

The genetics of recolonization: an analysis of the stock structure of grey seals (*Halichoerus grypus*) in the northwest Atlantic

S.A. Wood, T.R. Frasier, B.A. McLeod, J.R. Gilbert, B.N. White, W.D. Bowen, M.O. Hammill, G.T. Waring, and S. Brault

Abstract: Although historically distributed along the northeast coast of the United States (US), grey seals (*Halichoerus grypus* (Fabricius, 1791)) were considered locally extinct until the late 1980s when three naturally re-established pupping colonies were discovered. Two large populations in Canada, the Gulf of St. Lawrence (GSL) and Sable Island (SI) seals, are possible sources of immigrants for the recovering US population. To assess the stock structure of grey seals in the northwest Atlantic, tissue samples were collected from Canadian and US populations for genetic analyses. We examined nine highly variable microsatellite loci ($n = 158$; mean number of alleles per locus = 7.22). When population differentiation was assessed without a priori inference of potential subpopulations, all individuals were placed into one population. Pairwise F_{ST} values showed little difference in allele frequencies between the SI and the GSL or the Canadian and the US samples. We sequenced a 319 bp segment of the mitochondrial control region and identified 25 haplotypes ($n = 163$). Nucleotide diversity was similar at SI, GSL, and the US sites. Based on mtDNA haplotypes, no significant difference was found between the SI and GSL populations or the Canadian and the US populations. Although grey seals are philopatric, our study demonstrated that the genetic structure of the northwest Atlantic grey seal population is not different from the null hypothesis of panmixia.

Résumé : Bien que répartis dans le passé le long de la côte nord-est des États-Unis (É.-U.), les phoques gris (*Halichoerus grypus* (Fabricius, 1791)) étaient considérés comme disparus localement jusqu'à la fin des années 1980 quand on a découvert trois colonies de mise bas rétablies naturellement. Deux importantes populations canadiennes, les phoques du golfe du Saint-Laurent (GSL) et de l'île de Sable (SI), sont des sources possibles des immigrants dans la population en voie de rétablissement aux É.-U. Afin de déterminer la structure des stocks de phoques gris dans le nord-ouest de l'Atlantique, nous avons prélevé des échantillons de tissus dans des populations canadiennes et américaines pour analyse génétique. Nous avons examiné neuf locus microsatellites très variables ($n = 158$; nombre moyen d'allèles par locus = 7,22). Lorsque la différenciation de population est évaluée sans présupposition a priori de sous-populations potentielles, tous les individus sont placés dans une seule population. Les valeurs appariées de F_{ST} montrent peu de différence de fréquences d'allèles entre les échantillons SI et GSL ou entre les échantillons canadiens et américains. Nous avons séquencé un segment de 319 pb de la région mitochondriale de contrôle et identifié 25 haplotypes ($n = 163$). La diversité des nucléotides est semblable aux sites SI, GSL et américains. D'après les haplotypes d'ADNmt, il n'y a pas de différence significative entre les populations SI et GSL, ni entre les populations canadiennes et américaines. Bien que les phoques gris soient philopatriques, notre étude démontre que la structure génétique de la population de phoques gris du nord-ouest de l'Atlantique n'est pas incompatible avec une hypothèse nulle de panmixie.

[Traduit par la Rédaction]

Introduction

Grey seals (*Halichoerus grypus* (Fabricius, 1791)) are found throughout the temperate north Atlantic Ocean (Davies

1957). Analysis of grey seal mitochondrial DNA (mtDNA) (Boskovic et al. 1996), in addition to cranial morphology (Rice 1998) and timing of the breeding season (King 1983), has supported the view of three discrete stocks: Baltic Sea,

Received 25 May 2010. Accepted 28 January 2011. Published at www.nrcresearchpress.com/cjz on 16 May 2011.

S.A. Wood and S. Brault. University of Massachusetts – Boston, Department of Biology, 100 Morrissey Boulevard, Boston, MA 02125-3393, USA.

T.R. Frasier, B.A. McLeod, and B.N. White. Natural Resources DNA Profiling and Forensic Centre, Department of Biology, Trent University, 1600 East Bank Drive, Peterborough, ON K9J 7B8, Canada.

J.R. Gilbert. University of Maine, Department of Wildlife Ecology, Orono, ME 04469, USA.

W.D. Bowen. Bedford Institute of Oceanography, Fisheries and Oceans Canada, 1 Challenger Drive, Dartmouth, NS B2Y 4A2, Canada.

M.O. Hammill. Maurice Lamontagne Institute, Fisheries and Oceans Canada, 850, route de la Mer, Mont-Joli, QC G5H 3Z4, Canada.

G.T. Waring. National Oceanic and Atmospheric Administration (NOAA), National Marine Fisheries Service (NMFS), Northeast Fisheries Science Center (NEFSC), 166 Water Street, Woods Hole, MA 02543, USA.

Corresponding author: S.A. Wood (e-mail: Stephanie.Wood@noaa.gov).

northeastern Atlantic, and northwestern Atlantic. The northwestern Atlantic population has two large, long-established pupping sites. Both are located in Canada, one on Sable Island, Nova Scotia, and the other on the ice and coastal islands in the southern Gulf of St. Lawrence and off eastern Cape Breton Island (Bowen et al. 2003; Hammill et al. 1998).

Archaeological studies demonstrate that grey seals were distributed along the Northeast coast of the US into the 17th century (Eaton 1898; Waters 1967; Ritchie 1969; Spiess and Lewis 2001). After that time, there was a period of decline, perhaps owing to native hunting pressure and local bounty systems throughout northeast US. Although rare, bounty records and local observations indicate that individual grey seals were found in Nantucket Sound (Massachusetts, USA) during the early to mid-20th century (Andrews and Mott 1967). Grey seal sightings in US waters were extremely rare from the 1950s until the late 1980s (Rough 1981, 1983). Since 1991, three small, but increasing, pupping sites have been identified in the northeastern US: Muskeget Island, Massachusetts; Green Island, Maine; and Seal Island, Maine (Wood LaFond 2009). The most pronounced increase has been observed on Muskeget Island, where approximately 6 pups were born in 1991 (Rough 1991) and a minimum of 2036 pups were born in 2008 (Wood LaFond 2009). This growth occurred in the span of approximately one seal generation (16 years; Graves et al. 2009). For all three US sites, a minimum of 2620 pups were born in 2008 (Wood LaFond 2009).

Prior to and during the time of grey seal re-establishment in the US, the grey seal populations in Canada have increased considerably. From population estimates on Sable Island, the annual rate of increase in pup production had been constant at 12.8% for more than 25 years (Bowen et al. 2003). Although this population is still growing, the rate of increase in pup production has declined from 2004 on (Bowen et al. 2007a, 2007b). Bowen et al. (2007b) estimated total Sable Island pup production in 2007 was 54 500 (SE = 1 288). The annual rate of increase in pup production for the non-Sable Island (Gulf of St. Lawrence and east coast of Nova Scotia) has been more variable, in part owing to unstable ice conditions (Hammill et al. 2007). Hammill et al. (2007) estimated total non-Sable Island pup production to be 12 964 (SE = 595) in 2007.

Extensive pup marking programs have taken place in the Gulf of St. Lawrence and on Sable Island (Lavigneur and Hammill 1993; Stobo et al. 1990). Of the animals marked on Sable Island, 26 individuals have been observed in the Nantucket Sound area (Rough 1991, 1992, 1993, 1994a, 1994b, 1994c, 1995, 2000; Wood LaFond 2009) and 13 juveniles marked in the Gulf of St. Lawrence were observed in the Gulf of Maine (Lavigneur and Hammill 1993). Some of the current breeders in the US have been observed with brands and tags indicating that they had been born on Sable Island. Thus, these two Canadian sites were both considered potential source populations for recolonized sites in the US.

We undertook genetic analyses of the northwest Atlantic grey seal population to assess the relationship between pupping sites in the US and Canada. We sought to identify the source population for the US grey seals; this required determining the relationship between the two potential source populations (Sable Island and Gulf of St. Lawrence). An RFLP (restriction fragment length polymorphism) analysis

(Boskovic et al. 1996) showed little or no difference in mtDNA haplotypes between these two populations, but their sample size was relatively small. In this study, we used nine microsatellite loci and sequenced a fragment of the mtDNA control region to provide a more comprehensive understanding of stock structure. In addition, we sought to determine the genetic diversity of seals pupping in the US compared with those pupping in Canada to understand whether the US population is the result of a single founder event or continual immigration from the Canadian populations.

Materials and methods

Sample collection and storage

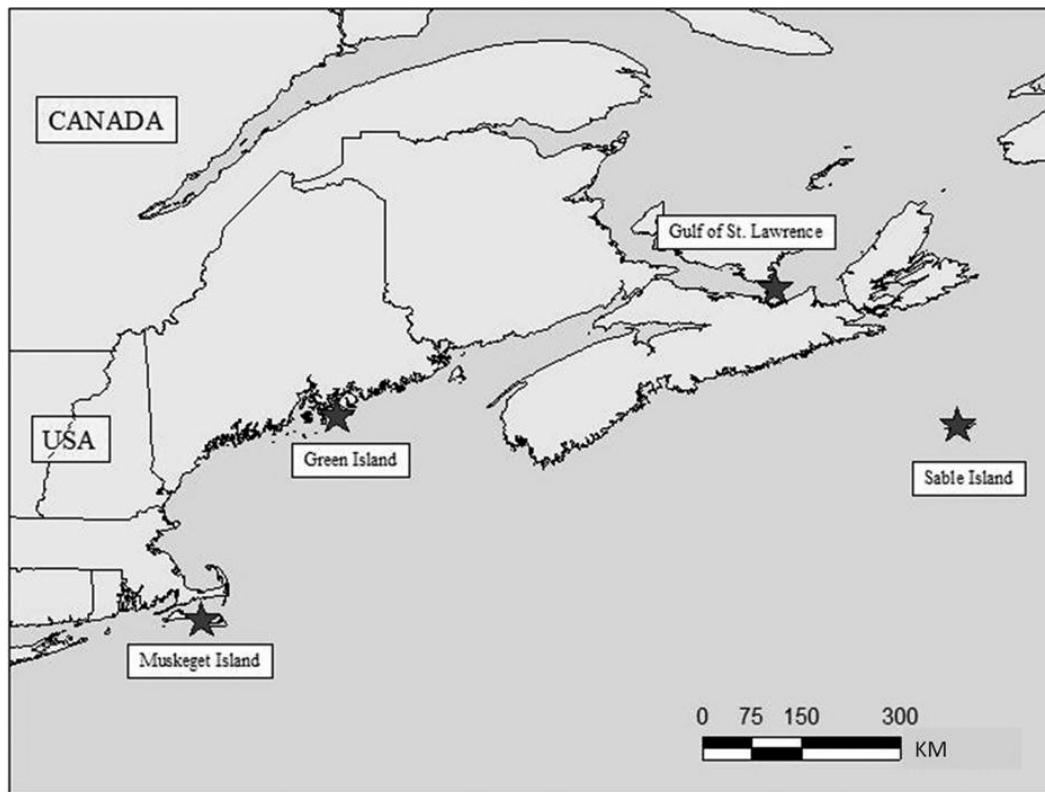
A total of 231 tissue samples were collected from grey seals at 4 pupping sites in the northwest Atlantic: Muskeget Island (US) ($n = 101$); Green Island (US) ($n = 15$); Sable Island (CAN) ($n = 65$); and Gulf of St. Lawrence (CAN) ($n = 50$) (Fig. 1). Tissue collection occurred during the pupping season (January and February) from 2002 to 2004. The majority of the tissue samples ($n = 204$; 79%) were collected from the hind flipper of live pups (Allen et al. 1995). The remainder was collected from beach carcasses or animals taken for scientific purposes. Tissue samples were stored in a 20% dimethylsulphoxide (DMSO) solution saturated with NaCl at room temperature (Seutin et al. 1991).

Extractions

Skin samples were prepared for DNA extraction using protocols commonly used for marine mammal tissues (Wang et al. 2008). For each sample, ~40 mg of tissue, was added to 400 μ L of lysis buffer and rotated at room temperature for ~5 days. Subsequently, three spikes of proteinase *K*, each of 2 U/mg of tissue (1 U \approx 16.67 nkat of enzyme), were added to each sample. After adding the first spike, the samples were rotated at room temperature overnight. After adding the second spike, the samples were placed in a 65 °C water bath for 1 h, then transferred to a 37 °C incubator in a tray with some of the 65 °C water, where they incubated for 1 h to slowly bring the sample temperature down to 37 °C. A third spike of proteinase *K* was subsequently added and the samples rotated overnight at room temperature. Lysis buffer was purchased from Applied Biosystems and the proteinase *K* was purchased from Roche Diagnostics Corp. Fifty microlitres from each sample were transferred into 96-well PCR (polymerase chain reaction) plates, the DNA was extracted using the magnetic beads approach (e.g., Rudi et al. 1997) using MagneSil paramagnetic beads (Promega), and eluted in 50 μ L of TE_{0.1}. The quantity of DNA obtained was estimated using PicoGreen (Singer et al. 1997), and the quality of DNA was examined by electrophoresis of 20 ng of DNA through 1.5% agarose gels stained with SYBR® Green I (Cambrex).

Microsatellite amplification and profiling

Samples were genotyped at nine microsatellite loci using the multiplex PCR protocol described in Table 2. The PCR cycling conditions used were as follows: (i) 5 min at 94 °C; (ii) 30 cycles of 94 °C for 30 s, annealing temperature (T_a) (Table 1) for 1 min, and 72 °C for 1 min; and (iii) a final extension step of 60 °C for 45 min. Reactions were carried out in 10 μ L volumes containing 1 \times PCR buffer (20 mmol/L

Fig. 1. Collection location of tissue samples from grey seals (*Halichoerus grypus*).**Table 1.** Sample sizes (*n*) for each region and analysis of grey seals (*Halichoerus grypus*).

	Green Island	Muskeget Island	Gulf of St. Lawrence	Sable Island	Northwest Atlantic total
Nine microsatellite loci	14	73	51	45	158
Mitochondrial control region sequence	12	63	51	37	163

Table 2. Amplification conditions and variability of the nine microsatellite loci from grey seals (*Halichoerus grypus*), included are the locus name, fluorescent label used, annealing temperature (T_a), concentration of primers used in polymerase chain reactions (PCR), reaction number (RXN No.), number of alleles detected, size range of alleles, observed heterozygosity (H_O), expected heterozygosity (H_E ; Nei 1978), polymorphic information content (PIC; Botstein et al. 1980), and reference for each locus.

Locus	Label	T_a (°C)	Primer ($\mu\text{mol/L}$)	RXN No.	Allele		H_O	H_E	PIC	Reference
					No. detected	Size range (bp)				
<i>SGPv9</i>	HEX	60	0.28	1	4	166–174	0.678	0.667	0.601	Goodman 1997
<i>SGPv11</i>	NED	60	0.1	1	8	163–177	0.662	0.683	0.626	Goodman 1997
<i>Hg3.6</i>	HEX	60	0.25	1	8	85–105	0.807	0.793	0.757	Allen et al. 1995
<i>Hgdii</i>	6FAM	60	0.4	1	7	118–138	0.725	0.697	0.662	Allen et al. 1995
<i>Hg6.3</i>	HEX	60	0.15	1	8	223–237	0.697	0.764	0.727	Allen et al. 1995
<i>Hg6.1</i>	NED	60	0.3	2	6	155–165	0.676	0.672	0.604	Allen et al. 1995
<i>Hg4.2</i>	HEX	60	0.05	2	7	146–160	0.717	0.708	0.658	Allen et al. 1995
<i>Hg8.10</i>	6FAM	60	0.5	2	4	187–193	0.713	0.698	0.642	Allen et al. 1995
<i>Hg8.9</i>	NED	60	0.4	2	9	197–213	0.762	0.705	0.677	Allen et al. 1995

Tris-HCl at pH 8.0, 50 mmol/L KCl); 0.05 U/ μL *Taq* polymerase (Invitrogen); 1.5 mmol/L MgCl_2 ; 0.2 mmol/L each dNTP (Invitrogen); and 10 ng of DNA. Primer concentrations varied for each locus (Table 2); primers labeled with 6FAM or HEX were purchased from Sigma-Genosys and primers labeled with NED were purchased from Applied Biosystems.

All PCR cycling was conducted on MJ Research PTC-225 DNA Engine Tetrad thermocyclers. After PCR amplification, products were desalted using 96-well G50 Sephadex plates, and size-separated and visualized on a MegaBACE™ 1000 (GE Healthcare). Allele sizes were estimated based on comparison with the MegaBACE™ ET550-R size standard that

Table 3. Natural logarithm of the probability of the data estimated by the program STRUCTURE, with the number of populations (K) ranging from 1 to 5 and 5 iterations of each population.

Iteration	K				
	1	2	3	4	5
1	-4393.5	-4402.3	-4447.6	-4487.4	-4789.0
2	-4392.2	-4482.3	-4427.9	-4479.5	-4411.8
3	-4391.8	-4575.2	-4491.1	-4403.1	-4476.2
4	4392.9	-4549.9	-4442.7	-4420.3	-4392.7
5	-4389.4	-4536.9	-4391.4	-4404.6	-4414.9
Mean	-4392.0	-4509.3	-4440.1	-4439.0	-4496.9

Note: Value in boldface type indicates the mean K with the highest probability.

was combined with each sample, and alleles were scored using the Genetic Profiler version 2.0 software (GE Healthcare); all scores were confirmed or edited by eye.

Allele frequencies, polymorphic information content (PIC) (Botstein et al. 1980), observed heterozygosity (H_O), and expected heterozygosity (H_E) (Nei 1978) were estimated using CERVUS version 2 (Marshall et al. 1998), and the data were tested for deviations from Hardy–Weinberg equilibrium based on the Markov chain Monte Carlo (MCMC) approach of Guo and Thompson (1992), as implemented in GENEPOP (Raymond and Rousset 1995a).

Population differentiation was assessed in two ways: (1) based entirely on genetic data, without a priori inference of potential subpopulations using the program STRUCTURE (Pritchard et al. 2000); and (2) by testing genetic differentiation between the putative subpopulations (Sable Island, Gulf of St. Lawrence, Muskeget Island, and Green Island). For the STRUCTURE analyses, the program was run with 100 000 MCMC steps as the burn-in time and 500 000 steps with recorded results, allowing for admixture and a correlation of allele frequencies between populations. The analyses were run allowing the sampled individuals to represent from one to five populations ($K = 1-5$), and five iterations of the analyses were performed for each K . The mean probability of the five runs for each K was taken as the probability (Table 3).

For the analyses based on a priori assumptions of population structure, pairwise F_{ST} estimates, and when possible exact tests of population differentiation, were obtained using GENEPOP (Raymond and Rousset 1995a, 1995b); otherwise MCMC methods were used (dememorization = 10 000; batch = 500; iterations = 10 000). To visualize the differentiation between grey seals from Sable Island and Gulf of St. Lawrence, the probability of identity (P_{ID} ; Paetkau and Strobeck 1994) was calculated for each individual based on (i) the allele frequencies in the Sable Island population and (ii) allele frequencies in the Gulf of St. Lawrence population. When plotted on a two-dimensional graph, these probabilities visualize how different the genetic profiles are in the two putative subpopulations and where individuals from Muskeget and Green islands fit in (see Wilson et al. 2000).

mtDNA amplification and sequencing

Primers LGL 283 and LGL 1115 (Bickham et al. 1996) were used to amplify a 319 bp fragment of the mitochondrial control region, which overlaps with the corresponding hyper-

variable region II of the human genome. PCR amplification conditions were the same as described for the microsatellite profiling, but with an annealing temperature of 50 °C. PCR products were purified using ExoSAP-IT™ (Dugan et al. 2002) and sequencing reactions were performed using the DYEnamic™ dye terminator kit (GE Healthcare). Products were size-separated and visualized on a MegaBACE™ 1000 (GE Healthcare).

All sequences were visually edited in Bioedit version 6.0.7 (Hall 1999) and aligned using ClustalX version 1.8 (Thompson et al. 1994). Estimates of the transition to transversion ratio and the α value of the gamma distribution for heterogeneity of mutation rates across sites were obtained with TREE-PUZZLE version 5.2 (Schmidt et al. 2002). Phylogenetic analyses were conducted using two methods: (1) the quartet-puzzling maximum likelihood approach implemented by TREE-PUZZLE (Strimmer and von Haeseler 1996) and (2) a distance-based approach using the F84 model of molecular evolution (formally described in Kishino and Hasegawa 1989), the Fitch–Margoliash tree-building algorithm (Fitch and Margoliash 1967), and 1000 bootstrapped data sets, as implemented in PHYLIP version 3.62 (Felsenstein 2004). In both analyses, two outgroups were used: one leopard seal (*Hydrurga leptonyx* (de Blainville, 1820)) and one Weddell seal (*Leptonychotes weddelli* (Lesson, 1826)). ARLEQUIN version 2.000 (Schneider et al. 2000) was used to estimate nucleotide diversity (π ; Nei 1978) and assess population differentiation (F_{ST} and Φ_{ST}) (Excoffier et al. 1992) based on the mitochondrial sequence data. The significance of these estimates was tested based on 1000 permutations.

The probability of finding a new haplotype was estimated to confirm that seals at the four pupping sites had been sampled sufficiently. The software MATLAB version 6.5.1 (The Mathworks, Natick, Massachusetts, USA) was used to estimate the probability of finding new haplotypes with increased sample size, from bootstrap resampling of individuals' haplotypes within each site (resample $n = 5000$).

Results

Microsatellites

All nine sites were polymorphic and the number of alleles per locus ranged between 4 and 9, with a mean of 7.22. All nine microsatellite loci were in Hardy–Weinberg equilibrium (Table 2). No significant population structure was found within the data set. A comparison of Sable Island vs. the

Fig. 2. Probability of identity (POI) is the likelihood that a specific genotype (based on the microsatellite loci) is found in a population, given the allele frequencies of that population. The axes represent the natural log (\ln) of the POI of the potential source populations: Sable Island and the Gulf of St. Lawrence. Most individual grey seals (*Halichoerus grypus*) fall along the 1:1 line, indicating that their genotype is almost as likely to be found on Sable Island as in the Gulf of St. Lawrence.

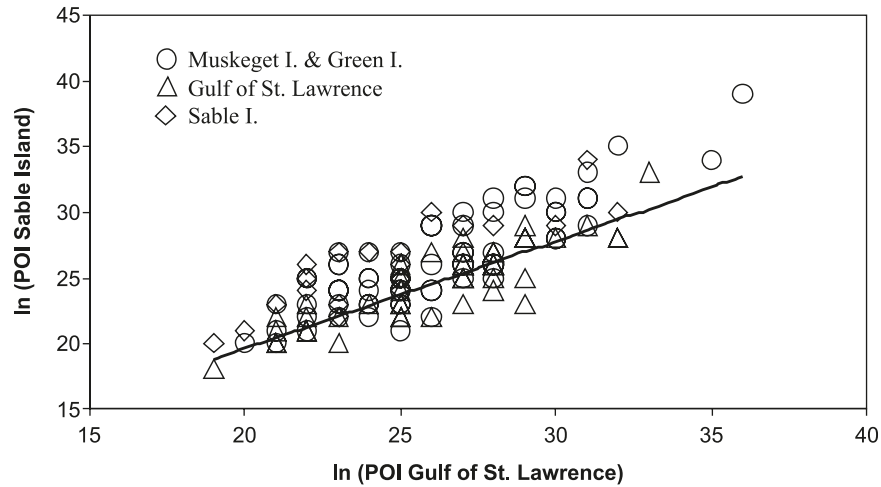


Fig. 3. Haplotype diversity at the four pupping sites of grey seals (*Halichoerus grypus*).

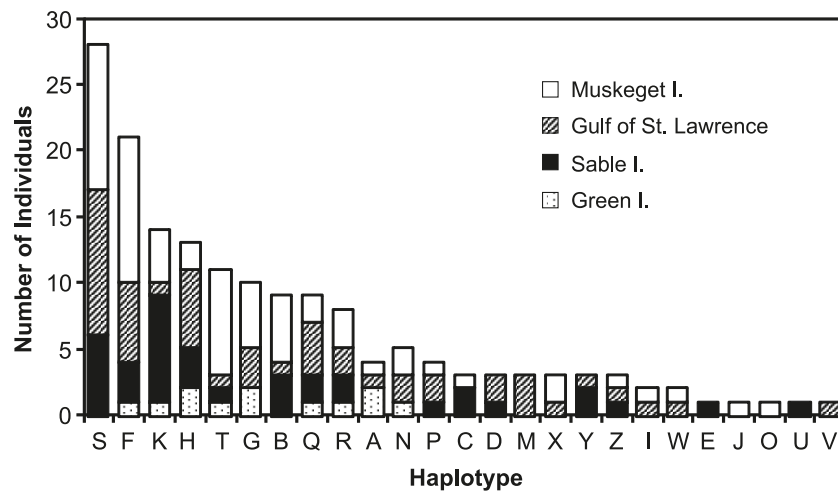


Table 4. Number of haplotypes and nucleotide diversity (π) from mtDNA from grey seals (*Halichoerus grypus*).

Site	No. of haplotypes	π
Sable Island (SI)	15	0.013750
Gulf of St. Lawrence (GSL)	20	0.010729
US	19	0.011567
Canada (SI and GSL)	25	0.012066

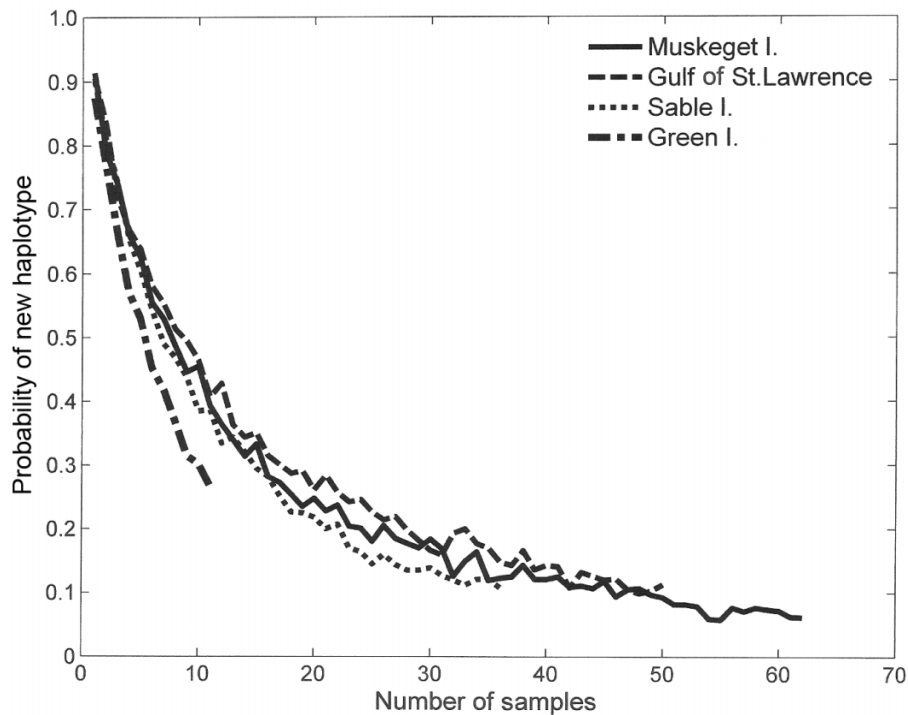
Gulf of St. Lawrence data sets gave a F_{ST} value of 0.0056 (MCMC, $p = 0.06493$). A comparison of the Canadian and US data set gave a F_{ST} value of 0.0009 (MCMC, $p = 0.02022$). The significant result of the MCMC test for the Canadian vs. US comparison appears to be the result of one locus (*Hg8.9*) being significantly different ($p = 0.00061$). The other eight loci were not different. When the program STRUCTURE was run with the entire data set, all individuals were consistently placed into one population providing further evidence that grey seals in Canada and the US are one interbreeding population.

Probability of identity (POI) is the likelihood that a specific genotype (based on the microsatellite loci) is found in a population, given the allele frequencies of that population. Our results show that most individuals fell along the 1:1 line, indicating that their genotype is almost as likely to be found in the Sable Island population as in the Gulf of St. Lawrence population (Fig. 2).

mtDNA

A 319 bp fragment of the mitochondrial control region was sequenced for 163 grey seals yielding 25 haplotypes. The most common haplotype was S, which occurred at a frequency of 17% (Fig. 3). Six of the 25 (24%) haplotypes were found in only one individual. Nucleotide diversity was similar at the Gulf of St. Lawrence, Sable Island, and the US (Muskeget and Green islands pooled) (Table 4). There was no significant difference between the Sable Island and the Gulf of St. Lawrence populations based on mtDNA haplotypes ($\Phi_{ST} = 0.01223$, $p = 0.15347$; $F_{ST} = 0.01199$, $p = 0.09983$). There was also no significant difference between the Canadian and the US populations ($\Phi_{ST} = -0.00242$, $p =$

Fig. 4. Probability of finding new haplotypes with increased sample size, from bootstrap resampling of haplotypes of individual grey seals (*Halichoerus grypus*) within each site.



0.55901; $F_{ST} = 0.00390$, $p = 0.19041$). A Fitch–Margoliash tree also did not show any geographic pattern among the 25 North American haplotypes.

Probability of finding new haplotypes

For three of the geographic areas (Sable and Muskeget islands and Gulf of St. Lawrence), the probability of finding new haplotypes if more samples were obtained is very low (Muskeget Island = 0.06; Sable Island = 0.11; the Gulf of St. Lawrence = 0.11). The probability of finding new haplotypes at the fourth area (Green Island) is 0.27, indicating that more samples are needed to capture the full genetic diversity at this site (Fig. 4).

Discussion

These analyses demonstrate that there is little genetic differentiation in the northwest Atlantic grey seal population. There is no significant difference between the Sable Island and the Gulf of St. Lawrence populations based on either mtDNA sequences or the nine microsatellite loci. Boskovic et al. (1996) used RFLP analyses to compare Sable Island and Gulf of St. Lawrence grey seals and also found no significant genetic difference between the two sites. Because these two Canadian populations were genetically indistinguishable from each other, it was not possible to specify one as the source population of the US seals. The POI analysis (Fig. 2) demonstrates that microsatellite allele frequencies were similar in the two potential source populations (Sable Island and Gulf of St. Lawrence), and that an individual seal's given genotype was as likely to be found in the Sable Island population as the Gulf of St. Lawrence population.

The microsatellite F_{ST} shows little genetic variation between the Canadian and the US populations. The analysis

using the program STRUCTURE consistently placed all individuals in the sample set into one population.

Mitochondrial DNA nucleotide diversity in the US population (Muskeget and Green islands) was comparable with that of the Canadian population (Sable Island and Gulf of St. Lawrence). The re-establishment of the US grey seal population shows no founder effect. US seals are not simply a small subset of immigrants but are a genetically representative sample of the northwest Atlantic population at large. The recolonization is best explained as a continuous process of immigration from the Canadian population, as evidenced by the fast and sustained increase in US pup counts over the last 20 years (Wood LaFond 2009).

What processes would be responsible for emigration to occur in a species known to be highly philopatric (Pomeroy et al. 1994; Twiss et al. 1994)? In their study of the pattern of grey seal colonization in the UK, Gaggiotti et al. (2002) used a combination of genetic data and field observations to demonstrate that the most important sources for new grey seal pupping colonies were populations of medium or large size with rate of increase close to zero. These results point to an important role of density-dependent emigration from the source colonies. Gaggiotti et al. (2002) hypothesized that adult females should show a preference for their natal site as long as there is sufficient pupping space.

In contrast to the UK, the source populations in the northwest Atlantic (Sable Island and the Gulf of St. Lawrence) have continued to increase during the period of recolonization of US pupping sites (Bowen et al. 2007a; Hammill et al. 2007). The rate of increase in pup production on Sable Island (accounting for 85% of pups born in Canada), declined from 12.8% to 7% per year between 2004 and 2007 (Bowen et al. 2007a) and to 4% between 2007 and 2010 (W.D. Bowen, unpublished data).

Furthermore, in comparing female cohorts from 1998 to 2002 to cohorts from the mid- to late 1980s, age at primiparity was significantly delayed in the latter cohorts compared with the former cohorts (Bowen et al. 2007a, 2007b). This shift indicates that life-history parameters for this population are changing. Thus, density dependence at the source colonies may be playing some role in the re-establishment of grey seal pupping sites in the US as it has in the UK. Nevertheless, recolonization in the US clearly began prior to the expression of density dependence at Sable Island. Therefore, other factors must have been responsible for initiating US recolonization. Range expansion associated with the rapidly growing large population of grey seals is one possible explanation. Although comparative data prior to recolonization are lacking, it is clear from satellite tagging of adult Sable Island grey seals since 1995 that as the population increased the at-sea distributional range also increased to encompass all of the Scotian shelf and areas off the northeastern US (e.g., Breed et al. 2006, 2009). Thus, population-size-mediated range expansion might account for the initial recolonization of US waters.

Acknowledgements

The project was funded by a National Marine Fisheries Service (NMFS) – Sea Grant Fellowship in Population Dynamics Program (to S.A. Wood), NOAA's NMFS–NEFSC, and the University of Maine. We thank all of the field researchers who collected samples on Muskeget, Green, and Sable islands and in the Gulf of St. Lawrence. Paul Wilson brought the mtDNA primers to our attention. Richard Merrick, Michael Rex, and two anonymous reviewers provided valuable comments on an earlier version of the manuscript.

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