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Phylogeography of 3 North Atlantic Wolffish species (*Anarhichas* spp.) with Phylogenetic Relationships within the Family Anarhichadidae

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Abstract

Phylogenetic analyses of all 4 wolffish species (Atlantic, Spotted, Northern, and Bering wolffishes) and the Wolfeel were assessed with both mitochondrial (D-loop and ND1) and nuclear (amplified fragment length polymorphism) DNA to resolve relationships within the family Anarhichadidae. Species-specific mitochondrial DNA (mtDNA) mutation rates were estimated based on 2 possible dates of divergence between the Pacific and Atlantic lineages. Phylogeographic patterns within each of the 3 North Atlantic wolffishes were investigated with Markov chain Monte Carlo simulations based on mtDNA to determine whether population size changes occurred following the last glaciation and where wolffishes likely survived glaciation. All 3 species of North Atlantic wolffishes showed evidence of postglacial expansion but did not show evidence of persistence in multiple refugia in both the eastern and western Atlantic Ocean. Rather, the data supported persistence in a single refuge, with postglacial expansion into the rest of the range. Nucleotide diversity, in particular, was low in wolffishes compared with other marine fishes, possibly related to reductions in population sizes during the last glaciation.

Key words: Anarhichas lupus, *denticulatus*, *minor*, *phylogeny*, *phylogeography*

Pleistocene glaciations are known to have had a profound effect on species distributions as well as on patterns of intraspecific genetic variation (Bernatchez and Wilson 1998; Hewitt 2000). During the most recent glaciation, ice reached its maximum extent between approximately 18–21 thousand years ago (ka) (CLIMAP 1976; Peltier 1994). Glacial ice reached as far south as 40°N in the western Atlantic and 50°N in the eastern Atlantic, with ice sheets extending well into the marine environment on both coasts (CLIMAP 1976; Peltier 1994). The combination of the greater extent of ice sheets and more compressed isotherms in the western Atlantic made this region less hospitable than the eastern Atlantic to many North Atlantic marine species during the last glaciation (Wares and Cunningham 2001). Nevertheless, a western Atlantic glacial refuge likely existed (Peltier 1994; Shaw et al. 2006), with suitable habitat for anadromous and marine fishes (Bigg et al. 2008). A growing number of marine species have revealed evidence of having survived glaciation on both the eastern and western coasts of the North Atlantic Ocean. This includes primarily near-shore species with limited dispersal potential (Bernatchez and Dodson 1991; Brunner et al. 2001; Wares and Cunningham 2001; Hickerson and Cunningham 2006; Dodson et al. 2007; Makinen and Merila 2008) but also includes Atlantic cod, *Gadus morhua*, which inhabits continental shelves (Bigg et al. 2008).

The Atlantic, Spotted, and Northern wolffishes (*Anarhichas lupus* Linnaeus, 1758, *A. minor* Olafsen, 1772, and *A. denticulatus* Krøyer, 1845, respectively) are found across the North Atlantic Ocean, with parts of their ranges extending into the Arctic (Scott WB and Scott MB 1988). The Bering wolffish (*Anarhichas orientalis* Pallas 1814) is the sole Pacific representative of the genus *Anarhichas*. Wolffishes and the Wolfeel (*Anarrhichthys ocellatus* Ayres 1855), also found in the Pacific Ocean, comprise the family Anarhichadidae (Scott WB and Scott MB 1988). Wolffishes are known for their sedentary behavior and limited larval dispersal and thus represent a life-history conducive to phylogeographic analysis. Atlantic wolffish, the best studied of the 3 North Atlantic species, builds nests on the hard substrate of continental shelves and males guard the eggs for up to 8 or 9 months (Pavlov and Novikov 1993). Tagging

studies of adults from all 3 species have shown that most migrations occur over short distances. Off Newfoundland, most Atlantic wolffish were recaptured <10 km from the tagging site after 5–7 years (Templeman 1984). Spotted wolffish were recaptured <30 km from the tagging site off West Greenland (Riget and Messtorff 1988), and <100 km from the tagging site in the Barents Sea (Ostvedt 1963). Nevertheless, occasional long-distance migrations (100 s of km) have been reported for all 3 species (Ostvedt 1963; Jønsson 1982; Templeman 1984; Riget 1986; Riget and Messtorff 1988).

Although wolffishes are not of major commercial importance, they have been negatively affected by commercial fisheries directed toward other species. Their sedentary nature and nest-building behavior have made them vulnerable to habitat degradation by bottom trawlers as well as by-catch (Collie et al. 2000; O'Dea and Haedrich 2000). The Committee on the Status of Endangered Wildlife in Canada has assessed these species with respect to probability of extinction as either "threatened" (Northern and Spotted Wolffish) or "special concern" (Atlantic Wolffish), and all 3 species are currently listed under the Canadian Species at Risk Act.

We have 3 main study objectives that we will present in the order in which they will be addressed. 1) We will assess phylogenetic relationships among the 5 extant species in the family Anarhichadidae. Relationships among the Atlantic, Spotted, and Northern wolffishes have been assessed with whole mitochondrial genomic analysis (Johnstone et al. 2007); however, this analysis included only one individual per species and no samples from the Bering wolffish or Wolfeel. We will evaluate phylogenetic relationships with both mitochondrial DNA (mtDNA) and amplified fragment length polymorphism (AFLP) to assess corroboration between the 2 marker types. Although AFLP data have only recently been applied to phylogenetic questions, this marker has proved effective, particularly for recently derived species (Mendelson and Shaw 2005a, 2005b; Altoff et al. 2007; Koopman et al. 2008). 2) We will assess phylogeographic patterns within the 3 North Atlantic wolffishes using mtDNA to determine if a) they show evidence of postglacial population expansion and b) they show evidence of having survived glaciation in 2 proposed refugia (in the eastern and western Atlantic Ocean). 3) We will place the findings of this research in a conservation framework. If deep phylogeographic divergences are found, this will be of importance for the conservation and management of these species (Crandall et al. 2000; Waples 2006).

Materials and Methods

Wolffish samples were collected with trawl nets or longlines, with the exception of North Sea samples which were taken from a fish market in Aberdeen and the Wolfeel which is from the Vancouver Aquarium (Figure 1, Table 1). DNA was extracted with either a glassmilk protocol (Elphinstone et al. 2003) or Qiagen DNeasy extraction kits. The D-loop

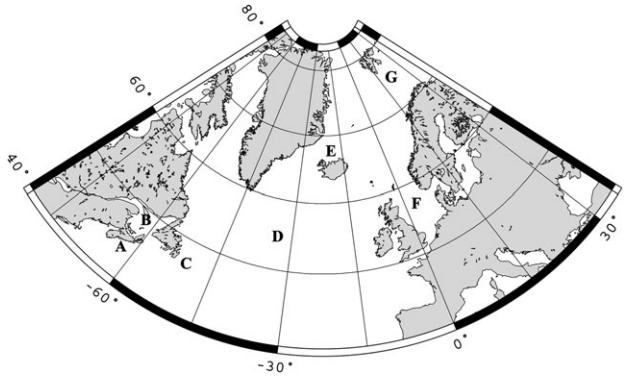

Figure 1. Sample sites for Wolffishes across the North Atlantic (see Table 1 for location names and sample sizes).

and ND1 regions were amplified with standard polymerase chain reaction (PCR) (10 μl volume), containing 1× ThermoPol buffer (10 mM Tris–HCl, pH 8.3; 50 mM KCl), 20–100 ng DNA, 2.0 mM $MgCl_2$, 50 μM each dNTP, 0.5 U *Taq* DNA polymerase (New England Biolabs), and 0.3–0.5 μM each primer. We developed primers from conserved regions in related species (see Supplementary material). Thermal cycling conditions for D-loop were as follows: 96 °C 1 min; 30 cycles of 95 °C 30 s, 60 °C 30 s, and 72 °C 1 min; 72 °C 5 min, and 4 °C hold. Conditions were identical for ND1, with the exception of a 58 °C annealing temperature. PCR products were purified with Omega 10 filter plates. Sequencing PCR was performed with dye terminator cycle sequencing kits (Beckman-Coulter) following manufacturer's instructions with a combination of the original primers and internal primers, developed after examining initial sequencing results. Samples were resuspended in 40 μl deionized formamide and sequenced with a Beckman-Coulter CEQ 8000. For the majority of individuals, both sense and antisense sequences were produced in order to verify results. Sequences were compiled with Sequencher v 4.2 (Gene Codes Corporation), and all unique D-loop and ND1 sequences were deposited in GenBank (accession numbers: EU095868–EU095896 for D-loop, EU095897–EU095934 for ND1 sequences).

AFLP analyses were performed on 2 individuals from each of Atlantic, Spotted, and Northern wolffishes, as well as one Bering wolffish and one Wolfeel. We followed methods outlined by Vos et al. (1995), as modified by Agresti et al. (2000), with *Xba*1 rather than *Eco*R1 as the rare cutter. Selective amplification was performed with all combinations of *Eco*R1-AGA, -AGC, -ATA, -ATC and Xba-GGA, -GGC, -GT, -GC, resulting in 16 primer combinations. PCR products were imaged with 6% Sequagel on an LI-COR system, and bands were scored to approximately 300 bp with Saga2 software (LI-COR Biosciences, Lincoln, Nebraska). Scoring errors were evaluated, following Bonin et al. (2007), with a blind duplicate of the Bering Wolffish.

Composite haplotypes were aligned in Clustal X (Thompson et al. 1997) with the slow-accurate alignment method, and a 15-point gap penalty. Phylogenetic

Table 1 Sample locations and sizes taken of species in the family Anarhichadidae across the North Atlantic Ocean

Map	Location	*n*	Gene diversity (SD)	Nucleotide diversity (SD)
Atlantic Wolffish				
A	Nova Scotia	18	0.63 (0.07)	0.00041 (0.00035)
B	Gulf of St. Lawrence	15	0.68 (0.10)	0.00045 (0.00038)
C	Newfoundland	15	0.91 (0.06)	0.00107 (0.00072)
E	Iceland	20	0.87 (0.05)	0.00094 (0.00064)
F	North Sea	23	0.89 (0.05)	0.00120 (0.00077)
G	Barents Sea	16	0.98 (0.03)	0.00160 (0.00099)
	Overall	107	0.87 (0.02)	0.00102 (0.00065)
Spotted Wolffish				
C	Newfoundland	21	0.54 (0.11)	0.00060 (0.00045)
E	Iceland	16	0.81 (0.06)	0.00068 (0.00051)
G	Barents Sea	17	0.79 (0.08)	0.00105 (0.00070)
	Overall	54	0.74 (0.05)	0.00078 (0.00054)
Northern Wolffish				
A,C	Atlantic Canada	24	0.66 (0.09)	0.00051 (0.00040)
D	Mid-Atlantic Ridge	14	0.49 (0.09)	0.00027 (0.00028)
E	Iceland	19	0.75 (0.09)	0.00057 (0.00044)
G	Barents Sea	16	0.67 (0.11)	0.00068 (0.00051)
	Overall	73	0.65 (0.05)	0.00051 (0.00036)

Gene diversity and nucleotide diversity are presented for each sample locality.

relationships among all mtDNA composite haplotypes were evaluated with parsimony and Bayesian analyses. Parsimony analysis was performed in PAUP 4.0 (Swofford 2002) with a heuristic search, tree bisection-reconnection branch swapping, treating gaps as a fifth base. Support for nodes was evaluated with 10 000 bootstrap replicates. Bayesian analyses were implemented in MrBayes (Ronquist and Huelsenbeck 2003). Sequences were partitioned by region (D-loop and ND1) and codon position (ND1). The evolutionary models chosen with Akaike information criterion in Modeltest (Posada and Crandall 1998) were K81uf+I for D-loop, HKY for first and second codon positions in ND1, and TrN+G for the third codon position in ND1. These were incorporated into the Bayesian analysis as $N_{st} = 6$ for D-loop and ND1 third position codons and $N_{st} = 2$ for ND1 first and second position codons. Posterior probabilities from MrBayes were determined from 10 million generations of Markov chain Monte Carlo (MCMC) simulations, after discarding the first 2.5 million generations as burn-in. Two analyses were run simultaneously with 4 Markov chains; trees were sampled every thousand generations. The Wolfeel was designated as the out-group in phylogenetic analyses.

Speciation timing and lineage-specific mtDNA mutation rates were then assessed with a Bayesian MCMC analysis in BEAST v1.4.7 (Drummond and Rambaut 2007). The most common composite haplotype was chosen for each species and analyzed with the Yule process. Data were partitioned by region (D-loop and ND1) and analyzed with the relaxed log-normal clock. The most general evolutionary model, GTR+I+G was chosen in BEAST, rather than HKY. The first analysis assumed the divergence between the Pacific and Atlantic Ocean lineages dated back to the Trans-Arctic Interchange 3.5 million years ago (Ma) (standard deviation [SD] = 0.5, 95% confidence interval [CI] of 2.5–4.5 Ma) (referred to as "DIV3.5"), which is arguably the most likely divergence time for this genus (see Briggs 1970; Vermeij 1991; Marincovich and Gladenkov 1999, see Discussion for more details). However, given uncertainty surrounding this estimate, a second analysis was performed assuming an older divergence time of 6 Ma (SD = 1, 95% CI of 4–8 Ma) (referred to as "DIV6") (see Collins et al. 1996; Marincovich and Gladenkov 2001; Nikula et al. 2007; see Discussion for more details). Each analysis was run multiple times, with a minimum of 10^7 iterations each. Convergence was evaluated with effective sample size (ESS) values and Trace plots in Tracer v.1.4.1, as well as by comparing runs. Runs were combined with LogCombiner after removing the burn-in; mean heights were taken in TreeAnnotator v. 1.4.8; and trees were plotted in FigTree v.1.1.2.

Phylogenetic relationships were estimated with AFLP data to assess congruence between mtDNA and nuclear DNA. No evolutionary model for AFLP has been developed for a likelihood analysis and parsimony methods appear to be either too restrictive (e.g., Dollo parsimony, which permits derived characters to evolve only once) or inappropriate (e.g., Wagner parsimony which assigns an equal probability of gain or loss of a fragment) (Koopman 2005). Distance-based approaches, particular those based on shared presence rather than shared absence, may be the most appropriate methods for assessing species relationships with AFLP (see Mendelson and Shaw 2005a, 2005b). We evaluated relationships with Nei and Li's (1979) genetic distance method with neighbor joining, implemented in Phylip (Felsenstein 2005). Support for nodes was based on 10 000 bootstrap replicates.

Phylogeographic Patterns within Species

We addressed 2 main questions with respect to intraspecific variation. First, we tested for evidence of postglacial population size change, and second, we evaluated the

evidence that wolffishes survived in 2 refugia or a single refuge during the last glaciaion. In order to assess relationships among composite haplotypes, haplotype networks were constructed for each species in Network v.4.2.0.1 (http://www.fluxus-technology.com) based on maximum parsimony. Second, standard statistics were generated from Arlequin v.2.0 (Schneider et al. 2000), such as gene diversity, nucleotide diversity, and Tajima's *D*. Geographic trends in diversity were evaluated with a least squares linear regression of both gene diversity and nucleotide diversity on longitude in SYSTAT (v.11). As natural selection can obscure phylogeographic patterns, selection was evaluated (for ND1) by comparing synonymous and nonsynonymous substitutions within and among North Atlantic species with a McDonald–Kreitman test in DNAsp (Rozas et al. 2003).

To assess evidence of population size changes, Bayesian Skyline plots were analyzed in BEAST v1.4.7 (Drummond and Rambaut 2007). Sequences were partitioned by mtDNA region (CR vs. ND1), with the GTR+I+G mutation model. A minimum of 10^7 iterations were run, with a burn-in of 10^6, assuming a linear model of population size change. Species trajectories were evaluated with the lineage-specific mutation rates from BEAST (see above). Convergence was assessed by evaluating ESS values and Trace plots in Tracer v. 1.4.1, as well as by comparing runs. Two independent runs were combined in LogCombiner. Skyline plots were visualized in Tracer v. 1.4 (Rambaut and Drummond 2007), and model parameters were converted to demographic parameters (years and N_e). Generation time was taken to be 8, 7, and 5 years for Atlantic, Spotted, and Northern wolffishes, respectively (O'Dea and Haedrich 2000, 2001a, 2001b), which were used in the estimate of N_e.

To test for evidence of persistence in 2 refugia during the last glaciation versus a single refuge, we evaluated divergence times between eastern and western samples with the program Isolation with Migration (IM) (Hey and Nielsen 2004). The last interglacial period was approximately 135–115 ka (Hewitt 1996), therefore our a priori expectation was that a divergence time of >100 ka would provide support for the 2 refugia model, whereas an estimate of <20 ka would provide support for the single refuge model. Samples were divided into western Atlantic (Nova Scotia, Gulf of St. Lawrence, Newfoundland) and eastern Atlantic (Iceland, Barents Sea, North Sea) samples. Mid-Atlantic Ridge (MAR) samples, which could not be grouped with either "eastern" or "western" samples with certainty, were removed from this analysis. A geometric heating scheme was used with a minimum of 20 chains and 20 swap attempts per step, and consistency across multiple runs was evaluated. Stationarity and convergence of parameters were evaluated by assessing ESS values, output graphs to assess mixing, and consistency across multiple runs after a minimum of a 10^5 burn-in period and 10^6 iterations.

Results

In total, 855 bp were sequenced from the D-loop (including indels) and 975 bp from the ND1 region, producing a composite haplotype of 1830 bp (see Supplementary material for more details). Two unusual ND1 haplotypes were found in Spotted wolffish, one in the Newfoundland sample and one in the Barents Sea sample, which differed from the most common haplotype by 5 mutations. These 2 haplotypes still clearly clustered with Spotted wolffish in a phylogenetic analysis (although both were basal; Figure 2) and the translated amino acid sequence was identical to other Spotted wolffish haplotypes. Reextracting the individuals and resequencing the region did not change the result, therefore these haplotypes remained in all analyses unless otherwise indicated.

AFLP analyses revealed 1157 polymorphisms among the 5 species, with 819 occurring within the 4 wolffish species and 528 occurring within the North Atlantic wolffishes. Scores for the duplicated Bering wolffish sample differed at 3.2% of bands, which is in the range typically found for AFLP studies (Bonin et al. 2007).

Phylogenetic Relationships among Species

Phylogenetic analyses provided support for the monophyly of each species, the monophyly of North Atlantic wolffishes as a whole, and a sister species relationship for Atlantic and Spotted wolffishes (Figure 2). Parsimony analysis produced comparable results with Bayesian methods at the species level (see Supplementary material) but was not as well resolved at the intraspecific level (Figure 2). The intraspecific resolution among haplotypes from the Bayesian analysis produced similar results to the haplotype network analysis (see below). Therefore, the Bayesian phylogeny most likely accurately depicts evolutionary relationships among haplotypes even at the intraspecific level.

Based on the Trans-Arctic Interchange ~3.5 Ma, (DIV3.5), lineage-specific substitution rates ranged from 0.69–1.52 $\times$ 10^{-8} substitutions per site per year across species (Table 2). With an earlier divergence model, substitution rates ranged from 0.46–1.02 $\times$ 10^{-8} substitutions site year (Table 2). Both models placed the divergence time among North Atlantic species to be on the order of 1–2 Ma (Table 2). These lineage-specific substitution rates were used for all subsequent analyses. The substitution rate for D-loop was consistently estimated to be approximately half that of ND1 based on analyses in BEAST.

Phylogenetic analyses of AFLP data resulted in 100% bootstrap support for the 2 samples from each species being monophyletic. Phylogenetic relationships among species supported results from mtDNA, with North Atlantic species being monophyletic (100% bootstrap support) and Atlantic and Spotted wolffishes as sister species (69% bootstrap support; see Supplementary material).

Phylogeographic Patterns within Species

Haplotype networks produced star-like patterns for all species but virtually no phylogeographic patterns. Given similarity to Bayesian phylogenetic analysis (Figure 2), haplotypes networks were not presented in this manuscript

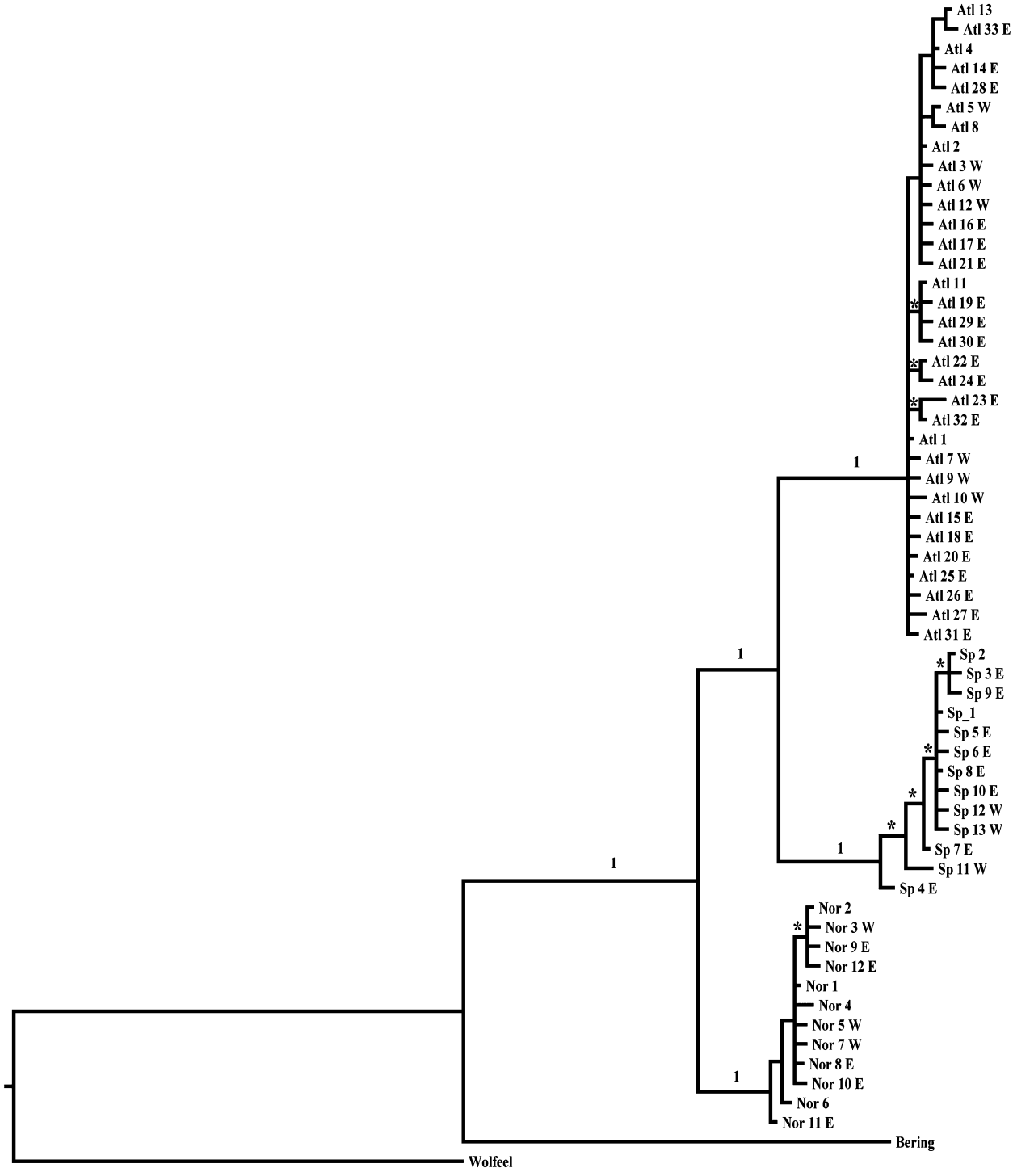

Figure 2. Phylogenetic analysis from MrBayes of composite haplotypes from Atlantic (Atl), Spotted (Sp), Northern (Nor), and Bering wolffishes, with Wolfeel as the out-group. Posterior probabilities for species-level relationships are indicated. Posterior probabilities greater than 0.9 are indicated by asterisks for intraspecific nodes. The sample location of haplotypes was indicated by an "E" (Eastern Atlantic), "W" (western Atlantic), or was left blank if the haplotype was found in both locations.

(but see Supplementary material). The most common haplotypes for each species were found on both coasts, although in general, more unique haplotypes were found in the eastern Atlantic compared with the western Atlantic samples. Atlantic Wolffish had the highest diversity of the 3 North Atlantic species (Table 1; Figure 2). In all 3 species, a general trend emerged of decreasing diversity from the eastern Atlantic to the western Atlantic, though this was

Table 2 Mutation rates (mutations per site per year) and divergence time estimates (Ma) based on the Yule process in BEAST

	DIV 3.5		DIV 6	
	Estimate	95% HPD	Estimate	95% HPD
Mutation rate				
Atlantic wolffish	1.52×10^{-8}	[0.02−3.21]	1.02×10^{-8}	[0.18−2.19]
Spotted wolffish	1.50×10^{-8}	[0.01−3.16]	1.01×10^{-8}	[0.18−2.19]
Northern wolffish	0.69×10^{-8}	[0.08−1.43]	0.46×10^{-8}	[0.05−0.97]
Divergence time				
Atlantic versus Spotted	0.83 Ma	[0.15−1.72]	1.37 Ma	[0.24−2.85]
Northern versus Atlantic/Spotted	1.34 Ma	[0.34−2.59]	2.21 Ma	[0.55−4.33]
Bering versus North Atlantic wolffishes	3.26 Ma	[2.25−4.29]	5.42 Ma	[3.22−7.49]
Wolfeel versus wolffishes	4.21 Ma	[2.11−7.24]	7.07 Ma	[3.07−12.34]

HPD, highest posterior density. Estimates are based on 2 estimates of divergence time between Pacific and Atlantic species: 3.5 Ma DIV3.5 and 6 Ma DIV6.

only significant for Atlantic Wolffish (relationship between nucleotide diversity and longitude was significant, $P = 0.015$, but not gene diversity, $P = 0.064$). Tajima's D values were negative and significant for Atlantic, Spotted, and Northern Wolffishes (−2.045, $P < 0.004$; −1.934, $P < 0.007$; –1.602, $P < 0.019$, respectively), suggestive of population expansion, although negative D values can also indicate natural selection. However, the McDonald–Kreitman test revealed no evidence of selection for ND1. Bayesian skyline plots revealed population expansion for all 3 species (Figure 3). The timing of expansion, derived from lineage-specific mutation rates, was consistent with a post-glacial time frame in all cases, with the exception of Northern wolffish, which had an estimated onset of expansion slightly prior to 20 ka (Figure 3).

Not all parameters in the IM analysis were well resolved; therefore, we presented results for divergence time only, which was the parameter of interest. In Atlantic wolffish, divergence time was estimated across 4 runs of 10^6 iterations, and we presented results for one of those runs with a divergence time comparable with the overall average (Figure 4). Atlantic wolffish was the only species for which the parameter "s," which estimates the proportion of the ancestral population that founded each of the 2 current populations, was well resolved. In this species, s was consistently low (<0.01) suggesting that a very small portion of the ancestral population founded the western Atlantic.

For Spotted wolffish, the 2 divergent haplotypes appeared to obscure results in IM. When all Spotted wolffish haplotypes were included, divergence time was estimated with multiple peaks. However, when the 2 outlier haplotypes were removed, posterior probability curves were defined and consistent. Therefore, we ran analyses without the 2 divergent haplotypes, reasoning that these potential holdovers from earlier glacial cycles obscured an otherwise clear signal of recent divergence in this species. As IM analyses took considerably less time for Spotted and Northern wolffishes, after assessing convergence across multiple runs of 10^6 iterations, a final run for each species was conducted with 10^7 iterations. Divergence time estimates for each species were based on the final run (Figure 4). In both cases, the final run was comparable with the overall average across runs. Divergence time estimates were relatively consistent across runs for all 3 species, with the greatest variance found in Northern wolffish (SD = 5 ka for Atlantic wolffish; SD = 1 ka for Spotted wolffish; SD = 13 ka for Northern wolffish). Likelihood curves showed that the probability density of preglacial divergence (100 ka or earlier) was generally low (Figure 4).

Discussion

Phylogenetic Relationships among Species and Mutation Rate Estimates

The opening of the Bering Strait in the late Cenozoic ended a period of isolation between Pacific and Atlantic marine taxa that had lasted for nearly 100 million years (Marincovich and Gladenkov 2001). The most widely cited date for the principal opening is 3.1–4.1 Ma (Briggs 1970; Vermeij 1991; Marincovich and Gladenkov 1999), in which 8 times as many species migrated from the Pacific to the Atlantic than the reverse direction, possibly due to a change in the direction of flow across the Bering Strait. However, evidence for both earlier and later marine migrations have been found, with the timing estimated at 4.8–7.3 Ma (Marincovich and Gladenkov 2001), 7–8 Ma (Collins et al. 1996), and 6–12 Ma (Nikula et al. 2007), as well as more recently (Palumbi and Kessing 1991; Dodson et al. 2007). We accounted for the possibility of an earlier migration of wolffish across the Arctic by running 2 analyses: 1 with the traditional date of 3.5 Ma and 1 with an earlier date of 6 Ma, which incorporated wide margins of uncertainty (4–8 Ma). The earlier migration date was chosen to provide a conservative estimate of mutation rates as slower mutation rates would have the potential to alter our main conclusion that wolffishes did not survive glaciation in 2 refugia. If divergence between Pacific and Atlantic wolffishes occurred more recently (with a more recent opening of the Bering Strait), estimated divergence dates would be pushed forward, providing more robust evidence for the single refuge hypothesis.

Our first objective in this study was to assess phylogenetic relationships among species in the family Anarhichadidae. This was effectively tested 3 times with mtDNA, all analyses

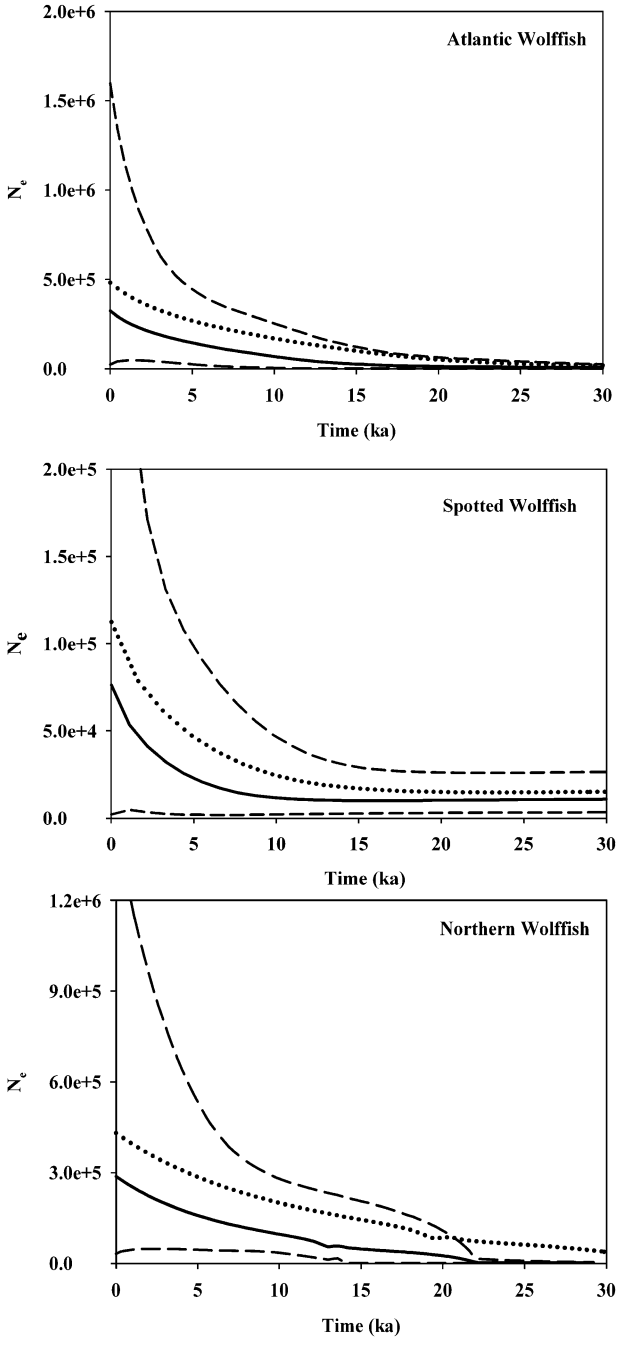

Figure 3. Bayesian skyline plots for Atlantic, Spotted, and Northern wolffishes from BEAST. Solid lines represent N_e estimates based on DIV3.5 (mutation rates based on divergence between Pacific to Atlantic species ca. 3.5 Ma), with upper and lower confidence limits represented by dashed lines. Dotted lines represent N_e estimates based on DIV6 (mutation rates based on divergence between Pacific to Atlantic species ca. 6 Ma).

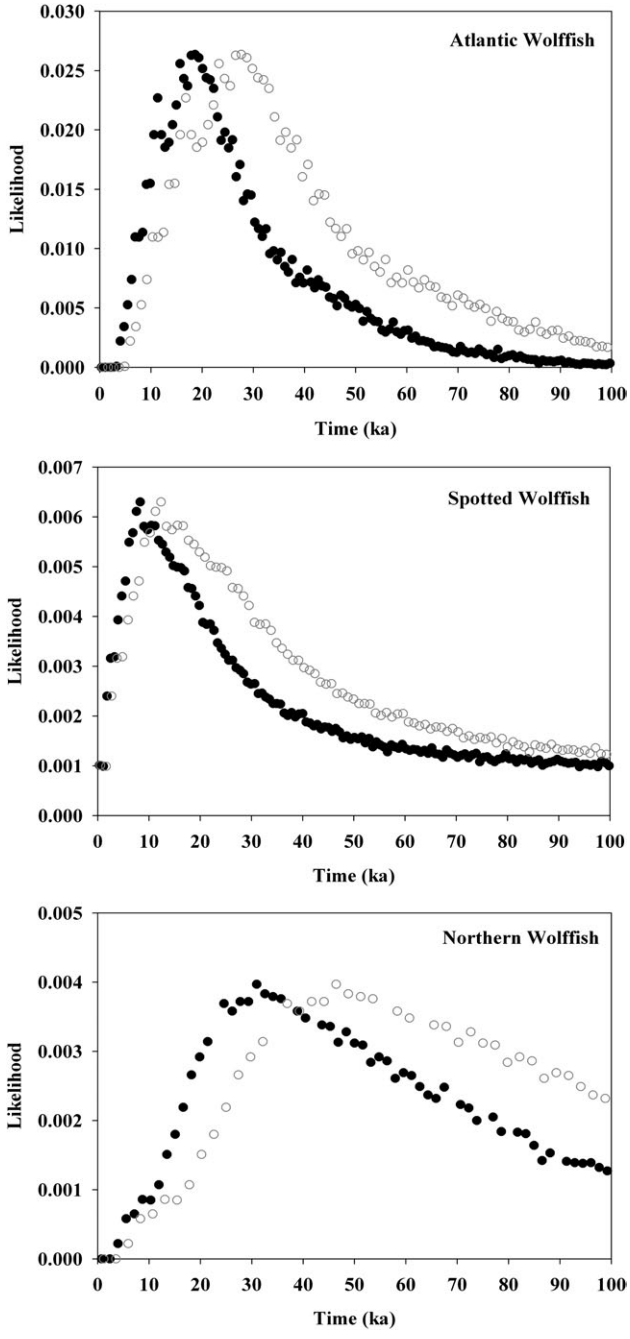

Figure 4. Posterior probability distributions of divergence time in thousand years (ka) from IM analyses for Atlantic (upper), Spotted (middle), and Northern (lower) wolffishes. Black circles represent probabilities based on DIV3.5 (mutation rates based on divergence between Pacific to Atlantic species ca. 3.5 Ma); open circles represent probabilities based on DIV6 (mutation rates based on divergence between Pacific to Atlantic species ca. 6 Ma).

were the same with respect to species-level topology, and strong support was found from posterior probabilities as well as bootstrap analyses (in fact, bootstrap support among was comparable with that found with whole mitochondrial genomic analyses; Johnstone et al. 2007). Although its haploid nature and maternal inheritance make mtDNA ideally suited to phylogenetic reconstruction (Zink and Barrowclough 2008), questions about selective neutrality

and reliability as a single locus remain (Moore 1995; Ballard and Whitlock 2004; Bazin et al. 2006). The congruence between AFLP and mtDNA data provided further support for this phylogeny. Low bootstrap support for AFLP data, relative to mtDNA, however, may be related to homoplasy, as well as slower lineage sorting for nuclear DNA compared with mtDNA. Finally, in contrast to results from many other species (Faber and Stepien 1997; Bowen et al. 2006), we found a lower mutation rate for D-loop than ND1, which was also supported by previous work (Johnstone et al. 2007), suggesting that D-loop may be under evolutionary constraint in these species.

Phylogeographic Patterns within Species

Although glaciers may be primarily associated with the terrestrial environment, glaciation has had a profound effect on marine organisms. Postglacial population expansion has been documented in numerous marine species (Nesbø et al. 2000; Hickerson and Cunningham 2006; Wilson 2006; Makinen and Merila 2008) likely due to reduced population sizes experienced by marine species during glaciation. Wolffishes also show strong evidence of postglacial expansion, suggesting striking postglacial expansions of population size and range size.

An emergent phylogeographic pattern for marine species in the North Atlantic Ocean is that of persistence in both eastern and western refugia during the last glaciation. Many near-shore species share a common pattern of reciprocal monophyly of haplotypes on both the North American and European coasts (Wares and Cunningham 2001; Hickerson and Cunningham 2006; Dodson et al. 2007; Makinen and Merila 2008). For more migratory species, less dependent on the near-shore environment, however, populations on the 2 coasts tend to share many haplotypes (e.g., bluefin tuna and swordfish [Bremer et al. 2005], Greenland halibut [Vis et al. 1997], mackerel [Nesbø et al. 2000], Atlantic cod [Bigg et al. 2008; Carr and Marshall 2008]), and phylogeographic patterns may be more subtle. In one such species, Atlantic cod, more sophisticated analyses (such as coalescent methods), were required to detect evidence of multiple refugia (Bigg et al. 2008). Nevertheless, we found no evidence that wolffishes persisted in 2 distinct refugia during the last glaciation. We found no phylogeographic structure in nearly 2000 bp (Figure 2), and IM analyses for all 3 species were not what would be expected for the 2 refugia hypothesis (~100 ka). We will explore the implications for each species separately.

Although reconstructions created by Bigg et al. (2008) for cod illustrate that suitable habitat likely existed for Atlantic wolffish on both sides of the Atlantic Ocean (Atlantic wolffish have comparable requirements to Atlantic cod in terms of depth), we found no evidence that Atlantic wolffish survived in 2 distinct refugia. IM analysis estimated divergence at <20 ka for DIV3.5, and only slightly older for DIV6. The overall probability curves argue against the 2 refugia hypothesis, although it leaves some questions as to why the estimate is so close to the timing of the last glacial maximum (LGM). One possibility is that the mutation rate is underestimated and that divergence is actually more recent, as has been estimated for other species (Palumbi and Kessing 1991; Dodson et al. 2007). Error inherent in estimation of mutation rates and in the IM analyses might account for this. If mutation rate was underestimated, all temporal estimates would move forward, well into a post-glacial time frame.

Nevertheless, one might speculate about whether divergence between eastern and western populations could have occurred "during" the LGM. If reconstructions of conditions by Bigg et al. (2008) are correct, it is conceivable that Atlantic wolffish could have gained access to suitable habitat off the North American coast when ocean levels were at their lowest (~130 m below what they are today; Mitrovica 2003). Lower sea levels may have allowed access to regions such as the MAR to Atlantic wolffish (where only the Northern wolffish is found today). The distance from where Northern wolffish were sampled on the MAR to where suitable habitat existed off Newfoundland is less than 500 km. Atlantic wolffish are mainly sedentary, but occasional long-distance migrations are known to occur. In one tagging study off Newfoundland, an Atlantic wolffish adult was documented migrating over 800 km (from the Flemish cap to the Labrador coast; Templeman 1984), albeit over continental shelf habitat. Thus, a transoceanic migration is conceivable, particularly if mid-ocean habitat was suitable to this species. Nevertheless, a conservative interpretation of the data is that Atlantic wolffish, which most likely required habitat on continental shelves, survived glaciation on the European coast and colonized the western Atlantic postglacially. The slight gradient in diversity from east to west supports the conclusion of survival in the eastern Atlantic and postglacial colonization of the western extent of the range (Table 1).

Spotted wolffish analyses are all consistent in their support of a postglacial divergence, implying persistence in one glacial refuge. No phylogeographic structure was found among haplotypes, and recent divergence was estimated by IM. Reconstructions of oceanic conditions and locations of suitable habitat for Atlantic cod suggest the eastern Atlantic would have been more likely than the western Atlantic (Bigg et al. 2008). The greater depth tolerance of the Spotted wolffish compared with Atlantic cod or Atlantic wolffish suggests that the species may have existed further away from continental shelves than Atlantic wolffish and may have been able to occupy a grater region in the northeastern Atlantic, south of Iceland, and west of the European coast. The occurrence of 2 unusual Spotted wolffish haplotypes in the eastern Atlantic and the western Atlantic suggests that they are unlikely related to events during the last glaciation. Selection does not appear to be a factor, and they may be a holdover from previous glacial episodes. A cursory assessment based on genetic distance, and the Spotted wolffish mutation rate estimated in this study, suggests that they originated over 100 ka.

Results for the Northern wolffish are the most equivocal of the 3 species. They do not fit the 2 refugia model well,

but divergence estimates between eastern and western samples was slightly earlier than expected for a postglacial divergence (Figure 4). Northern wolffish has the greatest depth tolerance of the 3 North Atlantic species, and samples from the MAR were found at ~1000 m deep. We might speculate that for organisms that do not require habitat on continental shelves (such as Northern wolffish) and can tolerate deep water, lower sea levels during glaciation may have made more of the ocean inhabitable, providing easier access across the ocean. Although speculative, a mid-glacial divergence time between eastern and western Atlantic samples is conceivable for the Northern wolffish. Nevertheless, as with Atlantic wolffish, an underestimate of the mutation rate and an overestimate of the divergence time from IM may be the best explanation for our findings, in which case a single eastern refuge would be proposed for this species.

In summary, we argue that none of the species fit the 2 refugia model. However, results for Northern wolffish, and to a lesser extent, Atlantic wolffish, would also be consistent with a mid-glacial divergence between eastern and western coasts. This would likely have required suitable habitat along the MAR and migration on the order of ~800 km to suitable habitat in the western Atlantic. A more conservative interpretation of the data is that mutation rates were slightly underestimated and that postglacial (<20 ka) divergence is more likely. Depictions of oceanic conditions during glaciation suggest that all 3 species were more likely to have survived in the eastern Atlantic than the western Atlantic.

Conservation Implications

Our stated goal was to identify ancient (preglacial) divergence in wolffishes for the purposes of delineating a fundamental unit of conservation. However, no evidence was found for pronounced phylogeographic structure in these species, therefore the conservation of glacial "races" does not appear to be a concern for these species. The next step is to assess genetic variation with more sensitive molecular markers to identify management units. Nevertheless, this study does illuminate some aspects of wolffish evolutionary history that may be important for the future conservation of these species. The lack of significant genetic divergence across haplotypes is typical for marine fishes that experienced fluctuations in population size, either due to glaciation or due to other perturbations (Grant and Bowen 1998). Wolffishes may represent an extreme case of this low diversity, as illustrated by a comparison of nucleotide diversity in wolffishes compared with other marine fishes (see Supplementary material).

The sensitivity of marine fishes to climate change is becoming more apparent given evidence for lower population sizes during glaciation. In the case of severe reductions of population size, marine fishes are likely not immune to the risks associated with low genetic variation, which include inbreeding depression and reduced adaptive potential (Frankham 2005; Willi et al. 2006). Whether marine fishes suffer from such potential problems remains unknown, but a cautionary approach should be taken with respect to exploitation of marine fishes.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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References

Agresti J, Seki S, Cnaani A, Poompuang S, Hallerman EM, Umiel N, Hulata G, Gall GAE, May B. 2000. Breeding new strains of tilapia: development of an artificial center of origin and linkage map based on AFLP and microsatellite loci. Aquaculture. 185:43–56.

Altoff DM, Gitzendanner MA, Segraves KA. 2007. The utility of amplified fragment length polymorphisms in phylogenetics: a comparison of homology within and between genomes. Syst Biol. 563:477–484.

Ballard JMO, Whitlock MC. 2004. The incomplete natural history of mitochondria. Mol Ecol. 13:729–744.

Bazin E, Glemin S, Galtier N. 2006. Population size does not influence mitochondrial genetic diversity in animals. Science. 312:570–572.

Bernatchez L, Dodson JJ. 1991. Phylogeographic structure in mitochondrial DNA of the lake whitefish *Coregonus clupeaformis* and its relation to Pleistocene glaciations. Evolution. 45:1016–1035.

Bernatchez L, Wilson CC. 1998. Comparative phylogeography of Nearctic and Palearctic fishes. Mol Ecol. 7:431–452.

Bigg GR, Cunningham CW, Otterson G, Pogson GH, Wadley MR, Williamson P. 2008. Ice-age survival of Atlantic cod: agreement between palaeoecology models and genetics. Proc R Soc Lond Biol Sci. 275:163–172.

Bonin A, Ehrich D, Manel S. 2007. Statistical analysis of amplified fragment length polymorphism data: a toolbox for molecular ecologists and evolutionists. Mol Ecol. 16:3737–3758.

Bowen BW, Muss A, Rocha LA, Grant WS. 2006. Shallow mtDNA coalescence in Atlantic pygmy angelfishes (genus *Centropyge*) indicates a recent invasion from the Indian Ocean. J Hered. 97:1–12.

Bremer JRA, Vinas J, Mejuto J, Ely B, Pla C. 2005. Comparative phylogeography of Atlantic bluefin tuna and swordfish: the combined effects of vicariance, secondary contact, introgression, and population expansion on the regional phylogenies of two highly migratory pelagic fishes. Mol Phylogenet Evol. 361:169–187.

Briggs JC. 1970. A faunal history of the North Atlantic Ocean. Syst Zool. 19:19–34.

Brunner PC, Douglas MR, Osinov A, Wilson CC, Bernatchez L. 2001. Holarctic phylogeography of Arctic charr *Salvelinus alpinus* L. inferred from mitochondrial DNA sequences. Evolution. 55:573–586.

Carr SM, Marshall HD. 2008. Intraspecific phylogeographic genomics from multiple complete mtDNA genomes in Atlantic cod *Gadus morhua*: origins of the codmother, transatlantic vicariance and midglacial population expansion. Genetics. 180:381–389.

CLIMAP. 1976. The surface of the Ice-Age Earth. Science. 191:1131–1137.

Collie JS, Hall SJ, Kaiser MJ, Poiner IR. 2000. A quantitative analysis of fishing impacts on shelf-sea benthos. J Anim Ecol. 69:785–798.

Collins TM, Frazer K, Palmer AR, Vermeij GJ, Brown WM. 1996. Evolutionary history of northern hemisphere *Nucella* Gastropoda, Muricidae: molecular, morphological, ecological, and paleontological evidence. Evolution. 50:2287–2304.

Crandall KA, Bininda-Emonds ORP, Mace GM, Wayne RK. 2000. Considering evolutionary processes in conservation biology. TREE. 15:290–295.

Dodson JJ, Tremblay S, Colombani F, Carscadden JE, Lecomte F. 2007. Trans-Arctic dispersals and the evolution of a circumpolar marine fish species complex, the capelin *Mallotus villosus*. Mol Ecol. 16:5030–5043.

Drummond AJ, Rambaut A. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol. 7:214.

Elphinstone MS, Hinten GN, Anderson MJ, Nock CJ. 2003. An inexpensive and high-throughput procedure to extract and purify total genomic DNA for population studies. Mol Ecol Notes. 3:317–320.

Faber JE, Stepien C. 1997. The utility of mitochondrial DNA control region sequence for analyzing phylogenetic relationships among populations, species, and genera of the Percidae. In: Kocher TD, Stepien CA, editors. Molecular systematics of fishes. New York: Academic Press. p. 129–143.

Felsenstein J. 2005. PHYLIP Phylogeny Inference Package version 3.6. Seattle (WA): Department of Genome Sciences, University of Washington.

Frankham R. 2005. Genetics and extinction. Biol Conserv. 126:131–140.

Grant WS, Bowen BW. 1998. Shallow population histories in deep evolutionary lineages of marine fishes: insights from sardines and anchovies and lessons for conservation. J Hered. 895:415–426.

Hewitt GM. 1996. Some genetic consequences of ice ages, and their role in divergence and speciation. Biol J Linn Soc. 58:247–276.

Hewitt G. 2000. The genetic legacy of the Quaternary ice ages. Nature. 405:907–913.

Hey J, Nielsen R. 2004. Multilocus methods for estimating population sizes, migration rates and divergence time, with applications to the divergence of *Drosophila pseudoobscura* and *D. persimilis*. Genetics. 167:747–760.

Hickerson MJ, Cunningham CW. 2006. Nearshore fish *Pholis gunnellus* persists across the North Atlantic through multiple glacial episodes. Mol Ecol. 15:4095–4107.

Johnstone KA, Marshall HD, Carr SM. 2007. Biodiversity genomics for species at risk: patterns of DNA sequence variation within and among complete mitochondrial DNA genomes of three species of wolffish *Anarhichas* spp. Can J Zool. 85:151–158.

Jønsson G. 1982. Contribution to the biology of catfish (*Anarhichas lupus*) at Iceland. Rit Fiskideildar. 6:2–26.

Koopman WJM. 2005. Phylogenetic signal on AFLP data sets. Syst Biol. 542:197–217.

Koopman WJM, Wissemann V, De Cock K, Van Huylenbroeck JV, De Riek J, Sabatino GJH, Visser D, Vosman B, Ritz CM, Maes B, et al. 2008. AFLP markers as a tool to reconstruct complex relationships: a case study in Rosa Rosaceae. Am J Botany. 953:353–366.

Makinen HS, Merila J. 2008. Mitochondrial DNA phylogeography of the three-spined stickleback *Gasterosteus aculeatus* in Europe—evidence for multiple glacial refugia. Mol Phylogenet Evol. 46:167–182.

Marincovich LJ, Gladenkov AY. 1999. Evidence for an early opening of the Bering Strait. Nature. 397:149–151.

Marincovich L, Gladenkov AY. 2001. New evidence for the age of the Bering Strait. Quat Sci Rev. 20:329–335.

Mendelson TC, Shaw KL. 2005a. Rapid speciation in an arthropod. Nature. 433:375–376.

Mendelson TC, Shaw KL. 2005b. Use of AFLP markers in surveys of arthropod diversity. Meth Enzymol. 395:161–177.

Mitrovica J. 2003. Recent controversies in predicting post-glacial sea-level change. Quat Sci Rev. 22:127–133.

Moore WS. 1995. Inferring phylogenies from mtDNA variation: mitochondrial-gene trees versus nuclear-gene trees. Evolution. 49: 718–726.

Nei M, Li WH. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci U S A. 76: 5269–5273.

Nesbø CL, Rueness EK, Iversen SA, Skagen DW, Jakobsen KS. 2000. Phylogeography and population history of Atlantic mackerel *Scomber scombrus* L.: a genealogical approach reveals genetic structuring among the eastern Atlantic stocks. Proc R Soc Lond B Biol Sci. 267:281–292.

Nikula R, Strelkov P, Vainola R. 2007. Diversity and trans-Arctic invasion history of mitochondrial lineages in the North Atlantic *Macoma balthica* complex Bivalvia: Tellinidae. Evolution. 614:928–941.

O'Dea NR, Haedrich RL. 2000. COSEWIC assessment and status report on the Atlantic wolffish *Anarhichas lupus* in Canada. vi + 21pp. Ottawa (Canada): Committee on the Status of Endangered Wildlife in Canada.

O'Dea NR, Haedrich RL. 2001a. COSEWIC assessment and status report on the northern wolffish *Anarhichas denticulatus*in Canada. vi + 21 pp. Ottawa (Canada): Committee on the Status of Endangered Wildlife in Canada.

O'Dea NR, Haedrich RL. 2001b. COSEWIC assessment and status report on the spotted wolffish *Anarhichas minor* in Canada. vi + 22 pp. Ottawa (Canada): Committee on the Status of Endangered Wildlife in Canada.

Ostvedt OJ. 1963. On the life history of the spotted catfish *Anarhichas minor* Olafsen. Rep Nor Fish Mar Invest. 13:54–72.

Palumbi SR, Kessing BD. 1991. Population biology of the trans-Arctic exchange: mtDNA sequence similarity between Pacific and Atlantic sea urchins. Evolution. 458:1790–1805.

Pavlov DA, Novikov GG. 1993. Life history and peculiarities of common wolffish (*Anarhichas lupus*) in the White Sea. ICES J Mar Sci. 50: 271–277.

Peltier WR. 1994. Ice age paleotopography. Science. 265:195–201.

Posada D, Crandall KA. 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics. 14:817–818.

Rambaut A, Drummond AJ. 2007. Tracer v1.4 [Internet]. Available from: http://beast.bio.ed.ac.uk/Tracer.

Riget F. 1986. Migrations of spotted wolffish *Anarhichas minor* Olafson in West Greenland. NAFO Scientific Council Res Doc 86/45.

Riget F, Messtorff J. 1988. Distribution, abundance and migration of Atlantic wolffish (*Anarhichas lupus*) and spotted wolffish (*Anarhichas minor*) in West Greenland waters. NAFO Sci Council Stud. 12:13–20.

Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics. 19:1572–1574.

Rozas J, Sanchez-DelBarrio JC, Messeguer X, Rozas R. 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics. 19:2496–2497.

Schneider S, Roessli D, Excoffier L. 2000. Arlequin version 2, a software for population genetics data analysis. Geneva, Switzerland: Genetics and Biometry Laboratory, University of Geneva.

Scott WB, Scott MB. 1988. Atlantic fishes of Canada. Toronto, Canada: University of Toronto Press.

Shaw J, Piper DJW, Fader GBJ, King EL, Todd BJ, Bell T, Batterson MJ, Liverman DGE. 2006. A conceptual model of the deglaciation of Atlantic Canada. Quat Sci Rev. 25:2059–2081.

Swofford DL. 2002. PAUP*: Phylogenetic analysis using parsimony (*and other methods). Version 4. Sunderland (MA): Sinauer Associates.

Templeman W. 1984. Migrations of wolffishes, *Anarhichas* sp., from tagging in the Newfoundland area. J NW Atl Fish Sci. 5:93–97.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25: 4876–4882.

Vermeij GJ. 1991. Anatomy of an invasion: the trans-Arctic interchange. Paleobiology. 173:281–307.

Vis ML, Carr SM, Bowering WR, Davidson WS. 1997. Greenland halibut *Reinhardtius hippoglossoides* in the North Atlantic are genetically homogeneous. Can J Fish Aquat Sci. 548:1813–1821.

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, et al. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. 23:4407–4414.

Waples RS. 2006. Distinct population segments. In: Scott JM, Goble DD, Davis FW, editors. The Endangered Species Act at Thirty: Conserving Biodiversity in Human-Dominated Landscapes. Washington (DC): Island Press. p. 127–149.

Wares JP, Cunningham CW. 2001. Phylogeography and historical ecology of the North Atlantic intertidal. Evolution. 5512:2455–2469.

Willi Y, Van Buskirk J, et al. 2006. Limits to the adaptive potential of small populations. Annu Rev Ecol Syst. 37:433–458.

Wilson AB. 2006. Genetic signature of recent glaciation on populations of a near-shore marine fish species Syngnathus leptorhynchus. Mol Ecol. 15:1857–1871.

Zink RM, Barrowclough GF. 2008. Mitochondrial DNA under siege in avian phylogeography. Mol Ecol. 17:2107–2121.

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