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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. alabamae* 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 different 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. alabamae*; *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

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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. lacustris*, 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: Alocytbf1 (CCT TCT AAC ATT TCA GTC TGA TG) and Alocytbr1 (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 AG) located in the 16S rRNA region and Alond1r1 (TTT CTA AGG AGC TGG GG) 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 CAA AG located in the tRNA-Ile). 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
List of sampled taxa and their location, including major river basin and country, a sample code (including taxon, location and the number of individuals sampled shown in brackets), geographical coordinates, and GenBank Accession Nos. (a.n.) for the 39 individuals analyzed in this study

Sampled taxa	River/basin (country)	Sample code (*N*)	Latitude	Longitude	GenBank a.n. (cyt *b*)	GenBank a.n. (ND1)
A. aestivalis	Miramichi/Atlantic (Canada)	A.aesti.Can (4)	47°06′N	65°13′W	DQ419777	DQ419804
					DQ419779	DQ419805
A. pseudoharengus	Miramichi/Atlantic (Canada)	A.pseud.Can (2)	47°06′N	65°13′W	DQ419777	DQ419804
					DQ419778	DQ419806
A. pseudoharengus	Lake Ontario (EUA)	A.pseud.Eua (2)	43°15′N	77°32′W	DQ419776	DQ419803
A. sapidissima	Connecticut/Atlantic (EUA)	A.sapid.Eua (4)	41°16′N	72°20′W	DQ419773	DQ419799
					DQ419773	DQ419800
					DQ419774	DQ419801
					DQ419775	DQ419802
A. alosa	Lima/Atlantic (Portugal)	A.alosa.Lim (2)	41°41′N	8°50′W	DQ419761	DQ419785
					DQ419763	DQ419784
A. alosa	Garonne/Atlantic (France)	A.alosa.Gar (1)	44°08′N	0°44′E	DQ419760	DQ419782
A. alosa	Dordogne/Atlantic (France)	A.alosa.Dor (1)	44°50′N	0°27′E	DQ419762	DQ419785
A. fallax fallax	Tywi/Atlantic (England)	A.f.fall.Tyw (2)	51°45′N	4°23′W	DQ419764	DQ419786
					DQ419765	DQ419786
A. f. fallax	Tejo/Atlantic (Portugal)	A.f.fall.Tej (1)	38°41′N	9°15′W	DQ419764	DQ419787
A. f. fallax	Sebou/Atlantic (Morrocco)	A.f.fall.Mar (2)	34°15′N	6°40′W	DQ419767	DQ419791
A. f. Killarnensis	Lake Leane/Atlantic (Ireland)	A.f.kill.Irl (1)	52°02′N	9°31′W	DQ419764	DQ419786
A. f. rhodanensis	Rhône/Mediterranean (France)	A.f.rhod.Rho (2)	43°20′N	4°50′E	DQ419764	DQ419789
					DQ419766	DQ419788
A. f. rhodanensis	Herault/Mediterranean (France)	A.f.rhod.Her (1)	43°17′N	3°26′E	DQ419766	DQ419788
A. f. lacustris	Lake Garda/Mediterranean (Italy)	A.f.lacu.Gar (1)	45°32′N	10°42′E	DQ419764	DQ419789
A. f. nilotica	Pinios/Mediterranean (Greece)	A.f.nilo.Gre (1)	39°51′N	22°31′E	DQ419764	DQ419790
A. immaculata	Danube Delta/Black Sea (Romania)	A.immac.Rom (1)	44°52′N	29°37′E	DQ419769	DQ419796
A. immaculata	Danube Delta/Black Sea (Romania)	A.immac.Rom (2)	45°11′N	28°48′E	DQ419770	DQ419794
					DQ419771	DQ419795
A. immaculata	Black Sea (Northeastern Turkey)	A.immac.Tur (3)	41°03′N	39°41′E	DQ419768	DQ419792
					DQ419768	DQ419792
					DQ419772	DQ419797
A. caspia	Danube Delta/Black Sea (Romania)	A.caspi.Rom (2)	45°06′N	29°16′E	DQ419772	DQ419796
					DQ419770	DQ419793
A. macedonica	Lake Volvi/Mediterranean (Greece)	A.maced.Gre (2)	40°40′N	23°27′E	DQ419772	DQ419796
					DQ419772	DQ419798
Sardinella sp.	Samandag/Mediterranean (Turkey)	*Sardinella* sp. (2)	34°04′N	35°56′E	DQ419780	DQ419807
					DQ419781	DQ419808

Cycle Sequencing protocols, using Alocytbf1 and Alocytbr1 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 against the uncorrected pairwise distances (*p* distances); and (ii) *p* distances against ML distances. Base frequency homogeneity across taxa was tested using a chi-square test (χ^2). Distance between groups of taxa (corrected for within-group variation) was calculated using the net nucleotide divergence (*Da*) in MEGA 3.1 (Kumar et al., 2004).

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 subsequently 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 reconnection (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* distances. 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 (MC^3) 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 1×10^6 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 reaching 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 accumulated 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 666 steps (CI = 0.7943; RI = 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 ($\alpha = 2.4993$). 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 subgenera (*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).

A

A.alosa.Gar(Aa1)
A.alosa.Lim(Aa2)
A.alosa.Dor(Aa3)
A.alosa.Lim(Aa4)
A.f.fall.Tyw(Af1)
A.f.kill.Irl(Af1)
A.f.fall.Tyw(Af2)
A.f.fall.Tej(Af3)
A.f.rhod.Her(Af4)
A.f.rhod.Rho(Af4)
A.f.nilo.Gre(Af5)
A.f.lacu.Gar(Af6)
A.f.rhod.Rho(Af6)
A.f.fall.Seb(Af7)
A.f.fall.Seb(Af7)
A.immac.Tur(Bs1)
A.immac.Tur(Bs1)
A.immac.Rom(Bs8)
A.caspi.Rom(Bs7)
A.immac.Rom(Bs6)
A.immac.Rom(Bs5)
A.immac.Tur(Bs4)
A.maced.Gre(Bs2)
A.maced.Gre(Bs3)
A.caspi.Rom(Bs3)
A.sapid.Eua(As1)
A.sapid.Eua(As2)
A.sapid.Eua(As3)
A.sapid.Eua(As4)
A.pseud.Eua(Po1)
A.pseud.Eua(Po1)
A.aesti.Can(Po2)
A.aesti.Can(Po2)
A.pseud.Can(Po2)
A.pseud.Can(Po3)
A.aesti.Can(Po4)
A.aesti.Can(Po4)
Sardinella sp.1
Sardinella sp.2
Sardinops melanostictus

100* 100* 90/96 65/92 94/100 89/87 85/100 84/87 100* 62/96 61/64 91/100 92/88 93/95 75/97 60/- 58/- 55/55 79/92 100* 100* 92/100 89/96 83/99 81/77 100* 90/97 62/84 100/100 94/99 100* 85/65 98/97 92/85 100/100 100*

A. alosa
A. fallax
Black Sea & Lake Volvi
A. sapidissima
Subgenus *Alosa*
Subgenus *Pomolobus*

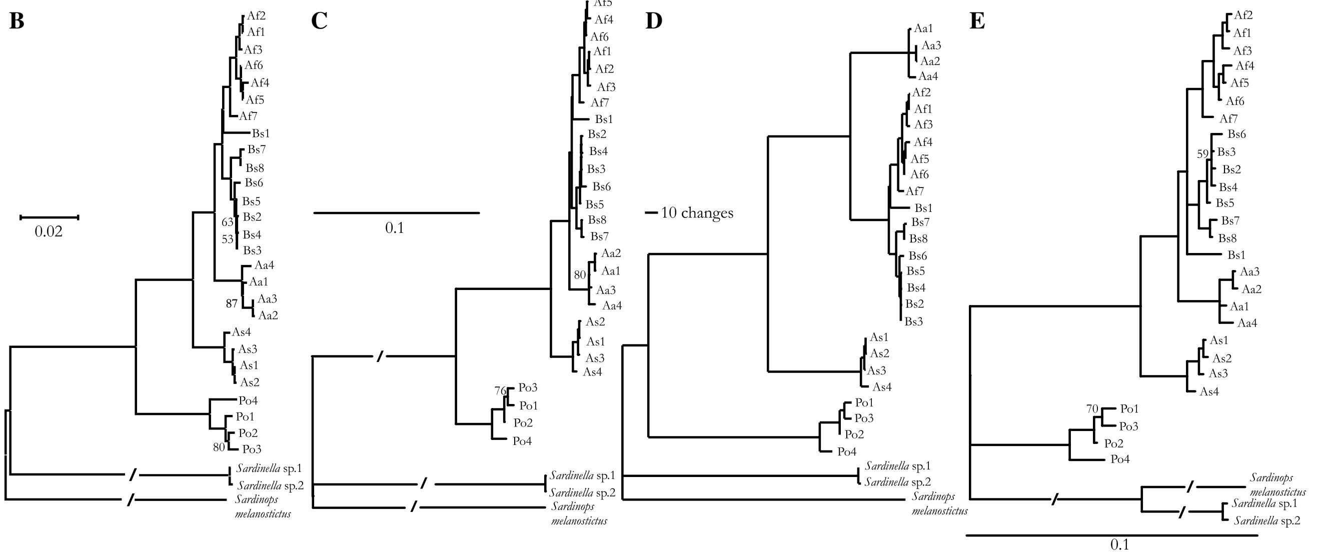

Table 2
Net nucleotide divergence between taxonomic groups (*Da*) based on cyt *b* (upper diagonal), ND1 (lower diagonal), and combined analyses (right)

	A. alosa	*A. fallax*	Black Sea[a]	*A.im.TurBs1*	*A. sapidissima*	*Pomolobus*	*A. alosa*	*A. fallax*	Black Sea[a]	*A. im.TurBs1*	*A. sapidissima*
A. alosa	—	0.013	0.012	0.015	0.020	0.060	—				
A. fallax	0.020	—	0.009	0.015	0.021	0.056	0.019	—			
Black Sea[a]	0.018	0.008	—	0.014	0.026	0.055	0.017	0.009	—		
A.im.TurBs1	0.024	0.012	0.013	—	0.030	0.066	0.022	0.013	0.014	—	
A. sapidissima	0.034	0.029	0.025	0.035	—	0.050	0.030	0.027	0.026	0.034	—
Pomolobus	0.066	0.066	0.066	0.070	0.068	—	0.064	0.063	0.063	0.069	0.063

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.im.TurBs1.

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).

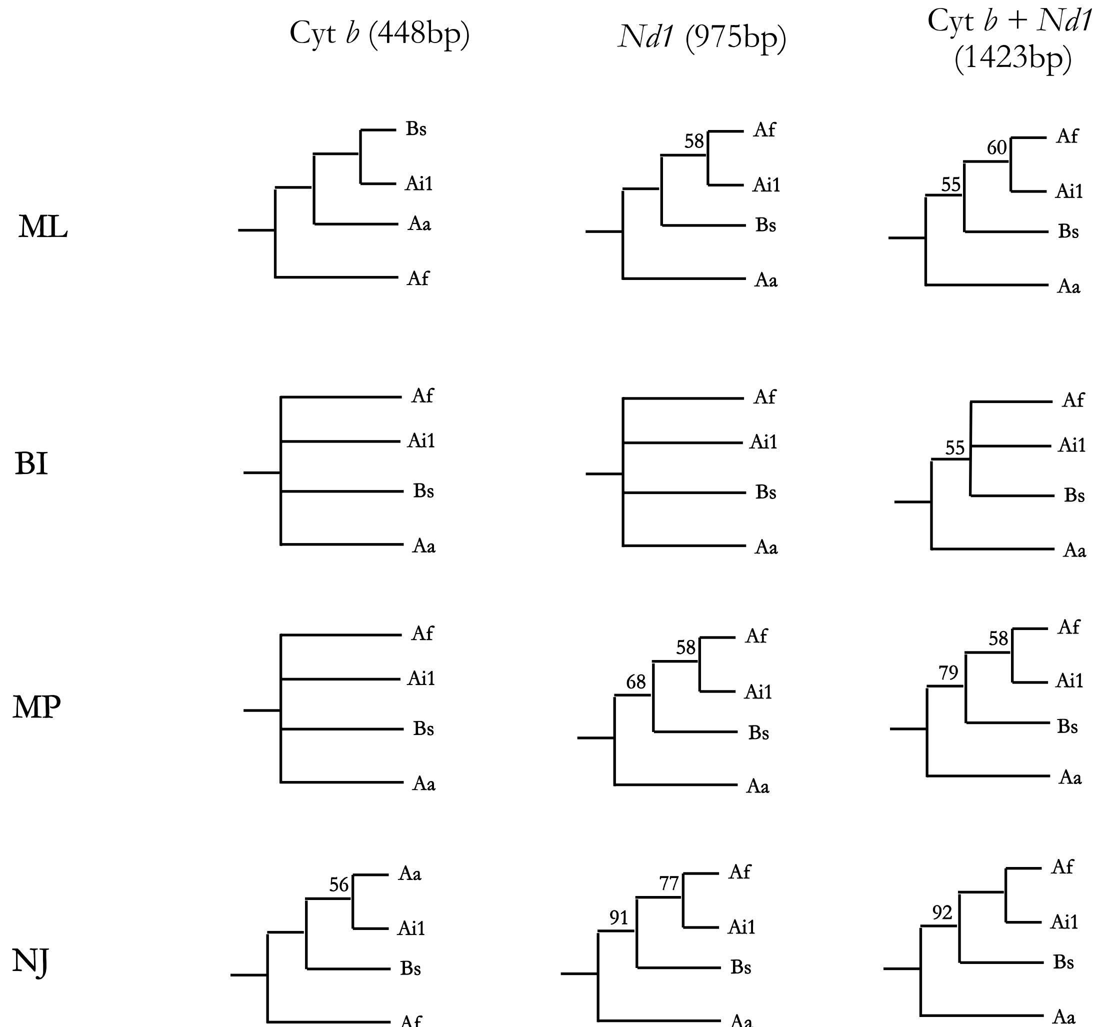

Fig. 2. Representation of the phylogenetic relationships between Eurasian representatives of the genus *Alosa* based on the different phylogenetic methods for each gene individually and combined. ML, maximum likelihood; BI, Bayesian inference; MP, maximum parsimony; NJ, neighbor-joining. Af, *A. fallax*; 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 Eurasian 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 included in a different subgenus, *Caspialosa* (Baglinière, 2000 and references therein). Fossil records from Serbia dated from the lower Miocene and also from Romania, Bulgaria, and Greece dated from the Pliocene, were attributed to *Caspialosa nordmanni*, which is actually a junior synonym of *A. caspia*. Based on this, Legall (2000) proposed that the split between the ancestor of the Ponto-Caspian and the species of subgenus *Alosa* preceded the split between *A. sapidissima* and the European *A. alosa* and *A. fallax*. However, our molecular data rather suggest a relatively recent separation of lineages found in the Black Sea/Lake Volvi. If we considered *Caspialosa* as a valid subgenus, the subgenus *Alosa* would become paraphyletic. Therefore, our molecular analysis supports the morphological based classification made by Svetovidov (1964).

Based on the morphological similarities described above, *A. macedonica* was considered to be more closely related to Black Sea species (Economidis and Sinis, 1986) rather than Atlantic species (Svetovidov, 1952 in Bobori et al., 2001). The high bootstrap support for the cluster formed by *A. macedonica* and Black Sea species as well as the low divergences between these taxa (0–0.8%, excluding A.immac.TurBS1), support 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 (Mezhzherin, 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.

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