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A molecular analysis of relationships and biogeography within a species complex of Holarctic fish (genus *Osmerus*)

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Abstract

Episodes of trans-Arctic faunal exchange and isolation between the north Pacific and Atlantic ocean basins have been implicated as important historic geological events contributing to extant patterns of genetic diversity and structure in Holarctic faunas. We made a further test of the significance of such biogeographic events by examining mitochondrial DNA (mtDNA) restriction fragment length and cytochrome *b* sequence polymorphism among north Pacific and Arctic, north-western Atlantic (north-eastern North American), and north-eastern Atlantic (European) regional forms of the boreal smelt, genus *Osmerus*. Our analyses also assessed whether the regional forms within this 'species complex': (i) represent a single widely distributed and polytypic species, or is composed of three geographically distinct species, and (ii) resulted from a single split from north Pacific ancestral *Osmerus* or two independent Pacific-Atlantic divergences. MtDNA sequence divergence estimates among forms ranged from 5.6–8.9% and from 6.1–8.5% based on restriction fragment and 300 base pairs of cytochrome *b* sequencing, respectively. Divergence within forms averaged less than 0.5% for fragment analysis and no differences were detected from sequence analysis. Provisional dating of lineage separations in *Osmerus* based on our sequence divergence estimates suggested a mid-Pliocene to early Pleistocene time frame for diversification among the forms. These estimated lineage separation dates support the idea that geological events in 'Beringia' and the surrounding trans-Arctic area (e.g. opening of the Bering Seaway, Pleistocene glacial advances), occurring over a similar time frame, have influenced radiation in *Osmerus*. Phenetic and parsimony analyses of the sequence divergence estimates and of sequence polymorphisms suggested that the north Pacific/Arctic form and the north-western Atlantic form shared a common ancestor more recently than either has with the north-eastern Atlantic form, thus supporting the hypothesis that the species complex has arisen from two independent Pacific–Atlantic divergences probably beginning during the mid-Pliocene.

Keywords: Holarctic zoogeography, mitochondrial DNA, *Osmerus*, species complexes

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Introduction

Vicariance biogeography embraces the idea that phylogenetic relationships among taxa should be reflected in the historical patterns of geographic isolation that have led to the diversification of the taxa in question. Indeed, some major historic geological events have been implicated as factors influencing evolutionary processes leading to extant patterns of interspecific genetic diversification and systematic relationships (Brundin 1965; Vawter *et al.* 1980; Bot *et al.* 1989). For instance, periods of trans-Arctic isolation and exchange between the north Pacific and Atlantic Ocean basins appear to have had a major impact on the genetic structure and relationships of ter-

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restrial, freshwater, and marine Holarctic faunas Although the present day Bering Sea was a land bridge over most of the last 7–10 million years, the north Pacific and Atlantic basins were subject to extensive faunal exchanges when the Bering Seaway first opened about 10–12 million years ago (Ma) and then again some 3–5 Ma (Durham & MacNeil 1964; Briggs 1970; Herman & Hopkins 1980; Grant 1987). Trans-Arctic exchange via the Bering Seaway has apparently been a major factor influencing genetic diversification and structure in sea urchins (Palumbi & Wilson 1990; Palumbi & Kessing 1991), pelecypods (MacDonald & Koehn 1988; Vermeji 1989), and several freshwater and marine fishes (Grant 1987; Billington *et al.* 1990; Ortí *et al.* 1994).

To assess the generality of Beringian and trans-Arctic geological events shaping patterns of diversification in Holarctic faunas we have studied levels of mtDNA divergence in smelts of the genus *Osmerus* (Pisces: Osmeridae). These freshwater and anadromous fishes have an almost complete circumboreal distribution (Nellbring 1989; Taylor & Bentzen 1993a) and consist of a 'species complex' (McPhail & Lindsey 1970) of three morphologically similar, but largely allopatric forms: a north Pacific and Arctic form, a north-western Atlantic (eastern North American) form, and north-eastern Atlantic (European) form (see Nellbring 1989 for distributional maps). Because the three regional forms of *Osmerus* span a geographic range potentially influenced by geological events occurring in Beringia, they provide a further test of the potential importance of trans-Arctic exchange/isolation in shaping genetic diversity in Holarctic faunas.

A molecular analysis of genetic diversity in *Osmerus* may also help resolve two outstanding issues in smelt systematics and zoogeography. First, because the forms are largely allopatric the genus has had a long history of systematic and phylogenetic uncertainty (Hubbs 1925; McAllister 1963; Klyukanov 1975; Luey *et al.* 1982) and there are competing zoogeographic scenarios for the evolutionary origins of the three regional forms of *Osmerus*. Klyukanov (1975) hypothesized that *Osmerus* had a north Pacific origin and that subsequent divergence of the Atlantic forms from one another occurred after an initial eastward, trans-Arctic migration by a north Pacific ancestral form into the north Atlantic near the close of the Pliocene. McAllister (1963) also hypothesized a north Pacific origin for *Osmerus*, but argued that the European form evolved after a westward migration of a north Pacific ancestor to the White Sea and into the Baltic before or during the early Pleistocene. McAllister (1963) further argued that the eastern North American form was derived from a north Pacific *Osmerus* after a more recent eastward trans-Arctic migration of the latter into the north-western Atlantic sometime during the Pleistocene. These zoogeographic models make distinct predictions about relative evolutionary affinity among the three forms of *Osmerus*. McAllister's (1963) model predicts that the two North American forms shared a common ancestor more recently than either has with the European form whereas Klyukanov's (1975) model predicts that the two Atlantic forms shared a common ancestor more recently than either has with the north Pacific form.

Secondly, and in terms of their systematic relationships it is unclear whether the three regional forms represent polymorphism within a single evolutionary lineage or whether there are three geographically separate, and evolutionary independent taxa. Taxonomically, this uncertainty is evidenced by unity of the three forms as *Osmerus eperlanus* or as three species [*O. dentex* (north Pacific and Arctic), *O. mordax* (eastern North American), and *O. eperlanus* (European)] based upon conflicting morphological, meristic, life-history, and biochemical genetic data (Hubbs 1925; McAllister 1963; Kliukanov 1969; McAllister *et al.* 1980; Luey *et al.* 1982; Haldorson & Craig 1984).

In this study we used restriction enzyme and direct sequencing analyses of the mitochondrial DNA (mtDNA) genomes of native populations of north Pacific and Arctic, eastern North American, and European smelt to: (i) provide an independent test of whether geological events occurring in Beringia relating to episodes of trans-Arctic exchange/isolation have influenced extant patterns of genetic diversity in Holarctic faunas, (ii) assess competing biogeographic scenarios for diversification within *Osmerus*, and (iii) assess interrelationships within the species complex. Assaying mtDNA variation has proven to be extremely useful for resolving phylogenetic relationships among closely related taxa (Moritz *et al.* 1987; Hewitt *et al.* 1991; Echelle & Dowling 1992). In the present context, the strengths of mtDNA as a phylogenetic marker lie: (i) in its maternal, non-recombining mode of inheritance that provides a powerful marker of matriarchal phylogeny (Wilson *et al.* 1985; Avise 1989 and (ii) its frequently elevated substitution rate relative to single copy nuclear loci (Brown 1983), which potentially results in more phylogenetically informative polymorphisms

Materials and methods

Sample collections

Anadromous and freshwater smelt were collected from the north Pacific and from watersheds tributary to the north-western (eastern North America) and north-eastern (Europe) Atlantic Ocean (Table 1). The north Pacific sample was obtained during a commercial herring fishery in Tokiak Bay (Bristol Bay) in Alaska and probably included fish from more than one spawning population.

Table 1 Taxa studied, life-history type, sample sizes, and collection locality for smelt used in the study

Species	Life-history type	Locality	*N*
Osmerus mordax (Mitchill)	Anadromous/freshwater	St. Lawrence River to Maine	651*
O. dentex Steindachner	Anadromous	Togiak Bay, Alaska	31
O. eperlanus (Linneaus)	Freshwater	IJsselmeer, The Netherlands	32
O. eperlanus (L.)	Anadromous	Loire River, France	28
O. eperlanus (L.)	Anadromous	Helsinki, Finland	14
O. eperlanus (L.)	Anadromous	Kyrönjoki River, Finland	36
Mallotus villosus (Muller)	Marine	NW and NE Atlantic	424†

*Data taken from Baby *et al.* (1991) and Taylor & Bentzen (1993a). †See Dodson *et al.* (1991).

Eastern North American smelt were from eight populations sampled by Baby *et al.* (1991) and 16 sampled by Taylor & Bentzen (1993a). European smelt were sampled from two Finnish, one Dutch, and one French population (Table 1). The Dutch sample was from IJsselmeer (the suffix 'meer' means lake in Dutch), the same population examined electrophoretically by Luey *et al.* (1982). Fish were collected in the winter/spring months of 1991 and were shipped whole on ice to Dalhousie or Laval. For convenience, below we refer to the north Pacific, eastern North American, and European forms by their specific (or subspecific) names: *dentex*, *mordax*, and *eperlanus*, respectively.

mtDNA extraction and restriction enzyme analysis

Mitochondrial DNA was extracted using protocols outlined by Taylor & Bentzen (1993a). Aliquots of mtDNA from each fish were digested separately with 15 restriction endonucleases: seven hexameric (*Pvu*II, *Apa*I *Bgl*I, *Dra*I, *Pst*I, *Eco*RV, *Bst*EII) six multihexameric (*Ava*I, *Ban*I, *Ban*II, *Hae*II, *Hin*cII, *Sty*I), and two multipentameric (*Nci*I, *Ava*II) enzymes. Digests proceeded for between 2 and 6 h under conditions specified by the vendors (Pharmacia or Boehringer Mannheim). Mitochondrial DNA fragments were resolved by electrophoresis in 0.8–1.2% agarose gels in Tris-acetate (TAE) buffer run at either 25 or 45 V overnight. A Bethesda Research Laboratories 1 kilobase pair (kbp) ladder or λ *Hin*dIII/*Eco*RI digested DNA were run in each gel as a molecular weight size standard. Mitochondrial DNA fragments were visualized with ethidium bromide staining and photographed under UV light.

When samples produced low or poor quality yields of mtDNA, the restricted fragments were alkali denatured and vacuum (at Dalhousie) or capillary (at Laval) transferred to nylon membranes (Amersham Hybond-N). At Dalhousie, mtDNA fragments bound to the nylon membranes were visualized by nonradioactive hybridization to mtDNA from American shad (*Alosa sapidissima*) as outlined by Taylor & Bentzen (1993a). The DNA hybrids were detected by chemiluminescence (Bronstein & McGrath 1989; Höltke *et al.* 1992) followed by exposure of the membranes to X-ray film (Kodak XOMAT-AR) at room temperature for between 2 and 16 h. Hybridization studies at Laval University were conducted using ^{32}P-labelled smelt mtDNA using methods outlined by Baby *et al.* (1991).

PCR amplification and sequencing of cytochrome b

Relative to an analysis of mapped restriction sites, our analysis of interform relationships based on fragment comparisons are subject to greater error particularly at high (i.e. > 5%) levels of sequence divergence (Swofford & Olsen 1990). To examine interform relationships more rigorously we sequenced a 300-bp region of the mtDNA cytochrome *b* gene from one individual from each of the major clades within *mordax*, *dentex*, *eperlanus*, and *Mallotus* (see below). We used derivatives of the 'universal' primers described by Kocher *et al.* (1989) to amplify a 360-bp fragment: 5′-CCATCCAACATCTCAGCATGATGAAA-3′ (light strand) and 5′-CCCCTCAGAATGATATTTGTCCTCA-3′ (heavy strand). Amplifications were carried out in 25-μL total volumes containing (final concentrations): 200 μM each of dATP, dGTP, dCTP, and dTTP, 800 nM of each primer, 0.4 U of *Taq* polymerase, 6.7 mM Tris-HCl (pH 8.8), 1.0 mM 2-mercaptoethanol 2 mM $MgCl_2$, and between 10 (purified mtDNA) and 1000 ng (genomic DNA) of template. Thirty cycles of amplification were performed with denaturation at 94 °C for 60 s, primer annealing at 45 °C for 60 s, and primer extension at 72 °C for 90 s. A final single extension step was performed at 72 °C for 5 min.

Successful reactions were purified using the Promega 'Magic' PCR Clean-Up Kit to a final volume of 50 μL of water. Double-stranded PCR products were sequenced in dideoxy sequencing reactions using the light-strand primer with the Sequenase 2.0 polymerase enzyme, reagents, nucleotide triphosphates (United States Biochemical) and [^{35}S]-dATP. Sequences were deposited in GENBANK under accession numbers U05594 and U05665-U05667.

Data analysis

Restriction enzyme fragment profiles were designated by a one-letter code (e.g. *Ava*I-A, B, etc.) in order of their appearance. Composite genotypes were designated by a 15-letter code with individual letters representing the fragment length polymorphisms for each of the 15 restriction enzymes. Within-form polymorphisms consisted of single or double site changes and maximum sequence divergence within all three *Osmerus* was about 1.0% (see Results). Our examination of between-form variation was therefore limited to the two most divergent (*mordax* and *dentex*) or three most common (*eperlanus*) mtDNA genotypes within each *Osmerus* for three reasons. First, there were too many restriction fragment differences among forms (over 20 for *Ava*II alone) to confirm fragment sharing among all genotypes by comigration on common gels. Secondly, we were interested in among-form relationships and examining only the most divergent or common genotypes within taxa provides reasonable control for within-form variability. Thirdly, phylogenetic variation using mtDNA restriction site information for eastern North American *Osmerus* is detailed elsewhere (Baby *et al.* 1991; Taylor & Bentzen 1993a). For eastern North American smelt we used composite genotypes 71 and 27 of Baby *et al.* (1991) which were the most common genotypes within the two major mtDNA lineages found in a survey of over 600 fish from the St Lawrence River, east to Newfoundland, and south to the Gulf of Maine (Baby *et al.* 1991; Taylor & Bentzen 1993a; Tables 2 and 3). North Pacific smelt were dominated by a single genotype (23/31 fish) with one other genotype represented by two fish and six others by single fish. Each of the four European populations was also dominated by a single composite genotype (61–78% occurrence within each sample) one of which predominated in both Finnish populations. Therefore, we used these three most common composite genotypes (one Dutch, one Finnish, and one French) as a measure of within-form variability for European smelt (Tables 2 and 3).

Table 2 Mitochondrial DNA composite genotypes that were used to estimate sequence divergence within and between *Osmerus* species and capelin (*Mallotus villosus*). Also given is the frequency of occurrence of each genotype within each sample. Upper-case letters represent fragment length polymorphisms for *Apa*I, *Ava*I, *Ava*II, *Ban*I, *Ban*II, *Bgl*I, *Bst*EII, *Dra*I, *Eco*RV, *Hae*II, *Hin*cII, *Nci*I, *Pst*I *Pvu*II and *Sty*I, respectively, which are illustrated in Table 3. Polymorphisms for *Mallotus villosus* are depicted in Dodson *et al.* (1991)

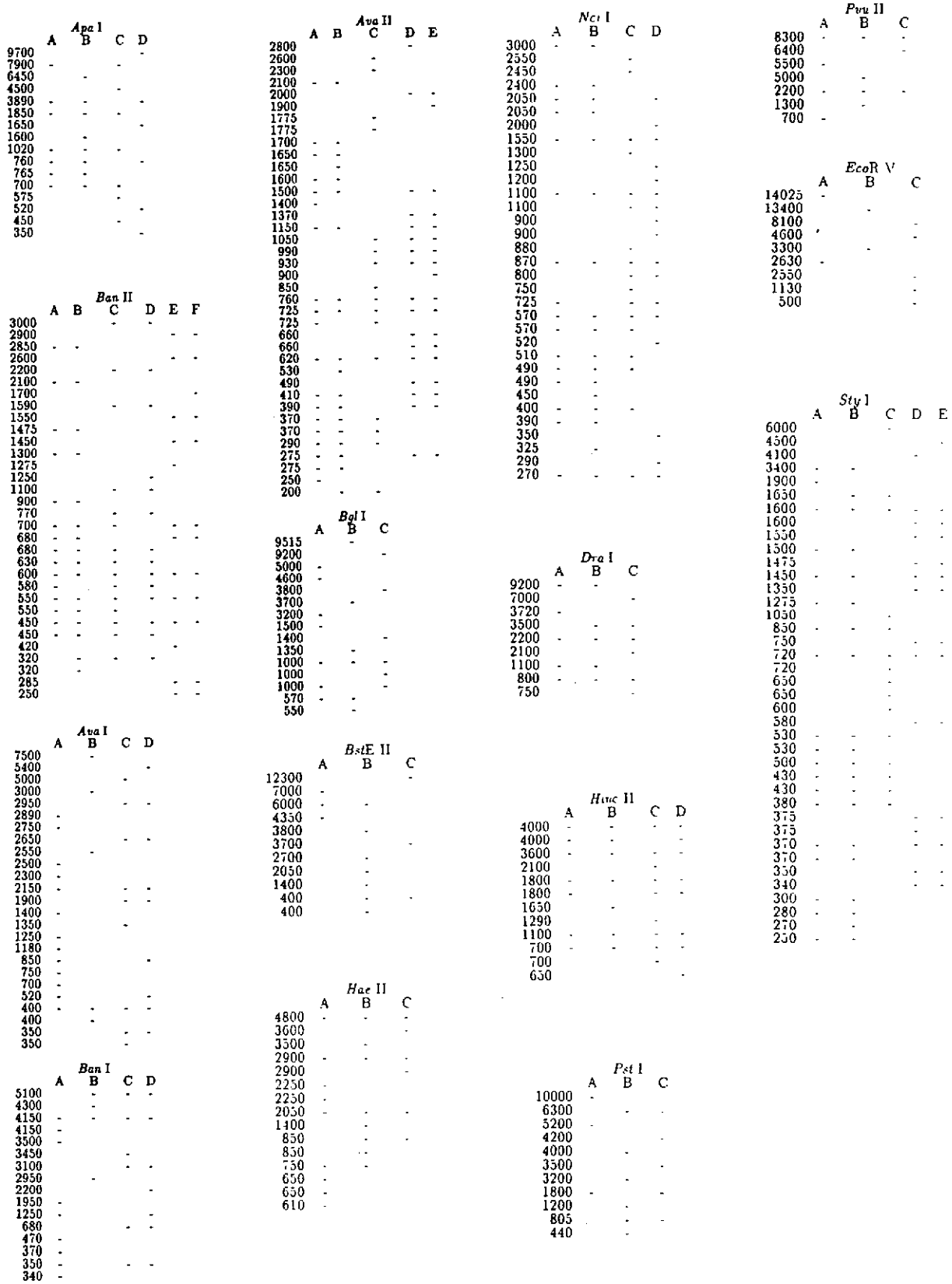
Composite genotype	Distribution	Frequency
1. AAAAAAAAAAAAAAA	*O. mordax*	0.24
2. BABABAAAAABBAAB	*O. mordax*	0.08
3. CBCBCBBBBBCCBBC	*O. dentex*	0.74
4. CBCBDBBBBBCCBBC	*O. dentex*	0.06
5. DCDCECCCCCDDCCD	*O. eperlanus*, Netherlands	0.78
6. DDEDFCCCCCDDCCE	*O. eperlanus*, France	0.61
7. DDDCECCCCCDDCCE	*O. eperlanus*, Finland	0.62
8. EEFEGDDDDDEEDDF	*M. villosus*, NW Atlantic	0.42
9. FFGFHEEEEEFFEEG	*M. villosus*, NE Atlantic	0.13

To place variation among *Osmerus* within a broader evolutionary context we used capelin (Osmeridae: *Mallotus villosus*) as an outgroup taxon in our analyses. Capelin are native to both the north Atlantic and Pacific oceans (McAllister 1963), but our samples were limited to north Atlantic fish examined by Dodson *et al.* (1991). We compared smelt restriction fragment profiles to those of the most common genotype within each of the two major mtDNA clades in north Atlantic capelin which differ in sequence by 3.4% (Dodson *et al.* 1991). Sharing of bands between capelin and smelt genotypes was evaluated by comigration of representative samples of each on common gels.

Fragment length polymorphisms among forms were sufficiently complex so we could not interpret the variants in terms of simple site changes. Our analysis was therefore limited to estimates of mtDNA sequence divergence based on a presence/absence matrix of restriction fragments. Estimates of the number of base substitutions per nucleotide site, d, and their standard errors among composite genotypes were calculated using the REAP software package (McElroy *et al.* 1992) based on algorithms developed by Nei & Li (1979) and Upholt (1977). Genetic diversity each collection was evaluated by calculating the nucleon or haplotype diversity index (h, Nei & Tajima 1981) using REAP.

To assess affinities among forms we clustered the estimates of sequence divergence using the unweighted pair-group method with arithmetic averages (UPGMA), a varying evolutionary rate branch-length method (Fitch & Margoliash 1967), and a Neighbor-Joining algorithm (Saitou & Nei 1987). All analyses were performed using programs found in the PHYLIP software package (Felsenstein 1990) with random input of genotypes repeated nine times, once for each of nine genotypes compared (two eastern North American, two north Pacific, and three European smelt genotypes, and two capelin genotypes). We assessed the stability of branch points in the Neighbor-Joining topology using the 'jacknife' procedure outlined by Lanyon (1985). We jacknifed across restriction enzymes by re-estimating sequence divergence among genotypes, but with the information from one enzyme eliminated in turn. We then clustered these jacknifed sequence divergence estimates for a total of 15 (= number of restriction enzymes) 'pseudoreplicate' analyses (i e. trees). We also jacknifed across composite genotypes re-running the analyses nine times, once for

Table 3 Restriction fragment length polymorphisms of *Osmerus* mtDNAs when digested with 15 endonucleases. Polymorphisms are those for the most divergent (*Osmerus mordax* and *O. dentex*) or the geographically most widespread (*O. eperlanus*) composite genotypes observed within each *Osmerus* (see Table 2)



each of the genotypes eliminated in turn. These manipulations simulated possible topological outcomes had we lacked information from any one enzyme or had we not sampled particular composite genotypes. In this way, jacknifing can detect internal branch point inconsistencies and thus provides a qualitative assessment of the stability of tree topology (Lanyon 1985; Swofford & Olsen 1990).

Cytochrome *b* sequences were aligned by eye and pairwise sequence divergences were calculated using Kimura's two-parameter algorithm found in DNADIST of PHYLIP. The sequence divergence data were then clustered by UPGMA and Neighbor-Joining. Maximum parsimony analysis of the sequence data was conducted using DNAPARS of PHYLIP. Confidence in the distance-based and parsimony dendrograms was assessed by bootstrap resampling of the sequence data (N = 500–1000 times) using SEQBOOT of PHYLIP and then recalculating sequence divergences, distance phenograms, and parsimony relationships for each of the replicate data sets. Majority-rule consensus trees were then constructed using CONSENSE of PHYLIP.

Results

mtDNA restriction fragment variation

We estimated the total molecular weight of *Osmerus* mtDNA genomes by averaging the sums of all restriction enzyme fragment profiles except those produced by *Ban*II which included too many fragments below 250 base pairs (bp) to score accurately. We estimated smelt mtDNA molecular weights of 16 838 ± 273 (SD), 16 711 ± 245, and 16 900 ± 335 bp for *mordax*, *dentex*, and *eperlanus*, respectively, values similar to mtDNA molecular weights for a variety of animal taxa assayed to date (cf. Brown, 1983; Billington & Hebert, 1991). We scored an average 125 mtDNA fragments per individual which represents 664 bp or about 3.9% of each *Osmerus* mtDNA genome.

Intraform variation

Variability in mtDNA restriction fragment profiles within eastern North American smelt is covered extensively by Baby *et al.* (1991) and Taylor & Bentzen (1993a). Briefly, after examining 209 smelt from the St Lawrence River and the northern Gulf of St Lawrence, Baby *et al.* (1991) identified 71 composite genotypes that clustered by UPGMA into two major genetic groupings at 0.83% sequence divergence. In 444 fish from the southern Gulf, Newfoundland, south-western Nova Scotia, New Brunswick, and Maine, Taylor & Bentzen (1993a) resolved 93 composite genotypes that clustered into the same two genetic groups identified by Baby *et al.* (1991) also at about 0.80% sequence divergence. Sequence divergence among the composite genotypes resolved by Taylor & Bentzen (1993a) averaged 0.32% (range 0.08–1.10%). Nucleon diversity (h) of eastern North American *Osmerus* averaged 0.642 ± 0.038 among 32 population samples, but varied widely among populations from 0.00 to 0.963 (Baby *et al.* 1991; Taylor & Bentzen 1993a).

The north Pacific smelt sample contained eight composite genotypes among 31 fish assayed, but 23 fish were characterized by a single genotype (h = 0.454). The genotypes differed by an average 0.38% sequence divergence (range: 0.02–1.19%).

The 110 smelt from European watersheds were characterized by 28 composite genotypes which differed by an average 0.31% sequence divergence (range: 0.05–0.88%). Both Neighbor-Joining and UPGMA analyses of the sequence divergence estimates among these genotypes suggested that fish clustered into groups that generally corresponded to the different sample localities (e.g. Fig. 1). Nucleon diversity in European smelt averaged 0.546 ± 0.067 and ranged from 0.343 in IJsselmeer (Netherlands) to 0.627 in the Kjrõnjoki River (Finland) sample. For each enzyme, restriction fragment differences between composite genotypes within *mordax*, *dentex*, and *eperlanus* were all interpretable in terms of one or two site changes (Table 3).

Interform variation

Pairwise sequence divergence estimates among the nine genotypes from four taxa (three *Osmerus* and *Mallotus*) averaged 7.2% with the greatest among *Osmerus* divergence observed between *dentex* and *eperlanus* genotypes (numbers 4 and 5, respectively, Table 4). After accounting for within-form variability (Nei & Li 1979) net divergences among *Osmerus* taxa were 5.7% (*mordax* vs. *dentex*), 6.8% (*mordax* vs. *eperlanus*) and 8.6% (*dentex* vs. *eperlanus*). Mean net divergence among *Osmerus* was 7.1% and net divergence between *Osmerus* and *Mallotus* was 7.8%

Although there were some differences in branching order of the three European smelt genotypes among themselves, concordant topologies of among *Osmerus* relationships were obtained using the various phylogenetic tree construction methods (i.e. UPGMA, Fitch-Margoliash, Neighbor-Joining). For example, the Neighbor-Joining tree (Fig. 2) indicates that north Pacific smelt and eastern North American smelt are more similar genetically to each other than either is to European smelt. As expected, the three *Osmerus* taxa form a monophyletic group relative to *Mallotus* (Fig. 2). Finally, whether we jacknifed across restriction enzymes or composite genotypes, the closer affinity of *mordax* and *dentex* genotypes to each other relative to *eperlanus* genotypes was supported in all 15 or nine 'pseudoreplicate' trees, respectively.

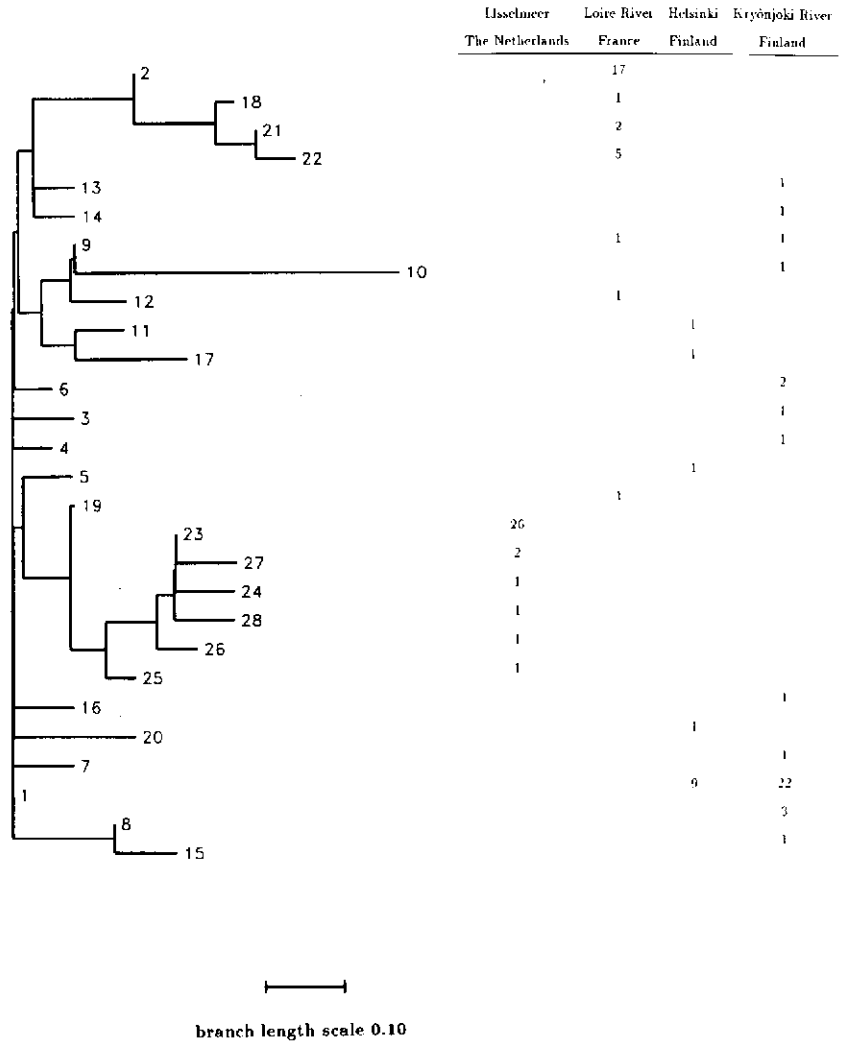


Fig. 1 Neighbor-Joining phenogram of genetic affinities among 28 European smelt mitochondrial DNA composite genotypes from populations sampled from the Netherlands, France, and Finland. Phenogram was inferred from a matrix of percentage sequence divergence estimates derived from restriction fragment length polymorphisms among genotypes. Also shown is the frequency distribution of genotypes across populations.

Table 4 Percentage sequence divergence ($d \infty 100$) (lower portion of matrix) and their standard errors (upper portion of matrix) among the nine composite genotypes used to estimate sequence divergence within and among *Osmerus* species and capelin, *Mallotus villosus*. Estimates were derived from a matrix of shared restriction fragments and the numbered genotypes are defined in Table 2

	Composite genotype								
	1	2	3	4	5	6	7	8	9
1	-	0.44	1.24	1.24	1.31	1.28	1.29	1.41	1.46
2	0.43	–	1.28	1.22	1.31	1.29	1.30	1.38	1.44
3	5.64	6.18	–	0.08	1.43	1.45	1.43	1.39	1.47
4	5.66	6.21	0.24	–	1.42	1.45	1.42	1.38	1.47
5	7.17	7.62	8.70	8.72	–	0.44	0.22	1.45	1.45
6	6.77	7.18	8.92	8.94	0.42	–	0.38	1.46	1.46
7	6.86	7.29	8.71	8.73	0.21	0.21	–	1.45	1.46
8	9.53	9.00	9.68	9.46	9.53	9.79	9.53	–	0.98
9	9.70	9.16	10.13	9.93	9.22	9.50	9.44	3.24	–

mtDNA sequence variation

We obtained 300 base pairs of sequence from the 5′ end of the mitochondrial DNA cytochrome *b* gene from North Atlantic capelin (clade 'A' of Dodson *et al.* 1991), and for single specimens of each of the major clades or geographic groupings within *mordax*, *dentex*, and *eperlanus* (Fig. 3). No base differences were observed between individuals representing the major groupings within each regional form of *Osmerus* in this region of cytochrome *b*. An average of 42 nucleotide positions varied between *Osmerus* and *Mallotus* (Table 5). Most differences were transitions (82%) with purine–purine differences being more common than changes between pyrimidines. Transversions accounted for the remaining 18% of the differences between the two genera. Sequence divergences in cytochrome *b* between smelt and capelin averaged 15.2 ± 0.35% (SE) and were similar when using Kimura's two-parameter or maximum likelihood algorithms. Cyto-

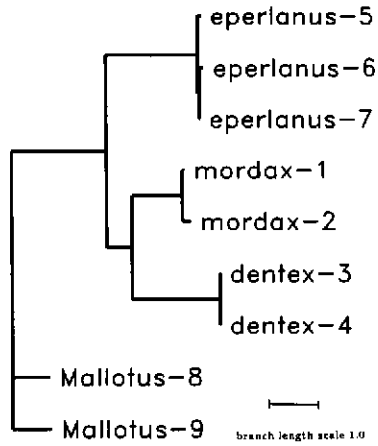


Fig. 2 Neighbor-Joining phenogram of genetic affinities among regional forms of *Osmerus* and *Mallotus*. Affinities were based on estimates of percentage sequence divergence inferred from restriction fragment length polymorphisms among *O. mordax*, *O. dentex*, *O. eperlanus*, and *M. villosus*. Numbers at terminal branches denote mtDNA composite genotypes defined in Table 2 and the phenogram is rooted at *Mallotus*–9.

chrome *b* sequence divergence between the genera, however, were considerably higher than estimates based on restriction fragment polymorphisms (Table 4), a situation commonly observed and one which might be attributable to restriction analysis missing multiple substitutions occurring at the variable sites (Beckenbach 1991; Geffen *et al.* 1992) or the reduced confidence in fragment sharing approaches as divergence levels increase (Swofford & Olsen 1990).

An average of about 21 (total = 33) nucleotide differences were observed between each pair of *Osmerus* sequence comparisons; all were transitions except for three tranversions between *mordax* and each of *eperlanus* and *dentex* (Fig. 3). All but three of the 33 total nucleotide differences were third position changes and no amino acid replacements were observed. Average sequence divergence (both for Kimura two-parameter and maximum likelihood estimates) among forms was 7.8 ± 0.6% (Table 5) which was slightly higher than our estimates from restriction fragment comparisons across the whole mtDNA genome. Neighbor-Joining and UPGMA analyses of the cytochrome *b* sequence divergences produced topologies identical to that from restriction fragments; *dentex* and *mordax* were more similar to each other than either was to *eperlanus* (Fig. 4). Confidence in this topology ranged from 79% bootstrap support (based on UPGMA analyses) to 52% support (Neighbor-Joining) For both sets of bootstrap analysis, the alternative topology of monophyly of *eperlanus* and *mordax* was supported in only 6% (UPGMA) to 22% (Neighbor-Joining) of the analy-

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Mallotus villosus   TTGGTTCTCTTCTTGGGCTTTGCCTCATTATTCAGATTCTTACAGGCCTA   50
Osmerus eperlanus   ....C..C.............T.............C..C......T..   50
O. dentex           .C..C..C......A....C..T.............C..C......C..   50
O. mordax           .T..C..C......A....C..C..A..........C..C......C..   50
                     -           -   -  -  -                       -

M. villosus         TTTCTGGCCATACATTACACTGCCGAGACTGCCACAGCATTCTCCTCCGT  100
O. eperlanus        ..C.....T.....C...........A.............T........  100
O. dentex           ..C..A..C..G..C...........G...........G..C........  100
O. mordax           ..C..A..T..A..C..T........G...........C..C........  100
                        -  -  -     -        -           -  -

M. villosus         GGTACACCTATGCCGTGACGTAAATTATGGCTGAATAATTCGAAACATGC  150
O. eperlanus        C..G.....C..T..G.....C..C..C......C....C..G.....A.  150
O. dentex           C..A.....C..C..A..T..C..C..C..T...C....T..G.....G.  150
O. mordax           C..A.....C..C..A..T..C..C..C..C...T....T..G.....G.  150
                      -        -  -  -           -  -    -        -

M. villosus         ATGCTAACGGAGCATCCTTCTTCTTTATTTGTATTTACCTCCATATCGGC  200
O. eperlanus        ............................C.........T..T.....T...  200
O. dentex           .C..........................C.........C..T.....T...  200
O. mordax           .C..........................C.........T..T.....T...  200
                     -                                     -

M. villosus         CGAGGGCTTTACTACGGCTCTTTCCTTTATAAAGAGACTTGAACCGTCGG  250
O. eperlanus        ..G..T.......................C..G..A..C....A.A.C..  250
O. dentex           ..G..T.......................C..G..A..C....A.A.T..  250
O. mordax           ..A..G..C....................C..A..A..C....A.A.C..  250
                      -  -  -                       -           -

M. villosus         CGTGGTCCTTCTCCTGCTAGTTATGATGACTGCCTTTGTAGGCTATGTCC  300
O. eperlanus        ...A..T........TT....C..A..A.........C............  300
O. dentex           ...A..C........CT....T..G..A.........C............  300
O. mordax           ...A..T..C.....TT....T..A..A.........C............  300
                        -  -      -     -  -
```

Fig. 3 Sequence of 300 base pairs from the 5′ end of cytochrome *b* for individuals representing the major clades or geographic groupings from *Mallotus villosus*, *Osmerus mordax*, *O. dentex*, and *O. eperlanus*. The 33 variable positions in the *Osmerus* sequences are underlined. Nucleotide positions indicated by a '.' are identical to that on the first line (*Mallotus* sequence).

Table 5 Percentage sequence divergences derived from 300 base pairs of cytochrome *b* (Kimura two-parameter *p* (lower portion of matrix)) and the number of nucleotide differences observed (upper portion of matrix) among *Mallotus* (*M.*), *Osmerus eperlanus* (*O.e.*), *O. dentex* (*O.d.*) and *O. mordax* (*O.m.*). No sequence differences were observed between individuals representing composite restriction fragment genotypes 1–2, 3–4, or 5–7 (Table 2) therefore, only one divergence value for cytochrome *b* sequences is presented for each pairwise comparison

Taxon	*M.*	*O.e.*	*O.d.*	*O.m.*
Mallotus	–	44	41	41
O. eperlanus	15.8	–	23	24
O. dentex	14.7	8.4	–	19
O. mordax	15.1	8.3	6.6	–

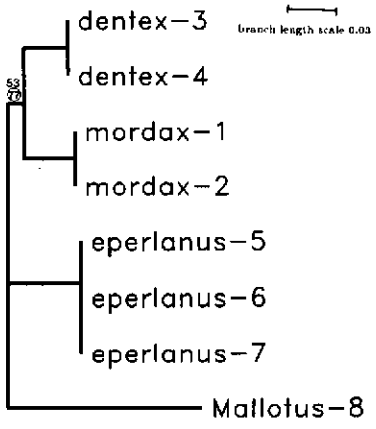

Fig. 4 Neighbor-Joining phenogram of relationships among *Osmerus* geographic forms and the outgroup *Mallotus* constructed from a matrix of Kimura's *p* measure of sequence divergence (%) inferred from variation among taxa in 300 base pairs of cytochrome *b* sequence. The number at the branch point between *O. dentex* and *O. mordax* indicates the percentage of bootstrap replicates that supported their monophyly from 1000 replications of the distance/Neighbor-Joining analyses (UPGMA bootstrap value is encircled). Numbers at terminal branches denote mtDNA composite genotypes defined in Table 2 whose cytochrome *b* sequences were identical within forms.

ses. Maximum parsimony analysis resolved five equally parsimonious trees each of which required 72 substitutions to explain relationships among the four distinct sequences. Nineteen steps separated *mordax* and *dentex* sequences, 24 *mordax* and *eperlanus*, and 23 separated *dentex* and *eperlanus*. The consensus phylogeny (Fig. 5) also identified a closer affinity between *mordax* and *dentex* than between either of these species and *eperlanus*. Bootstrap analysis of the sequences ($N = 500$ replications), however, indicated only weak support for monophyly of *dentex* and *mordax* (43%, Fig. 5), but as in the distance-based bootstrap analyses, monophyly between *eperlanus* and *mordax* received even lower support (26%).

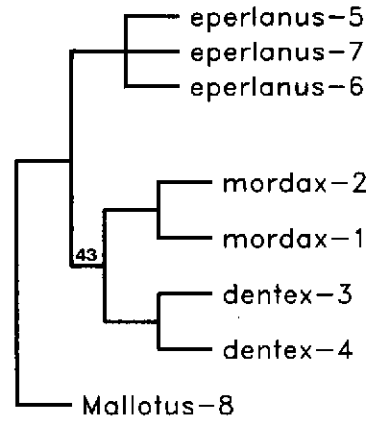

Fig. 5 Majority-rule consensus phylogenetic tree of relationships among *Osmerus* geographic forms and the outgroup *Mallotus* constructed using maximum parsimony criterion to organize variation observed in 300 base pairs of cytochrome *b* sequence. The number at the branch point between *O. dentex* and *O. mordax* indicates the percentage of bootstrap replicates that supported their monophyly from 500 replications of the parsimony analysis. Numbers at terminal branch points denote mtDNA composite genotype defined in Table 2. Sequences were identical within each *Osmerus*.

Discussion

mtDNA divergence in smelt and the trans-Arctic exchange

Our estimates of among-*Osmerus* mtDNA sequence divergence averaged about 7 6% (Tables 4 and 5). A 'conventional' nucleotide divergence rate between vertebrate mtDNA genomes has been estimated at about 2% per million years (p.m.y.) in a variety of taxa (e.g. Brown 1983; Wilson *et al.* 1985; Shields & Wilson, 1987) and has been widely used to approximate lineage separation times in fishes (e.g. Bermingham & Avise 1986; Bernatchez *et al.* 1991; Bernatchez & Dodson 1991; Billington *et al.* 1990). More recently, Ortí *et al.* (1994) provided an approximate fossil-based calibration for cytochrome *b* sequence divergence in the Gasterosteidae of about 2.8% per million years between genomes, reasonably close to the whole molecule 'conventional' rate. The lack of an *Osmerus*-specific calibration of mtDNA divergence rate and the uncertainty concerning the applicability of a general rate of mtDNA evolution in cold-blooded vertebrates, including fishes (cf. Hillis & Moritz 1990; Avise *et al.* 1992; Martin *et al.* 1992) make estimates of lineage separation times in *Osmerus* based on the conventional mtDNA clock provisional. Nevertheless, we estimated divergence of *dentex* and *mordax* at 2.8 million years ago (Ma), *mordax* and *eperlanus* at 3.4 Ma, and *dentex* and *eperlanus* at 4.3 Ma based on the restriction fragment divergence estimates (Table 6). Our cytochrome *b* observed divergences and the 2.8% rate of divergence p.m.y. cited

	Species comparison		
	O.m. vs. *O.d.*	*O.m.* vs. *O.e.*	*O.d.* vs. *O.e.*
Nei's *D*	0.48*	0.17*–0.39†	0.58*
Estimated divergence time (Ma)	2.4–9.1	0.87–3.3, 1.9–7.4	2.9–11.0
mtDNA fragment *d* (%)	5.7	6.8	8.6
Estimated divergence time (Ma)	2.8	3.4	4.3
cytochrome *b* sequence *p* (%)	6.6	8.4	8.3
Estimated divergence time (Ma)	2.4	3.0	2.9

*From Luey *et al.* (1982).
†From Vuorinen *et al.* (1991)

Table 6 Estimates of species divergence times (in millions of years before present, mybp) based on allozyme and mtDNA molecular 'clocks'. Estimates of divergences based on allozymes are ranges representing rates of 5 (Nei 1987) and 19 (Vawter *et al.* 1980) mybp per unit of Nei's genetic distance, *D*. mtDNA estimates are based on a rate of 2% sequence divergence per million years (pmy; Brown 1983) for whole molecule divergence (restriction fragments estimates) or 2.8% pmy (cytochrome *b* divergence; Ortí *et al.* 1994). *Osmerus eperlanus* (*O.e.*), *O. dentex* (*O.d.*) and *O. mordax* (*O.m.*)

by Ortí *et al.* (1994) yields separation dates of 2.5 Ma between *mordax* and *dentex* and about 3.5 Ma between the common ancestor of these species and that of *eperlanus* (Table 6). Our estimates suggest that divergence of *dentex* and *mordax* from a common ancestor occurred during the early Pleistocene, perhaps associated with glacial advances or by cooling trans-Arctic water temperatures (McAllister 1963; Briggs 1970; Grant 1987), and divergence between the common ancestor of these taxa and *eperlanus* occurred somewhat earlier near the end of the Pliocene. These estimates of lineage separation dates are broadly consistent with time frames associated with episodes of faunal exchange and isolation occurring in Beringia and the trans-Arctic region between the north Pacific and Atlantic (Briggs 1970; Grant 1987) and suggest that such events were important to radiation within *Osmerus*. Divergence times between *Mallotus* and *Osmerus* were estimated at between 4.0 (restriction fragments) and 5.4 (cytochrome *b*) Ma consistent with divergence between the genera within the mid-early Pliocene.

Two further arguments can be cited that support a Pliocene–Pleistocene time frame for *Osmerus* radiation. First, a Pacific origin for Osmeridae is strongly suggested by the presence of all six genera in the north Pacific, while only *Mallotus* and *Osmerus* also inhabit the north Atlantic (McAllister 1963; Berra 1981). Further, the north Pacific and Atlantic oceans were isolated from one another from the late Mesozoic (200 Ma) until the late Cainozoic when two periods of trans-Arctic exchange occurred. The first took place about 10–12 Ma during a brief Bering Seaway connection between the two ocean basins (Briggs 1970). The second occurred during the most recent Bering Seaway opening some 3–5 Ma (Briggs 1970). During both periods of trans-Arctic connection, there was extensive faunal exchange between the north Pacific and Atlantic oceans, particularly from the Pacific to the Atlantic (Briggs 1970; Herman & Hopkins 1980; Vermeij 1991). Assuming a Pacific origin of osmerids and given the geological constraint of isolation between ocean basins, Atlantic forms of *Osmerus* (or *Mallotus*) could not therefore have evolved (or colonized) earlier than the late-Miocene when the Bering Strait was first formed.

Secondly, our mtDNA sequence estimates of divergence times are within the range of estimates produced from allozymes which also suggest radiation of *Osmerus* beginning no earlier than the mid-Pliocene (Table 6). Further, average divergence time for north Atlantic and north Pacific herring (*Clupea harengus* and *C. pallasi*), cod (*Gadus morhua* and *G. macrocephalus*), and halibut (*Hippoglossus hippoglossus* and *H. stenolepis*) was estimated at 4.9 million years by Grant (1987) compared to the average mtDNA estimate of divergence between Pacific and Atlantic *Osmerus* at 3.7 million years (Table 6). The concordant patterns of divergence between species within these varied north temperate genera, including *Osmerus*, provide strong evidence for the importance of vicariant events (specifically those occurring in Beringia) to the diversification and phylogenetic history of Holarctic faunas (Durham & MacNeil 1964; Briggs 1970; Grant 1987).

The substantial levels of genetic divergence observed among the regional forms of *Osmerus* (and between most other north Pacific and Atlantic congeneric species and conspecific populations of fish, invertebrates, and seaweeds, e.g. Grant 1987; MacDonald & Koehn 1988; Bot *et al.* 1989) is strikingly different from the high degree of mtDNA sequence similarity observed between Pacific and Atlantic populations of the sea urchin *Strongylocentrotus droebachiensis* (Palumbi & Kessing 1991). Genetic similarity between trans-Arctic populations of *S. droebachiensis* was suggested to result from current or very recent gene flow between oceanic populations which might stem from a greater tolerance of cold Arctic water temperatures by urchin larvae relative to fish (Palumbi & Kessing 1991). North-eastern North American smelt are limited in their distribution to points south of approximately 54–55°N latitude and larvae from some populations are reported to avoid water temperatures below 2 °C (Nellbring 1989). By contrast, there are reports of urchin adults in Arctic waters of Canada and of development of larvae at temperatures as low as 0 °C (see Palumbi & Kessing 1991). These observations illustrate how life-history parameters may interact with historic

geological events to shape extant patterns of genetic diversity and structure in different taxa inhabiting a similar geographic range.

Evolutionary isolation among regional forms of smelt

Our results indicate that there is substantial genetic differentiation among the three geographic forms of *Osmerus*, in spite of their general morphological similarity (McAllister, 1963). For instance, although we used only a subset of composite genotypes in our phenetic and parsimony analyses, each form (north Pacific, eastern North American, and European) was characterized by a unique assemblage of composite genotypes resolved in our study and by Taylor & Bentzen (1993a). In fact, only a single genotype was shared among the three forms; one of 29 North Pacific *Osmerus* had a *Pvu*II genotype characteristic of all European smelt.

Furthermore, the inter-*Osmerus* sequence divergences of 5–9% documented in our study are in broad agreement with values documented for congeneric species in a variety of animal taxa. Restriction enzyme and sequence analyses of mtDNA have produced intrageneric divergence estimates ranging from 3.7 in some primates to 16.2% in rodents, 6.0–11.3% in echinoids (Vawter & Brown 1986), 4.4–16.7% in decapods (Brasher *et al.* 1992), and from 1.2 to 25% in various fish genera (Avise & Saunders 1984; Becker *et al.* 1988; Bentzen 1988; Bernatchez *et al.* 1991; Billington *et al.* 1990; Grewe *et al.* 1990; Ovenden *et al.* 1988; Thomas *et al.* 1986). By contrast, intraspecific mtDNA sequence typically averages below 1% (cf. Ferris & Berg 1987; Becker *et al.* 1988; Billington & Hebert 1991) as was observed within each *Osmerus* (Table 4).

Morphological differences among *Osmerus* are comparatively minor except for a single, nonclinal meristic trait (pored lateral line scales) that suggests long term isolation between European smelt and the two North American forms (McAllister 1963). The available allozyme evidence also suggests long-term evolutionary isolation among forms. Luey *et al.* (1982) examined 13 loci and reported genetic distances between north Pacific smelt and the two Atlantic forms that were similar to interspecific comparisons in other taxa, but a much lower distance between the two Atlantic forms. Vuorinen *et al.* (1991), however, assayed allozyme variation at 15 loci between *mordax* and *eperlanus* and documented a genetic distance between these forms 2.4 times greater than that reported by Luey *et al.* (1982) The higher of the two estimates of allozyme divergence between *mordax* and *eperlanus* is probably more reliable because: (i) Vuorinen *et al.* (1991) sampled a greater range of populations, (ii) Luey *et al.* (1982) examined only 3–8 individuals per population, and (iii) Luey *et al.* (1982) examined an introduced population of *mordax* which probably did not adequately sample genetic variation within this form (cf. Schreiner *et al.* 1984).

Taken together, our mtDNA results and those based on allozymes provide concordant documentation in independent genetic systems of substantial divergence among all three *Osmerus* that is probably the result of millions of years of evolutionary isolation among them (Brown 1983; Nei 1987; Meyer 1993). Genetic distances inferred from variation at enzyme-coding loci and mtDNA cannot, however, readily be used in making taxonomic decisions particularly for allopatrically distributed forms (Templeton 1980; Futyuma 1986). Nevertheless, the substantial mtDNA and allozyme divergence among the three forms and low mtDNA divergence within regional forms, patterns of genetic variation similar to interspecies comparisons in a wide variety of taxa, are at least compatible with the probable status of the forms as evolutionarily independent lineages (i.e. species) whether they are recognized taxonomically as such or not.

It should also be noted, however, that Taylor & Bentzen (1993a,b) described two lakes in north-eastern North America containing sympatric populations of smelt that are morphologically, ecologically, and genetically distinct. These sympatric reproductively isolated populations, therefore appear to be acting as good biological species, yet in both lakes the forms are separated by no more than 0.17% net sequence divergence between their mtDNAs (Taylor & Bentzen 1993b). It is therefore possible that valid biological species may exist at low levels of genetic divergence within each of the regional forms of *Osmerus*.

Substantial mtDNA and allozyme divergence among regional forms of *Osmerus* stands in stark contrast with their general morphological and meristic similarity (McAllister 1963). High levels of mtDNA sequence divergence accompanied by apparent morphological stasis was recently documented for a lineage of African cichlids (*Tropheus*) in Lake Tanganyika by Sturmbauer & Meyer (1992). These authors suggested that the morphological similarity among genetically divergent cichlids may have resulted from stabilizing selection for similar phenotypes in 'tightly packed communities'. The morphological similarity among genetically distinct regional forms of *Osmerus* might also result from parallel morphological evolution imposed within similar environmental and life-history selective regimes. For instance, the three geographic forms of *Osmerus* have a similar range of environmental tolerances and life-history patterns. In the north Pacific, eastern North America, and in Europe, smelt are pelagic carnivores feeding on zooplankton and small fishes in freshwater, estuarine, and near-shore marine environments (Nellbring 1989). Furthermore, overall life-history similarity among regional forms of *Osmerus* is suggested by parallel similarities in within-form mtDNA divergence. Sequence divergence within-forms is low

and population genetic structure within regions appears to be organized and less by geographic proximity among component populations than by life-history differences within forms (Fig. 1; Baby *et al.* 1991; Taylor & Bentzen 1993a).

Zoogeography and evolution of the species complex

Our data on mtDNA variation and relationships within *Osmerus* coupled with information on genetic differences between other north Pacific and Atlantic fishes tend to support McAllister's (1963) scenario of the evolutionary and zoogeographic origin of the species complex. For instance, our distance-based molecular phenograms suggest that north Pacific and eastern North American smelt shared a common ancestor more recently than did eastern North American and European smelt (Figs 2 and 4). Qualitatively, our phylogeny is strongly supported by the stability of the branch points during all jacknifed replicates of the Neighbor-Joining tree based on shared restriction fragment data, and received from strong (UPGMA) to majority (Neighbor-Joining) bootstrap support in the cytochrome *b* sequence divergence phenograms (Fig. 4). The parsimony-based analysis produced the same topology as in the distance-based analyses, but bootstrap replication of the data failed to provide statistical substantiation for closer affinity between *mordax* and *dentex* relative to *eperlanus*. Although further sequence data may stabilize the parsimony-based topology, it is also possible that the lack of resolution in the parsimony analysis results from rapid speciation within *Osmerus*. For all trees, the lengths of internodal branches are short relative to terminal branch lengths (Figs 2 and 4). Furthermore, sequence divergences, based on fragment sharing or sequence data, are quite similar among the three species (Tables 4 and 5). Short internodal branch lengths and similar sequence divergences among taxa have been associated with rapid phyletic radiation within a genus of pocket gophers *Orthogeomys*, and in four families of Pecoran ruminants where intrageneric and interfamilial relationships, respectively, remained largely unresolved (Kraus & Miyamoto 1991; Sudman & Hafner 1992).

Our mtDNA distance-based topology from fragment and sequence analyses (Figs 2 and 4) conflicts with that produced from allozymes by Luey *et al.* (1982) which suggested most recent ancestry between *mordax* and *eperlanus*. For the reasons discussed above, however, we feel that genetic variation within each *Osmerus*, and in particular the eastern North American form, was not adequately sampled by Luey *et al.* (1982). Furthermore, the allozyme survey of Vuorinen *et al.* (1991) did not include the north Pacific form. In summary, our mtDNA data represent the most thorough survey of genetic variation in *Osmerus*, both within and between regional forms, and an assessment of concordance between our topology and that based on nuclear genes must await further allozyme or nuclear DNA studies.

Morphological and meristic variation among regional forms of *Osmerus* is consistent with our hypothesized relationships based on mtDNA. Notwithstanding morphological and meristic overlap among the three regional forms, nonclinal variation in the number of pored lateral line scales suggests closer affinity between *mordax* and *dentex*, but marked differentiation between the two North American forms and European *eperlanus* (McAllister 1963; Copeman 1977).

Finally, the more recent divergence between *dentex* and *mordax* is supported by mtDNA phylogenies between Atlantic basin forms in other fishes. Avise *et al.* (1986) reported mtDNA sequence divergence between American and European forms of eel (*Anguilla*) at 3.7%. North-western and north-eastern Atlantic capelin mtDNA sequences are distinct at 3.4% (Dodson *et al.* 1991). The two north Atlantic groupings both within *Mallotus* and *Anguilla* may have diverged during cooling periods associated with Pleistocene glaciations (Avise *et al.* 1990; Dodson *et al.* 1991). MtDNA sequence divergence between Atlantic forms of *Osmerus*, however, is about twice that reported between north-western and north-eastern Atlantic populations in capelin and eel (Tables 4 and 5). The discordant level of divergence between Atlantic forms of smelt relative to capelin and eel suggests that fundamentally different phylogenetic and zoogeographic events have influenced divergence in Atlantic basin *Osmerus*, i.e. eastern North American and European smelt, have probably had independent origins from a north Pacific ancestor (cf. McAllister 1963).

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