square test (ML distances. Base frequency homogeneity across taxa was
determined using a chi-square test (likelihood ratio test).

Maximum parsimony (MP), maximum likelihood (ML),
neighbor-joining (NJ), and Bayesian analysis were used for
phylogenetic reconstruction with each gene alone and for
both genes concatenated. We used Modeltest 3.06 PPC
(Posada and Crandall, 1998) under the Akaike Information
Criterion (AIC) (Posada and Buckley, 2004) to obtain a
most likely model of nucleotide substitution that was subse-
uently used in the ML and Bayesian inference. Maximum
parsimony (MP) analysis was carried out in PAUP*4.0b10
using an heuristic search and the tree bisection and recon-

rection (TBR) branch swapping method. Searches started
with stepwise addition trees and were replicated 1000 times,
with each replicate beginning with a random order of
sequences. The software PHYML 2.4.4 (Guindon and
Gascuel, 2003), which implements an algorithm that adjusts
tree topology and branch lengths simultaneously, departing
from an initial tree constructed using BIONJ (Gascuel,
1997) was used to perform ML analysis. The software MEGA
3.1 was used to perform NJ analysis based on p dis-
rstances. Robustness of all trees was assessed by bootstrap
analysis involving 1000 pseudoreplicates. The Bayesian
analysis was implemented in MrBayes 3.0 (Huelsenbeck
and Ronquist, 2001), using a Metropolis-coupled, Markov
chain Monte Carlo (MC3) sampling approach. Parameters
were estimated with four Markov chains incrementally
heated with the default heating values. All analyses
started with randomly generated trees and ran for 1 × 106
generations, saving one tree in every 100 generations. The
log-likelihood values of the sample points were plotted
against the generation time and all the trees prior to reach-
ing stationarity were discarded. Combining the remaining
trees, a 50% majority rule consensus tree was generated.

3. Results and discussion

The final alignment yielded 448 bp for cyt b and 975 bp
for ND1, with no indels. No significant differences in base
frequencies across taxa were found. Individuals classified as
Sardinella sp. presented 1% mean divergence at the cyt b
gene with Sardinella maderensis (GenBank Accession No.
AF472583). Pairwise sequence divergence within the
ingroup ranged from 0 to 7.1% for cyt b, and up to 8.0% for
ND1. Mean divergence between the ingroup and outgroup
ranged from 16.8% for cyt b to 19.8% ND1, while the
mean divergence between Sardinella sp. and Sardinops
melanostictus ranged from 20.2% (cyt b) to 22.6% (ND1).
The mean pairwise sequence divergence ratio of ND1 to cyt
b was 1.28:1 (SD = 0.40), concordant with the report that
ND1 is more variable than cyt b in Alosa (Chapman et al.,
1994). In both genes, absolute number of transitions accu-
mulated rapidly and in a linear manner, while transversions
accumulated slowly. Little evidence of saturation was
found except for ND1 at high divergences (above 17%
divergence, data not shown), and thus had little effect on
resolving ingroup relationships.

Excluding the outgroup, there was a total of 46 variable
sites in cyt b and 132 in ND1, of which 36 (cyt b) and 106
(ND1) were parsimony informative. No stop codons were
detected except at the end of the ND1. For cyt b, six amino
acid changes were detected within the ingroup (L39M;
A67V; L108V; N114T; V118I and N172S), five of which are
synapomorphies for the subgenus Alosa and the sixth
(N114T) for all Eurasian shads. For ND1 there were two
amino acid changes within the ingroup (M6L; T173I).

For each gene, the different phylogenetic approaches
produced nearly identical tree topologies. As the clades are
generally better resolved with the two genes combined, only
the concatenated data set is presented (Fig. 1). MP revealed
eight equally parsimonious trees of 66 steps (C1 = 0.7943;
R1 = 0.8706). The best-fit model selected by Modeltest was
TrN+I+G with an estimate of invariable sites (I = 0.6348)
and a discrete approximation of the gamma distribution
(α = 2.4993). The four different phylogenetic methods
resulted in similar tree topologies and in general the clades
are well supported (Figs. 1A–E). Starting with the most
interior node, the monophyly of the genus Alosa is very well
supported as is the monophyly for each of the two subgen-
era (Alosa and Pomolobus). Our divergence estimate
between these two groups (Da = 5.6%) supports a relatively
recent common ancestor compared to that proposed based

Fig. 1. (A) Strict consensus tree of the eight most parsimonious trees based on the concatenated data. For the major clades, support values (over 50%) are shown for ML (above left), Bayesian (above right), MP (below left), and NJ (below right). 100* means that all bootstrap values are equal or higher than 95. (B) NJ tree based on the p distance. (C) ML tree using the TrN+I+G model. (D) One of the eight most parsimonious trees. (E) Bayesian 50% consensus tree. All the trees were rooted with the outgroup. In trees (B), (C), and (E), branch lengths of the outgroup were not proportional to the scale presented and therefore were marked with (/). Classification of the subgenus was made according to Svetovidov (1964).
on fossil evidence (37 to 25 Myr) (Gaudant, 1991), assuming conventional divergence rates for teleost fishes and a molecular clock (0.5–2% per Myr). This incongruence was noted by Bentzen et al. (1993), who reported 6.5% divergence between these subgenera, and discussed that this would result in an extremely low mtDNA divergence rate (0.22% per Myr) if the fossil record was taken as a calibration point. Together with doubts on the *Alosa* fossil classification recently presented (Zaragüeta, 2001), the fossil-based estimate of divergence must be regarded as very improbable.

All trees as well as net divergence estimates further support that *A. sapidissima* is phylogenetically closer to Eurasian taxa than to the other North American species, suggesting two biogeographic events involving the separation of lineages from both continents (Fig. 1 and Table 2).

<table>
<thead>
<tr>
<th>Taxonomic Groups</th>
<th>Cyt b (448bp)</th>
<th>Nd1 (975bp)</th>
<th>Cyt b + Nd1 (1423bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. alosa</td>
<td>—</td>
<td>0.013</td>
<td>0.012</td>
</tr>
<tr>
<td>A. fallax</td>
<td>0.020</td>
<td>—</td>
<td>0.012</td>
</tr>
<tr>
<td>Black Sea</td>
<td>0.018</td>
<td>0.009</td>
<td>—</td>
</tr>
<tr>
<td>A. im.TurBs1</td>
<td>0.024</td>
<td>0.012</td>
<td>0.013</td>
</tr>
<tr>
<td>A. sapidissima</td>
<td>0.034</td>
<td>0.029</td>
<td>0.025</td>
</tr>
<tr>
<td>Pomolobus</td>
<td>0.066</td>
<td>0.066</td>
<td>0.066</td>
</tr>
</tbody>
</table>

Black Sea/Lake Volvi haplotypes were considered as one lineage, as well as the divergent *A. immaculata* haplotype. Calculations were performed considering only the different haplotypes within each taxonomic group.

a Group including the haplotypes found in the Black Sea and Lake Volvi (Greece) with the exception of *A. immac.TurBs1*.

**Table 2**

Net nucleotide divergence between taxonomic groups (*Da*) based on cyt *b* (upper diagonal), ND1 (lower diagonal), and combined analyses (right).

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Fig. 2. Representation of the phylogenetic relationships between Eurasian representatives of the genus *Alosa* based on the different phylogenetic methods for each gene individually and combined. ML, maximum likelihood; BI, Bayesian inference; MP, maximum parsimony; NJ, neighbor-joining. Af, *A. fallax*; Aa, *A. alosa*; BS (Black Sea and Lake Volvi excluding *A. immac.TurBs1*); Ai1, *A. immac.TurBs1*. Bootstrap values above 50 are shown.
Within Pomolobus, A. aestivalis shares a haplotype with A. pseudoharengus but also reveals divergence up to 2%, suggesting either incomplete lineage sorting (Chapman et al., 1994) or introgressive hybridization.

Within Alosa, two well supported clades are observed: one representing A. sapidissima and the other the Eur-Asian representatives of the genus. Within the latter group, A. alosa forms a very well supported clade, while A. fallax and Black Sea/Lake Volvi form a large diverse clade. Additionally, the net distance between these groups together with the high bootstrap support (92) observed with NJ (Fig. 1 and Table 2), supports a closer relationship between the A. fallax and Black Sea/Lake Volvi clades than either to A. alosa. However, the bootstrap support for this clade is inconsistent among reconstruction approaches and there is a lack of reciprocal monophyly between A. fallax and the Black Sea/Lake Volvi clades (Fig. 1), due to the one highly divergent haplotype from Turkey (A.immac.TurBs1). This haplotype may represent introgression or ancestral polymorphism involving unsampled taxa from the Black or Caspian Sea, and therefore reflects another mtDNA lineage. Overall, however, the weak support for the branching order of these four groups (A. alosa, A. fallax, Black Sea/Lake Volvi, and A.immac.TurBs1) (Fig. 2) reflects a relatively rapid radiation with respect to the phylogenetic power of the data, and thus and increased sequencing effort is required to better support an evolutionary hypothesis.

Ponto-Caspian species constitute a distinct biogeographic entity, and exhibit morphological differences, like the presence of teeth in the palatine and vomer (Bobori et al., 2001), promoting the view that they could be the presence of teeth in the palatine and vomer (Bobori et al., 2001), promoting the view that they could be a relatively recent colonization of Lake Volvi by Black Sea lineages, as suggested by Bobori et al. (2001). Low divergence between A. caspia and A. immaculata was also found (not higher than 0.8%, excluding A.immacTurBS1), suggesting also a very recent split with incomplete lineage sorting or hybridization between them. However, the relationship between these Black Sea species may be much more complex, as a variety of forms have been described (Mezhzerin, pers. comm.). The low divergences among species of the Black Sea/Lake Volvi clade, as well as the fact that the Ponto-Caspian region harbors half of the described Alosa species and numerous subspecies and morphotypes, may reflect that a rapid diversification occurred after the colonization of Black and Caspian Seas.

Divergences among putative A. fallax subspecies ranged from 0 (between A. fallax fallax vs. A. f. killarnensis, and between A. fallax lacustris vs. A. fallax rhodanensis) to 0.8% (within the same subspecies, A. f. fallax). Moreover, even considering the validity of subspecies, A. f. fallax is paraphyletic. Currently, there are no clear diagnostic traits for these taxa and usually geographic location is used for a priori designation. We suggest that unless unambiguous diagnostic criteria can be established, populations of A. fallax should be viewed as representing a polytypic species presenting a broad range of morphological, ecological, and physiological variability. Alosa are extremely vulnerable to anthropogenic changes, especially related to access and quality of their spawning grounds. Therefore, these locally adapted populations of A. fallax should be preserved, regardless of their formal classification.

To conclude, we provide a molecular phylogenetic overview of the genus Alosa showing that in some cases the species boundaries are not well defined by the mtDNA molecule. These data could serve as an objective template to aid systematic revision, or more detailed local studies on the status of specific taxa. We suggest that such studies should include multiple molecular markers to ensure that some of the typical caveats of mtDNA based phylogenies are considered (Ballard and Whitlock, 2004), and the integration of morphological information.

Acknowledgments

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