Further analysis of stock structure for BCB bowhead whales using genetic data

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Abstract

An extensive set of microsatellite and mitochondrial DNA data is analysed with the aim of investigating potential population substructure in the Bering-Chukchi-Beaufort stock of bowhead whales. We found no significant temporal pattern in the genetic data from migrating bowheads along the Alaskan coast, as was found previously in a subset of the data. However, different strata of the microsatellite DNA data deviate from Hardy-Weinberg genotype expectations and display significant spatial genetic differentiation. These observations indicate that the BCB stock does not represent a single, randomly mating population, although the precise structure of the stock remains unclear.

Introduction

Until recently available data were judged consistent with the notion of one single biological population of bowhead whales (*Balena mysticetus*) inhabiting the Bering-Chukchi- and Beaufort (BCB) Seas (reviewed by Rugh *et al.* 2003). Since genetic data became available, statistical analyses (Givens *et al.* 2004, Jorde *et al.* 2004, Pastene *et al.* 2004) reported inhomogeneities that could indicate a more complex population structure of BCB bowhead whales. In particular, Jorde *et al.* (2004, 2007), found significant fluctuations in genetic similarity as a function of number of days between captures when analysing pair-vise genetic differences among whales migrating past Point Barrow, Alaska. The pair-wise genetic differences increase to a maximum at about seven days apart, and then decline. The existence of such a temporal "bump" demonstrates genetic heterogeneity in the data from Barrow and, if reflecting a real biological phenomenon, seems at odds with the notion of a single homogeneous population of BCB bowhead whales.

More extensive genetic data have recently been gathered to elucidate possible structures in the population of bowhead whales in the Bering- Chukchi- and Beaufort Seas. After the initial genetic findings further DNA microsatellites were developed to increase statistical power and to overcome some concerns about the quality and reliability of the original markers. All the original samples, along with new captures, have been genotypes for 25 new microsatellites loci in addition to the original 12 (Huebinger *et al.* 2006; Givens *et al.* 2007). In the present paper, we analyse the available data for possible genetic structures relevant for management of the aboriginal subsistence whaling that these bowhead whales are subject to.

Material & Methods

Material: Data for the present analyses refers to the version received Feb. 9. 2007 through the Data Availability Agreement procedure of the IWC. This data set contains a total of n=457 bowhead whales, with microsatellite DNA genotypes at up till 37 loci and mitochondrial DNA haplotypes. The material can be decomposed as follows. Of the 457 whales, n=344 are from the BCB stock, represented by Barrow (n=260), other Alaskan communities (n=36), Chukotka, Russia (n=16), St. Lawrence Island (n=29), and Commander Island (n=4), most of which were collected during aboriginal whaling, from 1983 through 2006. The remaining bowhead whales are from Canada (n=48) and the Okhotsk Sea (n=64), and one individual of unknown origin.

The 35 microsatellites comprise a set of 11 loci previously analysed for a subset of the individuals (Givens *et al.* 2004; Jorde *et al.* 2004, 2007) and a new set of 24 loci not

previously analysed. There were originally 12 loci, but one of them (Tv18) was found to exhibit a very strong deficiency of heterozygotes relative to Hardy-Weinberg expectations, in a pattern consistent with short allele dominance, and was therefore judged unreliable and excluded from further consideration both in the original as well as in the previous analyses. There were apparently 25 new loci, but locus Bmy47 was found on the X-chromosome (Givens *et al.* 2007) and is not included in the data set. Two of the new loci that are included (Bmy38 and Bmy 44) both display excessive deficiencies of heterozygotes: a phenomenon most likely arising as an artifact of null-alleles and/or PCR stuttering (Givens *et al.* 2007). Both Bmy38 and Bmy44 were judged unreliable and excluded from further consideration. The analyses are thus based on 33 microsatellite loci (Table 1).

Not all whales were scored at all 33 loci. First, the samples from Canada and Okhotsk Sea were not scored at all for the 11 original loci. Second, the Canadian sample was not scored for the new Bmy2 locus. Third, many individuals failed to amplify for one or more of the microsatellites because of technical problems. Many samples are quite old (from 1983 onwards) and probably contain partially degraded DNA. Because of concerns over the quality (i.e., correctness) of the genotypes that were scored for such samples, an upper limit of 3 missing microsatellites (2 in the case of the Canada and Okhotsk samples) were imposed to ensure a high quality data set, following the recommendations of the AWMP SWG (IWC 2007). Thus, individuals with more that 10% genotyping failures were excluded from the analyses, which are based on the following numbers: Barrow (n=206); Chukotka (n=15); Gambell (n=9); Kaktovik (n=12); Little Diomede (n=1); Nuiqsut (n=5); Point Hope (n=4); Savoonga (n=16); Wainwright (n=4); Canada (n=47); Okhotsk Sea (n=62). Note that a few individuals from Barrow are excluded in our analyses (see below) because they lack sex and length information (5 biopsied individuals and individual 04B6), or because they are fetuses whose mothers are included (4 individuals: 95B8F, 96B5F, 00B3F and 00B5F). The final 206 individuals from Barrow include 96 that were sampled during the spring migration and 110 in the autumn. 111 of the individuals from Barrow were included in the previous analysis by Jorde et al. (2004, 2007), and 95 are new (either caught after the 2003 spring season, or genotyped after the previous analysis). Hence, the present data set represents a substantial increase, both of individuals and loci, relative to the previous analysis of migrating whales off Barrow.

The mitochondrial DNA (mtDNA) is substantially less prone to genotyping errors than are the microsatellites, and no quality restriction was imposed on individuals for statistical analysis of this genetic marker. Instead, we used all available data from Barrow (n=254), including 126 individuals collected during the spring and 128 during the autumn migration.

Methods: Microsatellite genotype frequencies were tested within locus for conformation to Hardy-Weinberg expectations within stock, locality, and sample year. Deviations from Hardy-Weinberg genotype proportions were estimated by F_{IS}, using Weir & Cockerham's (1984) estimator *f*, which takes a positive value when the proportion of heterozygotes is less than expected, and a negative value when greater. Because population admixture and many genotyping problems with microsatellites are expected to result in an apparent heterozygote deficit, we carried out the tests on various data strata.

First, we tested deviations from Hardy-Weinberg in the combined sample from all BCB localities, and compared the test results with those obtained from the Canada and the Okhotsk Sea samples for the same loci. This comparison was done to check for potential problematic loci, on the supposition that problems with genotyping should yield similar deviations from Hardy-Weinberg in different populations. We tested the null-hypothesis of no deviation from Hardy-Weinberg genotype proportion (two-sided tests) by two methods. One test is based on the multinomial contingency table of genotypes, as implemented in the GENEPOP software (version 3.4: Raymond & Rousset 1995) with exact calculations of p-values. The other test is a binomial test based on the number of homo- and heterozygotes, pooling over alleles, as carried out by Jorde *et al.* (2004).

Furthermore, we tested each annual sample at Barrow separately with respect to Hardy-Weinberg equilibrium, in order to check for potential biological significant deviations (e.g., Wahlund effects) that may be infrequent and could be hidden when combining samples over the long sequence of sample years (from 1983 to 2006),

The degree of genetic differentiation between geographic localities were estimated using Weir & Cockerham's (1984) estimator for F_{ST} between pairs of samples considered separately. Differentiation was tested for significance using exact tests for allele frequency homogeneity within sample pairs, as implemented in the GENEPOP software. Tests results (p-values) for each locus were summarized in a joint test for genetic differentiation, following Fisher's summation procedure (i.e., summing twice the negative logarithm of the p-values for each single-locus test and evaluating the sum against the appropriate chi-square table).

Cluster analyses of individual whales were done on the basis of the 33 microsatellite loci using softwares STRUCTURE and BAPS, to test for potential population substructure within migrating whales off Barrow (n=206: spring and autumn combined). STRUCTURE (version 2.1: Pritchard *et al.* 2000) was run assuming that allele frequencies among potential populations are correlated and allowing for population mixture (the default options). We tried from one to six populations, and calculated log-likelihood of the data given each assumed number. BAPS (version 4.14: Corander *et al.* 2006) was run on the same data set with option "Clustering of individuals" and a

maximum of six populations. This software returns the most likely number of populations in the data.

Within each migratory season we also carry out a temporal analyses of bowheads as described in Jorde *et al.* (2007). The analysis is limited to Barrow, which is the only village with from which a reasonable large sample is available, and was carried out separately for whales sampled during the spring and autumn migrations. For each pair of individuals that were sampled in the same season and the same year, an estimate of pair-wise genetic difference (*a*) was calculated following Rousset (2000). This measure was plotted against days apart (*d*) between the two dates of sampling for the pair. A GAM analysis was then carried out to test for variation in genetic differences during the course of the migration run, using the fact that different pairs of individuals are caught different numbers of days apart. The plot was pooled over sample years to provide a reasonable number of pairs, covering the migration run. In the GAM analysis, we controlled for difference in birth year (*y*) and mean birth year (*m*), using age estimates that are based on length and sex. For individuals *i* and *j* in a given season and year, we thus fit the GAM model

$$a_{ij} \sim s(d_{ij}) + s(y_{ij}) + s(m_{ij}),$$

where s() are smoothing spline functions. In this model, a_{ij} is the estimated genetic difference between two individuals, *i* and *j*, using average differences over 33 microsatellite loci (Rousset 2000) and, in a separate analysis, mtDNA haplotype difference (equals 0 if *i* and *j* carry the same haplotype and 1 if different). y_{ij} and m_{ij} are the age difference and mean age, respectively, of the two individuals. The control variables were included to capture potential genetic differences among age classes (cf. Givens *et al*. 2004). Age information was obtained indirectly, from measured lengths and estimated sex-specific growth patterns (Rosa *et al.* 2004). The significance of the effect of days apart is calculated as explained in Jorde *et al.* (2007).

Results

Hardy-Weinberg tests: As judged by the multinomial test there is a significant departure (at the 5% level) from Hardy-Weinberg proportions in 6 out of 33 microsatellite loci in the entire BCB material (Table 1), and there is an over all departure from Hardy-Weinberg equilibrium (P=0.018). By the binomial test, however, these results do not hold up, and there are by this test no overall significant deviations from Hardy-Weinberg genotype proportions in the data from either of the three putative bowhead stocks, BCB, Canada, and Okhotsk Sea (Table 1). For both methods, the single-locus p-values were generally high, and the few test that come out as significant did so only at the 5% level, and no locus was significant in more than one stock. There is no indication that any

particular locus tended to deviate from Hardy-Weinberg in the same direction consistently among the three stocks. Of the 21 loci that were scored in all stocks, only 4 loci (Bmy14, Bmy54, Bmy55, and Bmy57) yielded F_{IS}-estimates with the same sign in all stocks. In the binomial tests, these 4 cases were all associated with moderate to high p-values, although 3 of them came out as significant in the BCB stock when using the multinomial test (cf. Table 1). There is a very poor correlation between the multinomial test results (p-values) and the estimated magnitude (F_{IS}) of Hardy-Weinberg deviations, however, indictating that the binomial test may be more appropriate for judgning deviations from Hardy-Weinberg common to all alleles within multi-allelic loci. At any rate, there are no obvious indications of problematic loci (when the previously identified loci Tv18, Bmy38 and Bmy44 are left out), as judged by these largely negative findings.

Within Barrow, we find no overall significant deviation from Hardy-Weinberg expectations (data not shown). There is a slight apparent deficiency of heterozygotes (average F_{IS} = 0.016), but it is not significant over loci (P=0.735 with the binomial test and P=0.088 with the multinomial test) and only two single-locus tests (4 using the multinomial tests) out of 33 were significant at the 5% level.

When testing the different sample years at Barrow separately a heterogeneity among years is apparent (Figure 1). Whereas most sample years at Barrow conform to Hardy-Weinberg expectations, the sample from 1992 does not. (Note, however that all sample sizes are small, n <=28, and power is low.) The average F_{IS} (0.198) for the sample from 1992 at Barrow is considerably higher than for any other year and the deviation involves many loci. Of a total of 33 microsatellites, 29 loci display an apparent deficiency of heterozygotes (positive F_{IS}-estimate) among whales sampled at Barrow this year, and the deviations are significant at three loci (Bmy10, Bmy18, and Bmy26: binomial test) and for the total over loci (P=0.02: data not shown), despite the diminutive sample size (n=8). (The significances are even more pronounced with the multinomial test in GENEPOP: 9 loci come out significant at the 5% level or better, and the joint test over loci is highly significant).

Spatial analysis: Both the Okhotsk Sea and the Canada sample differ significantly from the individual samples of bowhead whales from the BCB stock (Table 2) in allele frequency in common microsatellite loci, at all but the two very small samples from Nuiqsut and Point Hope (n = 4-5). Hence, there is at least three genetically differentiated bowhead whales populations, with the Okhotsk Sea stock clearly beeing the most divergent one, as judged by the magnitude of allele frequency differences (F_{ST}). Within the BCB area, we find little evidence for geographic differentiation, and the only significant pair-wise comparison refers to Barrow and Savoonga, on the St Lawrence Island (P=0.03).

Cluster analyses: Based on the sample from Barrow, the cluster analyses performed by STRUCTURE and BAPS gave limited and somewhat conflicting results. STRUCTURE yielded slightly higher log-likelihood of the data under the assumption of one single population at Barrow, whereas BAPS preferred two populations. However, the two putative populations identified by BAPS were highly dissimilar in size, with one of them containing only two individuals (individuals 92B3 and 96B21), caught during the autumn migrations in 1992 and 1996, respectively.

Temporal analysis at Barrow: Figure 2 gives the plot of the partial effects of days between captures on pair-wise genetic difference in whales caught off Barrow during the spring and autumn migrations, controlling for age difference and mean age (cf. GAM model above). Clearly, the "bump" that was found earlier (Jorde *et al.* 2004, 2007) during the autumn migration is no longer significant in the aggregated data. Neither is there a significant pattern in the spring, nor for microsatellites or mtDNA haplotypes.

Discussion

Although the genetic data we have been given access to are substantial compared to genetic data for other populations of cetaceans, the present data have their limitations for investigating substructure in the BCB stock of bowhead whales. It is particularly the imbalance in spatial and temporal coverage that makes it difficult to infer structure in these highly migratory whales.

In the absence of adequate geographic sampling coverage, statistical analyses are largely limited to test for and explore genetic inhomogeneities within samples from a single location, at Barrow, Alaska. Such inhomogeneities include deviations from Hardy-Weinberg genotype proportions (i.e., non-random assortment of alleles), but the statistical power in detecting such deviations as a result of population admixture (the so-called Wahlund effect) is low, and reliable detection is only feasible when genetic differentiation is strong.

As a numerical example, consider two hypothetical populations that are both in Hardy-Weinberg equilibrium and that have the same level of genetic differentiation as found between the BCB and Canada stocks (F_{ST} =0.006: Table 2). If these hypothetical populations were to appear mixed in a sample, the expected deficiency of heterozygotes (F_{IS}) in this mixed sample equals F_{ST} between the constituent populations (0.006), or less if they appear in uneven proportions in the mixture. This level of heterozygote deficieny would be very hard to detect with any statistical significance, and is actually less than the point estimate within Barrow alone (F_{IS} =0.016: above), which was not significant from zero. In other words, a mixture of whales from the Canada and the BCB stock would probably not be detectable by Hardy-Weinberg tests alone, and certainly not if the

mixture was uneven. Statistical cluster techniques, as implemented in the BAPS and STRUCTURE software, rely on deviations from Hardy-Weinberg proportions in population admixtures, and such techniques are quite powerless unless genetic differentiations is strong (Waples and Gaggiotti 2006). The negative results from BAPS and STRUCTURE analyses when applied to the Barrow sample cannot therefore be given much weight.

The method of testing for genetic inhomogeneities among whales caught during migration (Jorde et al. 2007) was developed in order to overcome the obvious limitations of the Hardy-Weinberg tests and derived techniques. By exploring the temporal aspect of the data, utilizing the fact that whales are caught various numbers of days apart, inhomogeneities in population composition may show up as fluctuations in the amount of genetic differentiation among whales. Such genetic differences are easier to detect statistically than are deviations from Hardy-Weinberg genotype proportions. There is a potential problem in implementing the Jorde *et al.* technique on the present bowhead data, however. Because few whales are caught each year, it was necessary to include whales caught in a great number of years (1983-2006) to improve sample sizes. If the timing of migration of different populations varies among years, or if the hunting does not hit the same phase of the migrations each year, this pooling over years will obscure any temporal genetic patterns expected from shifting population affinities of individuals during the migration season. Such annual variations must surely be common, no doubt depending on variation in ice cover and other variable factors, and could effectively destroy the power of the technique, and the problem may actually increase when more sample years are included in the analysis.

The variability in F_{IS}-values over the years at Barrow (Figure 1) might reflect genetic segregation, and that different subunits are differentially sampled over the years. This annual variability might explain why we in the present, expanded data set do not find the significant "bump"-pattern reported previously. Another possibility for a lack of a significant bump in the present analysis is that there were some unrealized problems with genotyping the "old" loci, and that the bump was a technical artifact. However, we tried repeating the GAM analysis with just the old loci on the new samples and found no bump or other significant pattern among them.

To summarize, there are different bits of evidence for the BCB stock of bowhead whales not representing a single biological population: (i) a "bump"-pattern in genetic differences during the autumn migration is highly significant in the part of the material considered by Jorde *et al.* (2007); (ii) the estimated F_{IS}-value varies substantially between sample years at Barrow (Figure 1); (iii) there is a tendency for heterozygote deficiency within the BCB stock (Table 1); (iv) the significant allele frequency difference between Barrow and Savoonga (Table 2). At present, we are however at short in providing a

comprehensive interpretation of these findings in terms of population structure within the Bering-Chukchi-Beaufort stock of bowhead whales.

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Table 1. Estimated deviations (F_{IS}) from Hardy-Weinberg genotype proportions at 33 microsatellite loci, and statistical tests of the null-hypothesis of no deviations. Two different tests were used: GENEPOP "exact" multinomial probability tests and a binomial test of the number of observed and expected homo- and heterozygotes with pooling over alleles. BCB refers to the combined sample from all Northwest Arctic samples. A dash indicates no data. (Note the difference in p-values for the two tests.)

	BCB (n=272)			Canada (n=47)			Okhotsk Sea (n=62)		
Locus		Genepop	Binimoal		Genepop	Binomial		Genepop	Binomial
	F_{IS}	p-value	p-value	F_{IS}	p-value	p-value	F_{IS}	p-value	p-value
New:									
Bmy1	0.001	**0.019**	0.989	-0.018	0.221	0.686	0.070	0.615	0.310
Bmy2	0.035	0.589	0.312	-	-	-	0.074	0.138	0.351
Bmy7	-0.027	0.769	0.364	0.099	0.183	0.262	-0.097	0.300	**0.038**
Bmy8	0.016	0.614	0.650	0.034	0.557	0.727	-0.023	0.999	0.626
Bmy10	0.028	0.715	0.129	-0.067	0.613	0.092	-0.056	0.708	0.169
Bmy11	0.018	0.326	0.484	-0.037	0.645	0.395	0.069	0.196	0.351
Bmy12	-0.016	0.358	0.294	-0.091	0.984	0.056	-0.040	0.737	0.396
Bmy14	0.078	0.066	0.167	0.017	0.696	0.960	0.137	0.429	0.324
Bmy16	-0.018	0.966	0.554	0.021	**0.013**	0.900	-0.055	0.411	0.381
Bmy18	0.025	0.179	0.244	-0.080	0.874	0.093	-0.021	0.872	0.687
Bmy19	0.006	0.611	0.877	-0.029	**0.049**	0.498	0.053	0.156	0.438
Bmy26	0.028	0.435	0.191	-0.072	0.912	0.080	0.032	0.330	0.610
Bmy33	0.022	0.064	0.495	-0.064	0.303	0.340	0.095	0.576	0.175
Bmy36	0.000	0.915	0.900	-0.021	0.404	0.438	0.000	0.769	0.867
Bmy41	0.011	0.389	0.630	0.039	0.234	0.562	-0.027	0.195	0.554
Bmy42	0.063	0.663	0.060	0.071	0.491	0.343	-0.043	0.825	0.395
Bmy49	-0.006	**0.015**	0.711	0.055	0.157	0.432	-0.018	0.904	0.581
Bmy53	-0.005	0.225	0.779	0.009	0.642	0.968	0.040	0.321	0.628
Bmy54	0.031	0.173	0.459	0.002	0.210	0.916	0.012	0.575	0.955
Bmy55	0.016	**0.019**	0.702	0.086	0.889	0.410	0.008	0.166	0.998
Bmy57	0.016	**0.037**	0.764	0.022	0.679	0.915	0.024	0.240	0.889
Bmy58	0.000	0.500	0.909	0.039	0.198	0.507	-0.049	0.728	0.197
Old:									
Ev1	-0.014	0.676	0.656	-	-	-	-	-	-
Ev104	0.027	0.953	0.365	-	-	-	-	-	-
Gata28	0.007	0.949	0.825	-	-	-	-	-	-
Tv7	0.086	**0.018**	**0.041**	-	-	-	-	-	-
Tv11	0.073	0.290	0.118	-	-	-	-	-	-
Tv13	-0.021	0.378	0.581	-	-	-	-	-	-
Tv14	0.036	0.600	0.433	-	-	-	-	-	-
Tv16	-0.015	0.673	0.773	-	-	-	-	-	-
Tv17	0.004	0.349	0.945	-	-	-	-	-	-
Tv19	0.074	**0.024**	**0.032**	-	-	-	-	-	-
Tv20	-0.011	0.575	0.772	-	-	-	-	-	-
All	0.016	**0.018**	0.637	-0.018	0.318	0.547	0.005	0.744	0.668

Table 2. Average amounts of genetic differentiation, F_{ST} (below diagonal), for microsatellite loci among pairs of sample localities, and tests of pairwise allele frequency homogeneity (above diagonal: multinomial "exact" p-values calculated using GENEPOP). Sample size is given for each spatial stratum. F_{ST}-estimates and p-values refer to 33 microsatellite loci for the BCB samples, 22 loci for comparisons involving the Okhotsk sample, and 21 loci for Canada.

	Barrow (n=206)	Chukotka (n=15)	Gambell (n=9)	Kaktovik (n=12)	Nuiqsut (n=5)	Pt Hope (n=4)	Savoonga (n=16)	Wainwright (n=4)	Canada (n=47)	Okhotsk (n=62)
Barrow	-	0.291	0.315	0.722	0.564	0.726	0.031	0.279	0.000	0.000
Chukotka	0.002	-	0.304	0.971	0.756	0.639	0.705	0.363	0.022	0.000
Gambell	0.005	0.005	-	0.442	0.369	0.215	0.696	0.458	0.011	0.000
Kaktovik	-0.001	-0.007	-0.002	-	0.958	0.771	0.668	0.865	0.026	0.000
Nuiqsut	-0.002	-0.006	0.006	-0.013	-	0.800	0.341	0.884	0.140	0.000
Point Hope	0.005	0.004	0.018	-0.001	-0.006	-	0.492	0.892	0.421	0.000
Savoonga	-0.001	-0.005	-0.005	-0.004	0.000	0.006	-	0.637	0.000	0.000
Wainwright	-0.011	-0.005	-0.005	-0.009	-0.018	0.000	-0.016	-	0.000	0.000
Canada	0.006	0.003	0.005	0.000	0.004	0.011	0.006	-0.009	-	0.000
Okhotsk	0.035	0.037	0.043	0.037	0.027	0.038	0.034	0.023	0.039	-

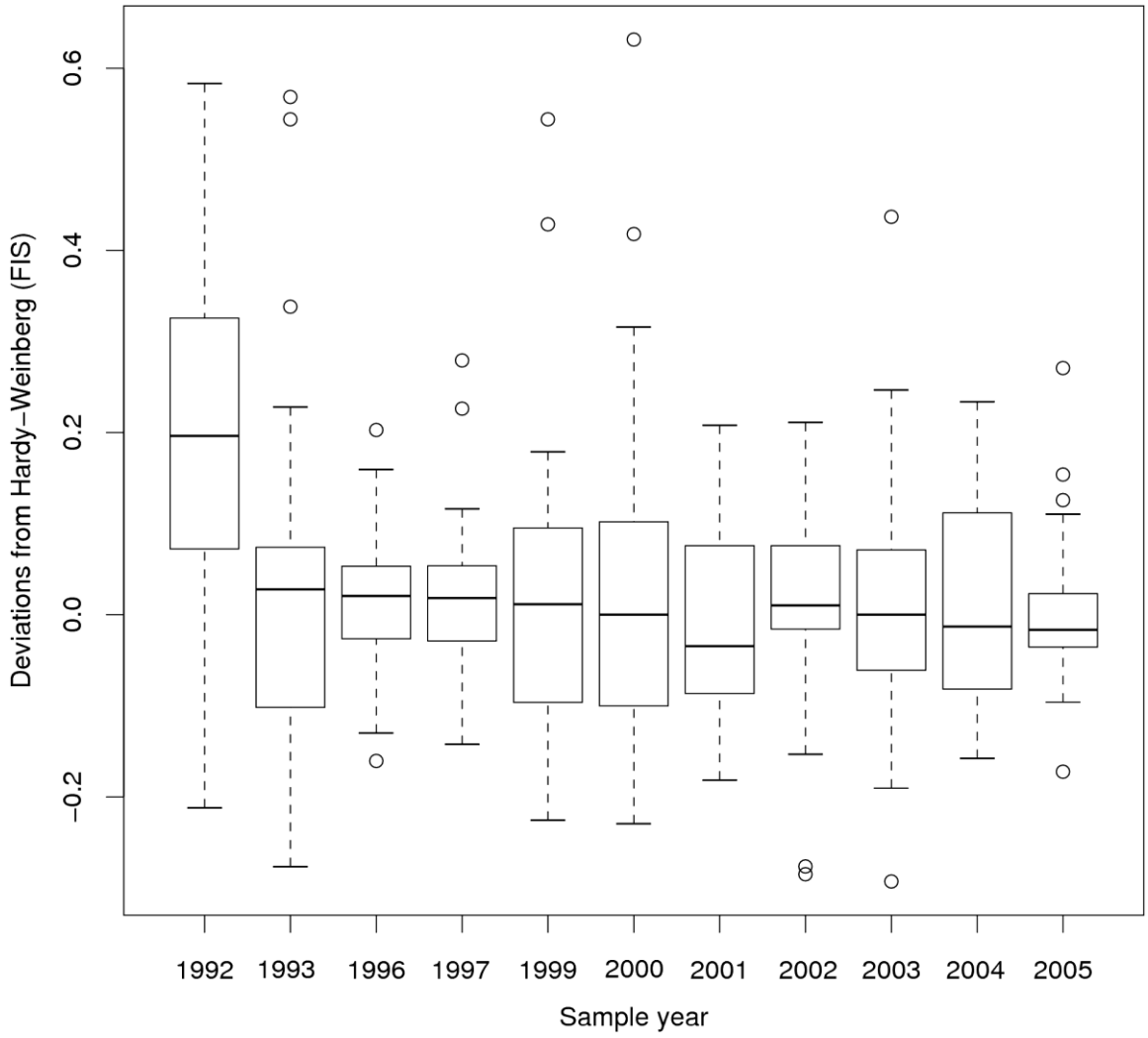

Figure 1. Boxplot of estimated deviations from Hardy-Weinberg genotype proportions (F_{IS}) in 33 microsatellite loci within sample years in bowhead whales sampled off Barrow. Each sample year includes 7 to 28 whales (autumn and spring pooled). Years with less than 4 whales are excluded from the plot. The horizontal bar indicates the median of the single-locus F_{IS}-values, the box covers the quartiles, and the wiskers cover all single-locus values, except outlying values plotted as circles.

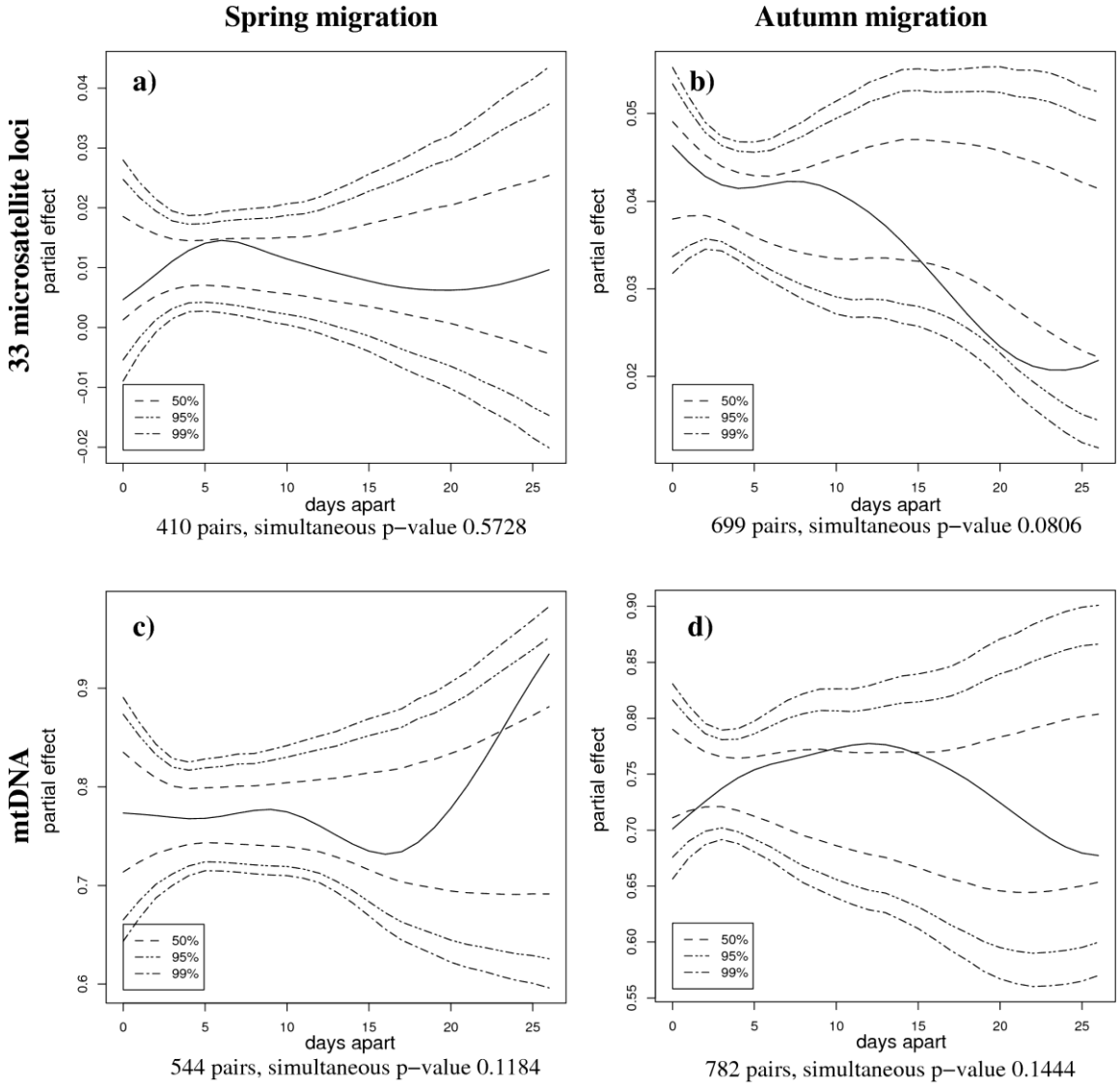

Figure 2. Pair-wise genetic difference between individuals taken the same season (spring at the left, autumn at right) versus days between sampling, when controlling for age effects. Upper panels (**a** and **b**): based on 33 microsatellite loci; lower panels (**c** and **d**): based on mtDNA haplotype data. Dotted lines represent simultaneous null bands of confidence, as indicated (50%, 95%, and 99%). Below the curves are indicated the total number of pairs of individuals uses, and the p-value representing the significance level for the curve.