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ORIGINAL ARTICLE

Extensive mitochondrial introgression in North American Great Black-backed Gulls (*Larus marinus*) from the American Herring Gull (*Larus smithsonianus*) with little nuclear DNA impact

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Recent genetic studies have shown that introgression rates among loci may greatly vary according to their location in the genome. In particular, several cases of mito-nuclear discordances have been reported for a wide range of organisms. In the present study, we examine the causes of discordance between mitochondrial (mtDNA) and nuclear DNA introgression detected in North American populations of the Great Black-backed Gull (*Larus marinus*), a Holarctic species, from the Nearctic North American Herring Gull (*Larus smithsonianus*). Our results show that extensive unidirectional mtDNA introgression from *Larus smithsonianus* into *Larus marinus* in North America cannot be explained by ancestral polymorphism but most likely results from ancient hybridization events occurring when *Larus marinus* invaded the North America. Conversely, our nuclear DNA results based on 12 microsatellites detected very little introgression from *Larus smithsonianus* into North American *Larus marinus*. We discuss these results in the framework of demographic and selective mechanisms that have been postulated to explain mito-nuclear discrepancies. We were unable to demonstrate selection as the main cause of mito-nuclear introgression discordance but cannot dismiss the possible role of selection in the observed pattern. Among demographic explanations, only drift in small populations and bias in mate choice in an invasive context may explain our results. As it is often difficult to demonstrate that selection may be the main factor driving the introgression of mitochondrial DNA in natural populations, we advocate that evaluating alternative demographic neutral hypotheses may help to indirectly support or reject hypotheses invoking selective processes.
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INTRODUCTION

Recent genetic studies have shown that at least 25% of plant species and 10% of animal species are involved in natural hybridization and potential introgression (Mallet, 2005). In birds, around 10% of species are known to hybridize (Grant and Grant, 1992), with some families having a strong propensity for hybridization. In Paradisaeidae (birds of paradise), for example, 43% of species hybridize in nature (Mallet, 2005). How these species can maintain their behavioral, phenotypic and ecological differences in the face of prevailing interspecific gene flow is an exciting question for speciation studies (for example, Pinho and Hey, 2010; Rice *et al.*, 2011). Relevant information regarding the consequences of interspecific gene flow on a species' genome can be gained from examining variations in the pattern of introgression among loci (for example, Barton and Hewitt, 1985; Barton and Gale, 1993; Gay *et al.*, 2007; Luttikhuizen *et al.*, 2012). Indeed, one general pattern that emerges from recent studies is that introgression rates among loci may greatly vary according to their location in the genome. In particular, several studies have reported cases of mito-nuclear discordances with introgression of mitochondrial DNA and low or even apparent absence of nuclear introgression (fish: Wilson and Bernatchez, 1998, birds: Bellemain *et al.*, 2008; Irwin *et al.*, 2009, mammals: Roca *et al.*, 2005; Good *et al.*, 2008; Melo-Ferreira *et al.*, 2009, amphibians: Zieliński *et al.*, 2013). The reverse situation, high nuclear introgression with low mitochondrial introgression or lack of mitochondrial introgression has also been observed in butterflies, a primate species and birds species (Steeves *et al.*, 2010, see also Petit and Excoffier, 2009 for a review).

Several factors have been postulated to explain these mito-nuclear discrepancies (reviewed by Toews and Brelsford, 2012). They can be divided between selection on mitochondrial DNA variants and demographic processes, such as sex-biased dispersal and sex-specific premating or postmating barriers. One key factor enabling researchers to disentangle demographic and selective factors shaping mito-nuclear discrepancies is the amount of nuclear introgression relative to mitochondrial introgression. Specifically, as autosomal loci are transmitted by both sexes, it is difficult to imagine how demographic

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processes such as sex-biased gene flow could result in substantial mitochondrial introgression without detectable traces of nuclear introgression (see Renoult *et al.*, 2009).

Among birds, Laridae are famous for their propensity to hybridize in nature (see Olsen and Larsson, 2004), especially the group known as 'the large white-headed gulls' (Sonsthagen *et al.*, 2012) or 'the Herring Gull complex' (Sternkopf *et al.*, 2010). These species include some of the best documented avian hybrid zones (Hoffman *et al.*, 1978; Bell, 1997; Good *et al.*, 2000; Good, 2002; Gay *et al.*, 2007, 2009; Neubauer *et al.*, 2009), and are remarkable for their low level of interspecific genetic differentiation compared with most biological species of birds, resulting from recent speciation events and on-going interspecific gene flow (de Knijff *et al.*, 2001; Crochet *et al.*, 2003; Vigfúsdóttir *et al.*, 2008; Sternkopf *et al.*, 2010; Sonsthagen *et al.*, 2012). Extensive introgression of mtDNA has been documented in several species (Crochet *et al.*, 2003; Gay *et al.*, 2007; Sternkopf *et al.*, 2010; Sonsthagen *et al.*, 2012). They thus constitute a promising model to address the causes of discordance between mtDNA and nuclear DNA introgression and the consequences of interspecific hybridization on the outcome of speciation processes.

Extensive mtDNA introgression has been documented between Great Black-backed Gull (*Larus marinus*) and American Herring Gull (*Larus smithsonianus*) in North America (Crochet *et al.*, 2003; Sternkopf *et al.*, 2010). Great Black-backed Gull (MARINUS hereafter) is a Holarctic species distributed in Western Europe, Greenland and eastern North America (Olsen and Larsson, 2004) and is a member of a clade comprising several western European gull species closely related to the Herring Gull (Crochet *et al.*, 2003; Liebers *et al.*, 2004; Sternkopf *et al.*, 2010). American Herring Gull (SMITHSONIANUS hereafter) is widely distributed in North America; it groups with the 'Arctic species clade', which is the sister group to the European species clade (Crochet *et al.*, 2002; Liebers *et al.*, 2004; Sternkopf *et al.*, 2010) and is thus not the closest relative of MARINUS. Genetic data suggest that MARINUS originated in Europe and colonized eastern North American coast where it came into secondary contact with SMITHSONIANUS (Crochet *et al.*, 2003; Sternkopf *et al.*, 2010). Currently, a large proportion of North American MARINUS carries SMITHSONIANUS mtDNA haplotypes (the SMI haplotype hereafter) (Sternkopf *et al.*, 2010). As only American MARINUS share SMI mtDNA haplotypes with SMITHSONIANUS, whereas SMI haplotypes are completely absent from Europe (even in other species of the Herring Gull complex), MARINUS populations in North America likely received SMI haplotypes by introgression rather than through the persistence of ancestral polymorphism in both species.

In this study, we aim to enhance our understanding of the evolutionary processes promoting mtDNA introgression by directly testing for the genetic contribution from SMITHSONIANUS to the nuclear gene pool of North American MARINUS. As discussed above, an agreement between the amount of nuclear and mtDNA introgression would indicate that neutral processes drive mtDNA introgression, while a large discrepancy in introgression rate would call for alternative explanations. We first assayed cytochrome *b* (cyt *b*) and control region fragments to measure mitochondrial introgression in samples of North American populations of SMITHSONIANUS and MARINUS. We then compare results of multivariate analysis (Factorial Correspondence Analysis (FCA)) and assignment methods (NewHybrids, based on Anderson and Thompson, 2002) between European and North American individuals of MARINUS using 12 microsatellite loci. If SMITHSONIANUS contributed to the nuclear gene pool of North American MARINUS, we would expect that (1) North American MARINUS are situated between European MARINUS and SMITHSONIANUS in bivariate plots of factorial scores and (2) assignment scores also differ between European and North American MARINUS, with North American MARINUS individuals having lower probabilities of assignment to MARINUS and higher to SMITHSONIANUS than European individuals. Field data suggest that contemporary hybridization is very rare in North America (Jehl, 1960; Andrle, 1972), suggesting introgression through historical hybridization events when MARINUS colonized North America probably during late Pleistocene or Holocene rather than contemporary gene flow. We first evaluated this hypothesis with NewHybrids, testing for the absence of individuals originating from recent hybridization events (F1 or backcrosses) between SMITHSONIANUS and MARINUS in our samples from North America. We also compared FCA scores and assignment probabilities of North American MARINUS specimens carrying SMI and MAR (the original MARINUS haplotype, see Crochet *et al.*, 2003) haplotypes: if they do not differ, then recent hybridization events contribute very little (at best) to current lineage sharing.

MATERIALS AND METHODS

We obtained tissue samples (blood, muscle or feathers, $n = 116$) from 49 North American *L. marinus* individuals, 15 European *L. marinus* individuals and 52 *L. smithsonianus* individuals were collected or obtained through museum tissue loans. Thirty-two tissue samples were collected from unrelated chicks at the Iles Sainte-Marie, Quebec, Canada. Other tissue samples were obtained mainly from adults that were collected in diverse localities spread over eastern and southern USA and do not necessarily correspond with birds sampled close to breeding locations. Information on sampling locations is given in the Table 1 and additional information on specimens and localities assayed in the study is reported in Appendix 1. In addition, 19 HVR-I sequences available in GenBank (see Appendix 1) were included in the analyses.

Table 1 Geographical distribution and frequency of specimens assayed in this study

Localities	*L. marinus*		*L. smithsonianus*
	European	*North American*	*North American*
Europe			
Denmark	1		
Faroe islands	9[a]		
Finland	6		
France	4		
Sweden	5		
Canada			
Quebec		12	20
Ontario			11[a]
United States			
Alaska			5
California			3
Louisiana		2	7
Maine		4	
Massachusetts		2	
Maryland		6	7
Missouri		2	
New York		16	8
New Jersey			2
North Carolina		2	
Virginia		3	

[a]Genbank sequences (Liebers *et al.*, 2004), see Appendix 1 for details.

Mitochondrial DNA (mtDNA) sequencing

We amplified a 391 base pair (bp) fragment of the HVR-I part of the control region using primers L15610 5′-TTACCCCCCCACAWCATCATGTGG-3′ (designed for this study) and H519 (Liebers *et al.*, 2001) or L15522 and H1816 (Sonsthagen *et al.*, 2012). A 389-bp cyt *b* fragment was amplified using primers L14967, H15503 or H15938 (Pons *et al.*, 2004). The amplification protocol was: 4 min at 94 °C, followed by 36 cycles of 94 °C for 40 s, 52–60 °C for 45 s, 72 °C for 40–50 s, with a final extension at 72 °C for 10 min. Cycle-sequencing reactions were performed using the CEQ Dye Terminator Cycle Sequencing kit (Beckman Coulter, Inc., Fullerton, CA, USA) or the Big Dye (Applied Biosystems Inc., Foster City, CA, USA) terminator chemistries kit using L15610 and H519 or H419 and H519 (Liebers *et al.*, 2001). PCR amplifications were sequenced on automated sequencers (CEQ2000 DNA Analysis System (Beckman Coulter Inc., Fullerton, CA, USA) or ABI3100 (Life Technologies, Carlsbad, CA, USA)). Sequences were aligned using Bioedit software (Hall, 1999). Alignment of the HVR-I sequences required the insertion of single-nucleotide gaps at one site, which were deleted before analyses. Cyt *b* sequences did not contain any unexpected stop-codons.

Microsatellites genotyping

Following preliminary testing on large white-headed gull species (see Sonsthagen *et al.*, 2012), 12 polymorphic loci were scored on all tissue samples: Hg16, Hg18, Hg25 (Crochet *et al.*, 2003), K16 (Tirard *et al.*, 2002), LarZAP12, LarZAP19, LarSNX24, LarZAP26 (Gregory and Quinn 2006), Rbg13, Rbg18, Rbg27, and Rbg29 (Given *et al.*, 2002). PCR amplifications and subsequent genotyping followed Sonsthagen *et al.* (2012). Ten percent of the samples were amplified and genotyped in duplicate for quality control, and no inconsistencies in genotype scores were observed between replicates.

Data analyses

Mitochondrial DNA. Standard diversity indices (number of substitutions, haplotype diversity (h), nucleotide diversity (π)) were calculated using Arlequin 3.1 (Excoffier *et al.*, 2005). Tajima's D statistic (Tajima, 1989) and Fu's F_S statistic (Fu, 1997) were computed in Arlequin 3.1 to test for selective neutrality. Analyses of molecular variance were performed using the Kimura two parameter model (K2P; Kimura, 1980); the closest nucleotide substitution model available in Arlequin 3.1 to the K2P with an invariant site parameter (K2P + I) model, which was selected as the best model for our HVR-I sequence data in Modeltest 3.4 (Posada and Crandall, 2001). To visualize the relationships among haplotypes and the sharing of haplotypes among species and geographical populations, median-joining networks based on HVR-I and cyt *b* haplotypes were constructed using Network 4.2 (Bandelt *et al.*, 1999).

Microsatellites. Measures of genetic diversity, population differentiation and departure from Hardy–Weinberg equilibrium were calculated in Genetix version 4.05 (Belkhir *et al.*, 1996–2004). As several loci exhibited significant F_{IS}, which can be due among other causes to null alleles, we examined our data with the software Microchecker (van Oosterhout *et al.*, 2004).

We used the software NewHybrids version 1.1 beta 3 (implementing a method described in Anderson and Thompson, 2002) to obtain assignment probabilities of every individual to each of the following categories: pure MARINUS, pure SMITHSONIANUS, F1 hybrids, and backcross to MARINUS and to SMITHSONIANUS.

We retained results based on uniform prior for the mixing proportions and the allelic frequencies (see NewHybrids documentation) as they gave more consistent results (smaller number of individuals misclassified based on *a priori* identification, see Discussion). We tried to use the z option, which allows specifying individuals of pure ancestry from each population, but results were generally less consistent. This is certainly because we had very few SMITHSONIANUS specimens sampled outside the zone of sympatry with MARINUS, and most of them were from Alaska. Substantial introgression from other large gull species into SMITHSONIANUS in Alaska has been well documented (Williamson and Peyton, 1963), and our Alaskan specimens had low probabilities of assignment to SMITHSONIANUS in all runs (with or without the z option), indicating that they had multilocus genotypes not representative of other North American SMITHSONIANUS. Labeling them as 'pure' SMITHSONIANUS therefore reduced the reliability of the results. The number of sweeps for burn in was 500 000, and the results were based on 1 000 000 sweeps.

As genetic differentiation between the two parental populations (SMITHSONIANUS and European MARINUS) is rather low, we tested for the reliability of NewHybrids with our populations by using Hybridlab (Nielsen *et al.*, 2001) to simulate random multilocus genotypes, based on observed allele frequencies. We generated virtual genotypes of pure European MARINUS, pure SMITHSONIANUS, F1 hybrids, backcross to SMITHSONIANUS and backcross to MARINUS (100 genotypes for each class) and analyzed them with NewHybrids, using the same options as for the real specimens.

For several individuals, genotypes were missing at one or more loci. As missing data could affect the reliability of the results, we first attempted to reduce the number of loci, hence reducing the number of missing data for some specimens. Two loci (Lar19 and Rbg18) did not exhibit any significant differentiation (F_{ST} values, respectively, 0.029, $P = 0.11$ and 0) between European MARINUS and SMITHSONIANUS and were excluded from subsequent analyses. We then performed separate NewHybrids analyses for individuals with at most four missing loci, for individuals with at most two missing loci and for individuals with no missing data and tested for differences in mean assignment scores between European and North American MARINUS for the three NewHybrids analyses.

We also used FCA of individual multilocus genotypes (FCA, in its modified version for diploid organisms: She *et al.*, 1987, Guinand, 1996) as implemented in Genetix version 4.05 to test whether North American MARINUS gene pool was influenced by contributions from SMITHSONIANUS. For analysis of assignment scores, we used the logit (= Log(probability/(1 − probability))) of the assignment probabilities, as assignment probabilities were not normally distributed. Number of missing loci was included in all models as a covariate, as it may affect assignment probabilities. FCA scores were analyzed directly using the same model (no logit transformation) as they were suitably distributed.

RESULTS

Mitochondrial DNA

All genetic diversity estimates highlighted that the European MARINUS population was more variable than their non-introgressed and introgressed North American counterparts. Nucleotide diversity and the mean number of pairwise differences were twofold higher in Europe (Table 2). Tajima's test of selective neutrality and Fu's test did not reject the neutral model neither for European MARINUS nor for introgressed and non-introgressed North American MARINUS. Mismatch distribution analyses supported the sudden expansion model (raggedness test, $P > 0.50$ for all populations).

Pairwise Φ_{ST} values indicated that the introgressed MARINUS population was highly differentiated from both the non-introgressed North American and European MARINUS ($\Phi_{ST} > 0.7$, Table 3). Conversely, introgressed MARINUS was weakly differentiated from SMITHSONIANUS with only 7% of the variance explained by differences between populations. Expectedly, European and non-introgressed North American MARINUS populations exhibited little differentiation (Table 3).

The HVR-I median-joining network clearly showed that MARINUS haplotypes were clustered in two clades separated by four nucleotide substitutions (Figure 1a). One clade comprised all European haplotypes as well as MARINUS haplotypes observed in America. The two haplotypes in highest frequency were represented by gulls residing on both sides of the Atlantic Ocean while most of the other haplotypes are only observed in Europe. The second clade comprised all SMITHSONIANUS haplotypes with the two most frequent haplotypes also represented by North American MARINUS individuals. This overall structure of two clades is also observed among cyt *b* haplotypes (Figure 1b). None of the European individuals clustered with the SMITHSONIANUS clade except one European MARINUS from Sweden. This bird possesses a haplotype also found in the sympatric species

Table 2 Molecular diversity and tests of neutral evolution of the control region HVR-I of introgressed (I) and non-introgressed (NI) North American *Larus marinus* individuals and European *L. marinus* individuals, including number of haplotypes (*H*), number of substitutions (*NS*), haplotype diversity (*h*), nucleotide diversity (π), (*d*) mean number of pairwise differences, Tajima's *D* and Fu's F_S

	n	H	NS	h *(s.d.)*	π *(s.d.)*	d *(s.d.)*	*Tajima's* D		*Fu's* F_S	
							D	P	F	P
Europe										
(NI)	24	11	17	0.877 (0.043)	0.0082 (0.0049)	3.20 (1.72)	−1.43	0.06	−2.75	0.08
North America										
(NI)	21	6	6	0.738 (0.081)	0.0038 (0.0027)	1.48 (0.93)	−1.06	0.18	−1.01	0.25
(I)	28	8	9	0.733 (0.069)	0.0048 (0.0031)	1.88 (1.11)	−0.59	0.33	−1.64	0.19

Table 3 Genetic differentiation at the mitochondrial DNA HVR-I D-loop segment (Φ_{ST}; above the diagonal) and 12 microsatellite loci (F_{ST}; below the diagonal) among non-introgressed mtDNA (NI) and introgressed mtDNA European (EU) and North American (NA) *L. marinus* and *L. smithsonianus* populations

	L. marinus (EU, NI)	*L. marinus (NA, NI)*	*L. marinus (NA, I)*	*L. smithsonianus*
L. marinus (EU, NI)	—	0.084**	0.713***	0.749***
L. marinus (NA, NI)	0.034*	—	0.812***	0.808***
L. marinus (NA, I)	0.037*	−0.007	—	0.071**
L. smithsonianus	0.237***	0.193***	0.169***	—

*$P<0.05$; **$P<0.01$; ***$P<0.001$.

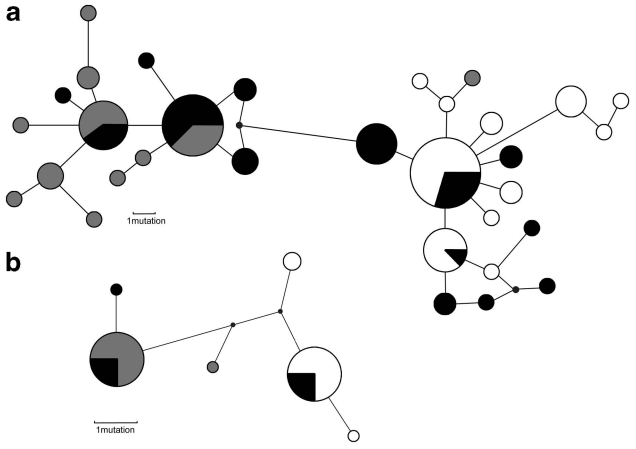

Figure 1 Median-joining networks illustrating relationships among mitochondrial DNA (**a**) HVR-I of the control region and (**b**) cyt *b* haplotypes. European *L. marinus* haplotypes are shown in grey (HVR-I $n=24$; cyt *b* $n=24$); North American *L. marinus* haplotypes in black (HVI non-introgressed $n=21$, introgressed $n=28$; cyt *b* non-introgressed, $n=11$, introgressed, $n=13$) and *L. smithsonianus* haplotypes in white (HVR-I $n=52$; cyt *b* $n=40$). The size of the node corresponds to the frequency of each haplotype and small black circles represent unsampled haplotypes.

L. argentatus (GenBank accession number AJ507814), and we hypothesize that it may have captured it by introgression from this species.

Out of the 49 North American MARINUS individuals included in this study, 57% possessed a SMI haplotype. Our sample did not allow us to test for geographical variation in the percentage of introgressed birds across America. It is nevertheless noteworthy that the proportion of introgressed individuals among MARINUS chicks ($n=12$) exceeded 80% in the breeding colonies of the Sainte-Marie islands located in Eastern Quebec. No specimens identified as SMITHSONIANUS were found carrying a MAR haplotype.

Microsatellites

Microsatellites diversity and genetic population structure. The amount of genetic variation within species (Table 4) is in line with previous estimates, partly based on the same samples (Crochet *et al.*, 2003). Genetic differentiation between SMITHSONIANUS and MARINUS (Table 3) is high compared with other species of large white-headed group (compare with Sonsthagen *et al.*, 2012), again consistent with previous estimates for the same species (Crochet *et al.*, 2003). There was a slight but significant differentiation between North American and European MARINUS, consistent with an expectation of reduced contemporary gene flow.

Significant departures from Hardy–Weinberg equilibrium were detected in the three main populations (SMITHSONIANUS, MARINUS Europe, MARINUS North America) as evidenced by the strongly significant global and positive F_{IS} value in all three populations (Supplementary Material S1). Such positive F_{IS} values indicate heterozygote deficiencies that can originate from population structure within species (Wahlund effect) but can also be generated by null alleles. Microchecker indeed suggested the presence of null alleles for five of the seven loci that exhibit significant F_{IS} for at least one population (Hg25, Lar12, Hg16, Rbg13 and HG18), which is supported by the high variance among loci for every population (Wahlund effect should show for all loci). As null alleles could affect results of assignment methods, we evaluated their performance by generating multilocus genotypes with the program Hybridlab.

Assignment of simulated individuals. Reliability of assignment by Newhybrids varied as expected, being very good for pure species and F1 hybrids but very poor for backcrosses. Using as classification criterion the category with the highest assignment probability, 90% of SMITHSONIANUS, 97% of F1 hybrids and, crucially, 100% of MARINUS were correctly assigned (see Supplementary Material S2). Backcrosses were correctly identified only in 25% (backcross with MARINUS) to 35% (backcross with SMITHSONIANUS) of the simulated genotypes. Misclassified backcrosses were assigned either to F1 hybrids or to the pure species most similar to the backcross type. To sum up, no pure MARINUS was misclassified; F1 hybrids would be readily detectable, but

only 60% of backcross with MARINUS were detected as 'non-pure' genotype (F1 or backcross).

Evidence for nuclear introgression. Due to amplification failures, one or more loci were missing for many individuals. The 'full' data set (at most four missing loci) included 104 of the 107 samples individuals, the 'max two missing' data set (at most two missing loci) included 92 individuals and the 'no missing loci' data set included 75 individuals. Both data sets with a variable number of missing data reveal a strong impact of missing data on the performance of the assignment method: effect of the number of missing loci on the assignment probability of MARINUS individuals to MARINUS $F(50,1) = 25.45$, $P < 0.00001$ and $F(41,1) = 5.24$, $P = 0.027$ for the 'full' and 'maximum two missing' data sets, respectively.

Most North American MARINUS were assigned to the category pure MARINUS with high probability values (Table 5, Figures 2a and b and Supplementary Material S2). Two individuals from Iles Sainte-Marie (Quebec, Canada) carried SMI haplotypes and were classified as pure SMITHSONIANUS with probability values typical of SMITHSONIANUS. More importantly, their assignment probabilities to either pure MARINUS or MARINUS-backcross were virtually zero. Their positions on the FCA plot (Figure 3, see below) were also completely outside the variation possible in North American MARINUS but fully typical of pure SMITHSONIANUS. As feathers of these two birds were obtained from chicks raised in a mixed colony where the two species bred (Chapdelaine G, personal communication), the most probable explanation for this odd result is a field identification error, and we have therefore removed these two individuals, which were most likely chicks of SMITHSONIANUS, from further analyses.

Table 4 Genetic variation at 12 microsatellites loci genotyped from European (EU) *L. marinus*, North American (NA) *L. marinus* (introgressed and non-introgressed individuals) and *L. smithsonianus* populations: sample size, average number of alleles per locus, observed and expected heterozygosity and F_{IS} (significance value based on 1000 permutations of alleles within populations)

Population	N	*Alleles/locus*	*Ho*	*He*	F_{IS}	P-*value*
L. marinus (EU)	15	3.5	0.335	0.398	0.202	0.001
L. marinus (NA)	47	5.5	0.357	0.460	0.236	<0.001
L. smithsonianus	49	6.6	0.490	0.576	0.159	<0.001

Table 5 Assignment of individuals (mtDNA non-introgressed (NI); mtDNA introgressed (I)) into one of the following categories: Pure *L. marinus*, Pure *L. smithsonianus*, first generation hybrid (F1), backcross to MARINUS (BCmar), and backcross to SMITHSONIANUS (BCsmi)

	Pure L. marinus	*Pure L. smithsonianus*	*F1*	*BCmar*	*BCsmi*
L. marinus (EU)	9	0	0	0	0
L. marinus (NA, NI)	16	0	0	0	1
L. marinus (NA, I)	18	2[a]	0	0	0
L. smithsonianus	0	43[b]	0	0	3[c]

Assignments are based on 10 microsatellites and individuals with two or fewer missing loci.
[a]Feathers for these two birds were collected from chicks raised in a mixed colony where the two species bred and likely represent a field sampling error (see text).
[b]Two birds, one from Alaska and one from Quebec, were assigned to this category with low PP values (0.52, 0.55). All other assignments received high PP values ranging from 0.65 to 0.99.
[c]These three birds were collected in Alaska where hybridization events have been noted between *L. smithsonianus* and other gull species (*L. vegae*, *L. glaucescens*). *L. marinus* does not occur in Alaska.

After removal of the two individuals misidentified in the field, there was no difference in assignment probabilities between European and North American MARINUS for the 'full' data set ($F(50,1) = 0.28$, $P = 0.602$) or 'maximum two missing' data set ($F(41,1) = 2.7565$, $P = 0.104$) but a slight difference for the 'no missing loci' data set ($F(30,1) = 4.44$, $P = 0.044$). In the last case, North American MARINUS have a slightly lower assignment probability to MARINUS than European birds (average assignment probability 0.96 in North America and 0.99 in Europe), with a small number of individuals in North America with rather low assignment probabilities (three individuals with assignment probabilities between 0.65 and 0.82, see Figure 2 and Supplementary Material S2). Note that the difference in the 'no missing loci' data set is not significant after correcting for multiple tests (that is, the same hypothesis was tested three times).

The first axis of the FCA on microsatellite data differentiated the two species, although a few individuals fall in an area of overlap (Figure 3). As for assignment probabilities, there was no difference in first axis scores between North American and European MARINUS ($F(50,1) = 1.58$, $P = 0.21$).

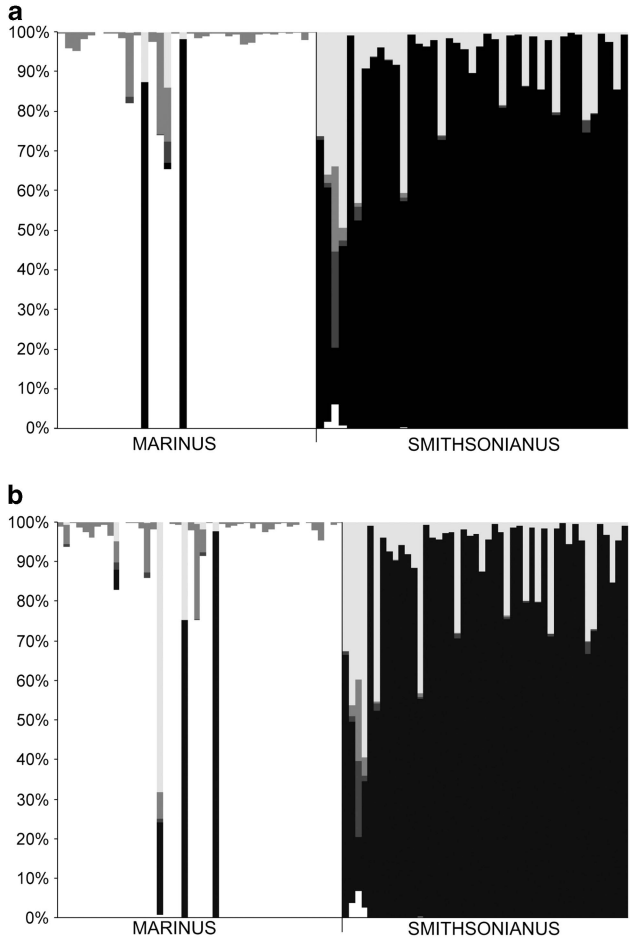

Figure 2 Assignment probabilities of North American *L. marinus* (MARINUS) and American Herring Gulls (SMITHSONIANUS) included in the study to the following categories: Pure *L. marinus* (MARINUS; white), Pure *L. smithsonianus* (SMITHSONIANUS; black), first generation hybrid (F1; dark gray), backcross to MARINUS (BCmar; light gray), and backcross to SMITHSONIANUS (BCsmi; light gray). Results of analyses of individuals with (**a**) no missing data at the 10 microsatellite loci and individuals with data (**b**) missing at two or fewer loci.

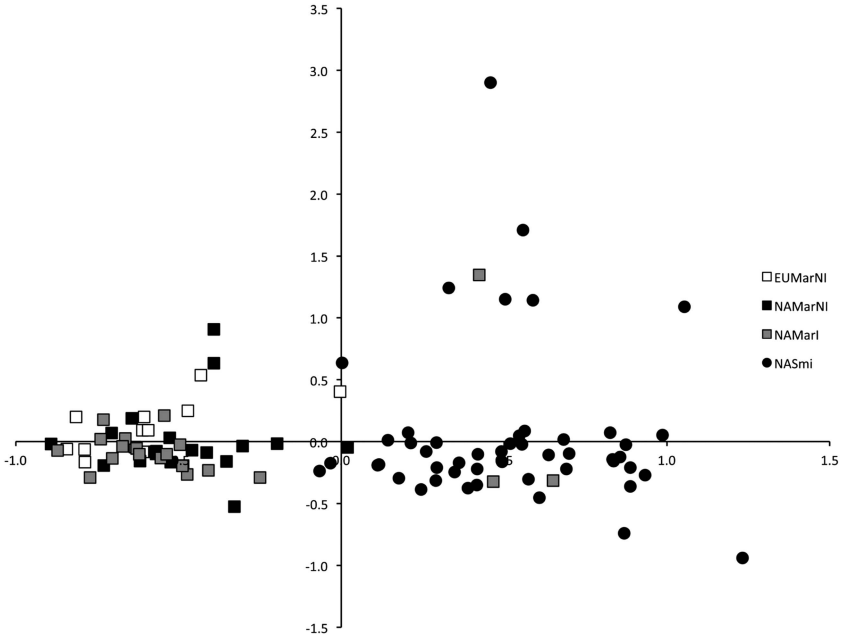

Figure 3 Scatter plot of the two-dimensional FCA performed on non-introgressed European *L. marinus* (EUMarNI, $n=11$), non-introgressed North American *L.marinus* (NAMarNI, $n=21$), mtDNA introgressed *L. marinus* (NAMarI, $n=20$) and *L. smithsonianus* (NASmi, $n=49$) individuals based on the 12 microsatellite data set.

Evidence for recent admixture in North America. There was no evidence that MARINUS individuals with the SMI haplotypes differ in nuclear genome from individuals with the MAR haplotype. Within North American MARINUS, the scores along the first axis of the FCA for individuals carrying the SMI mtDNA overlapped fully with individuals carrying the original MAR haplotypes and European birds, and there was no difference in the distribution of the scores of these two groups (analysis of variance: $F(49,2)=0.87$, $P=0.43$). Accordingly, there was no significant differentiation in microsatellite allelic frequency between North American MARINUS carrying the MAR or SMI haplotypes (Table 3). Thus, we did not detect any evidence that MARINUS individuals with the SMI haplotypes have recent co-ancestry with SMITHSONIANUS.

For examination of individual assignment probabilities, we restrained the data set to individuals with two or fewer missing loci, because individual assignments were unreliable for more incomplete genotypes. For example, two MARINUS from Finland, sampled as fully grown birds by very experienced gull ringers and carrying the typical MAR haplotypes but with four and three missing loci were identified as pure SMITHSONIANUS or backcross with SMITHSONIANUS (SMITHSONIANUS has never been recorded in Finland).

For individuals with two or fewer missing loci, assignment analyses with NewHybrids produced a similar pattern as FCA and F_{ST} values: most North American MARINUS are assigned to the pure MARINUS category with high probability, and none are identified as F1 or backcross with MARINUS, regardless of which mtDNA lineage they possess. The only exception is a specimen from North Carolina with a MAR haplotype; both FCA scores and assignment probability were incompatible with a pure MARINUS (most likely a backcross to SMITHSONIANUS; assignment probability to backcross with SMITHSONIANUS: 0.68, to pure SMI: 0.23). Accordingly, there was no difference in assignment probabilities between North American MARINUS carrying the MAR or SMI haplotypes (max. two loci missing: $F(32,1)=0.84$, $P=0.37$; no missing loci: $F(24,1)=0.11$, $P=0.74$). All SMITHSONIANUS individuals were unambiguously classified as pure SMITHSONIANUS, except four birds collected in Alaska (see the 'Methods' section).

DISCUSSION

Unidirectional introgression of American Herring Gull mitochondria into North American Great Black-backed Gull mirrors mate choice patterns

Mitochondrial lineage sharing between these two species in North America is best explained by unidirectional introgression from *L. smithsonianus* into *L. marinus*. First, ancestral polymorphism would result in the sharing of ancestral haplotypes rather than derived haplotypes, as can be seen from Figure 2 in Sternkopf *et al.* (2010). Second, lineage sharing only occurs in North America where they are sympatric and never in Europe where *L. smithsonianus* does not occur. As the shared HVR-I and cyt *b* haplotypes are observed at high frequency in these two species (Crochet *et al.*, 2003, Sternkopf *et al.*, 2010), it is unlikely that the North American MARINUS population was introgressed by gull species other than *L. smithsonianus*.

Phenotypic (such as large size) and behavioural (foraging and breeding) differences distinguish *L. marinus* from other members of the Herring Gull complex (Burger and Gochfeld, 1996). Due to these phenotypic and behavioural differences, *L. marinus* is considered relatively reproductively isolated from co-occurring gull species (Liebers *et al.*, 2004). However, several putative hybrids have been observed on both sides of the Atlantic (*L. marinus* × *L. argentatus*, in Europe or *L. marinus* × *L. smithsonianus,* in Canada: Pierroti, 1987, McCarthy, 2006), suggesting that reproductive barriers between this species and other gull species are still not completely hermetic. Because female preference for larger mates has been observed in several gull species (for example, Tinbergen, 1953, Neubauer *et al.*, 2009, personal observation), most hybrids are expected to result from the pairing of SMITHSONIANUS females with males of the larger MARINUS. Repeated backcrossing of female hybrids carrying SMITHSONIANUS mtDNA with male MARINUS would introduce SMITHSONIANUS mtDNA

into MARINUS populations. Conversely, introgression from MARINUS into SMITHSONIANUS (which we did not observe based on haplotypic data) would require pairing of large MARINUS females with smaller SMITHSONIANUS males and subsequent backcrossing of female hybrids with males of the smaller species. Indeed, known heterospecific pairs comprising *L. smithsonianus* and *L. marinus* always involved a male *L. marinus* and a female *L. smithsonianus* (McCarthy, 2006), consistent with the direction of mtDNA introgression observed here.

No nuclear differentiation between individuals carrying introgressed and non-introgressed mtDNA

If North American MARINUS individuals carrying SMITHSONIANUS mtDNA had acquired these alien (SMITHSONIANUS) lineages through recent (a few generations ago) hybridizations, we would expect differentiation between individuals carrying the SMI and MAR haplotypes in North America (significant F_{ST} and differences in FCA scores) and lower assignment probabilities of individuals carrying the SMI haplotype. Differences between MARINUS possessing the SMI and MAR haplotypes were not observed; therefore we conclude that most of the MARINUS individuals carrying SMI haplotypes acquired them through ancient hybridization. The SMI and MAR haplotypes are now segregating in the MARINUS population in North America, with recent hybridization events contributing very little to the lineage sharing of SMITHSONIANUS and MARINUS. Three individuals from the mixed colony of Sainte-Marie islands (Quebec, two MARINUS and one SMITHSONIANUS) have relatively low assignment probabilities to their respective species (see Supplementary Material S2). The probability to have this geographic clustering of ambiguous individuals by chance is small and suggests that recent hybridization is likely occurring in this area, albeit resulting in little contribution of haplotype sharing between these two species.

Homoplasy is a well-known drawback of microsatellites; it originates from a combination of high mutation rate and constraint on their evolution, such as size constraint that limit the number of possible repeats (for example, Hedrick, 1999, Balloux *et al.*, 2000). The main effect of such homoplasy is to reduce the level of genetic differentiation between populations. In the case of large white-headed gulls, homoplasy does not seem to contribute much to the low level of divergence between species, as similar levels of divergence have been observed with nuclear markers that are less subject to homoplasy, such as allozymes or AFLP (see Crochet *et al.*, 2003 for more details). However, the important point here is that if homoplasy reduces genetic differentiation between MARINUS and SMITHSONIANUS, it will make the two parental species more difficult to separate but will not affect introgressed individuals differently. Homoplasy could thus explain part of the low genetic divergence between SMITHSONIANUS and MARINUS but not affect any of our conclusions on nuclear introgression, which take into account the limited genetic divergence of the two species.

Extensive introgression of mtDNA from American Herring Gull into Great Black-backed Gull without significant nuclear introgression: evidence of selection of mtDNA variants?

In accordance with the results from Sternkopf *et al.* (2010), we detected an extensive introgression of mtDNA from SMITHSONIANUS into North American MARINUS. The discrepancy between our observed frequency of SMITHSONIANUS haplotypes into MARINUS (57%) and the frequency (34%) of the study from Sternkopf *et al.* (2010) might result from a simple sampling variance, but it is also possible that there is a geographical variation in SMITHSONIANUS haplotype frequency in MARINUS, with north-westernmost populations harbouring fewer of the introgressed haplotypes. When combining samples from both studies, 37 MARINUS specimens from North America out of the 79 (hence 47%) carry mitochondria inherited from SMITHSONIANUS parentage.

This extensive mitochondrial introgression is not mirrored by a similar amount of nuclear introgression. Although lack of diagnostic alleles at our suite of nuclear loci precludes equivalent estimation of introgressed alleles frequencies, none of the measures of nuclear introgression indicated a substantial contribution of SMITHSONIANUS alleles to the nuclear gene pool of MARINUS in North America: North American MARINUS were not closer to SMITHSONIANUS than European MARINUS along the FCA axis, and the difference in assignment probabilities was very low and not fully significant after correction for multiple testing. Although we are not aware of any study formally linking genomic admixture and position of individuals along FCA axis (often also called Multiple Correspondence Analysis, see Guinand, 1996) as has been done with Principal Component Analysis (PCA; for example, McVean, 2009), the close analytical similarities of FCA and PCA ensures that admixture would result in a shift of an individual's position on the FCA axis, as it has been observed in several empirical studies (for example, Johanet *et al.*, 2011, Lefebvre *et al.*, 2008). Note that individual genotype data (multiple allelic states at several loci, such as microsatellite data) are not suitable for PCA analyses, hence the use of the FCA here, which is the classical method in this context (Guinand, 1996). Our results thus demonstrate a lack of substantial admixture in the nuclear genome of North American MARINUS, indicating a strong discrepancy in the relative levels of mtDNA and nuclear DNA introgression. In line with the present study, Sternkopf *et al.* (2010) also observed high levels of mtDNA introgression in Nearctic *L. marinus* individuals from *L. smithsonianus* but were 'unable to discern a shared autosomal genetic component between these two gull taxa', although these results are not shown in their paper. Similar mito-nuclear discordances in the amount of introgression has been documented for a wide range of animal species, in some cases leading to the complete replacement of the mtDNA of the introgressed species by the mtDNA of the other species without any evidence of nuclear introgression (reviewed in Toews and Brelsford, 2012). The pattern we uncovered in North American MARINUS is, for example, similar to the savanna elephant (*Loxodonta africana*) where mtDNA from the closely related forest elephant (*L. cyclotis*) reached a high frequency, but none of the three biparentally inherited loci contained any foreign alleles (Roca *et al.*, 2005). Several hypotheses have been proposed to explain such mito-nuclear discrepancies in levels of introgression in vertebrates; they can broadly be divided between selective and neutral (demographic) processes (listed in Hedrick, 2010). Demographic processes include unidirectional mating and backcrossing, differential gene flow between organelles and nuclear genes and drift in small populations. Selective hypotheses propose stronger selection against alien nuclear alleles than mitochondrial haplotypes (Funk and Omland, 2003) and positive selection on introgressed mtDNA variants. We will examine these different hypotheses below.

Unidirectional mating and subsequent backcrossing has been invoked to explain some striking instances of discordant mito-nuclear introgression (for example, Roca *et al.*, 2005, see also Hedrick, 2010). As rightly noted by these authors, hybridization of a species A female with a species B male and subsequent backcrossing of female progeny with species B males produce individuals with species A mtDNA but with essentially species B nuclear background, because the ancestry of species A in the nuclear genome halves at each generation of backcrossing. This phenomenon, however, is unable to account for extensive mtDNA introgression without comparable nuclear

introgression. The frequency of the alien mtDNA in the introgressed species would remain very low unless there is sufficient mtDNA gene flow to result in an increase of the alien mtDNA frequency. Such substantial female-mediated gene flow would inevitably have consequences on the frequency of alien nuclear alleles (as females transmit both mtDNA and nuclear DNA). Unidirectional mating and subsequent backcrossing thus explains unidirectional gene flow between species (in our case, the introgression of SMITHSONIANUS in MARINUS but not the reverse) but is not sufficient alone to explain how SMITHSONIANUS mtDNA has reached such high frequency in MARINUS populations without detectable nuclear introgression.

Random drift in small populations, combined with unidirectional backcrossing, could generate the pattern we observe, however. Although the chances to observe the SMITHSONIANUS mitochondria rise in frequency by chance in a large population of MARINUS are remote, several generations of backcrossing of female hybrids in MARINUS (thus creating individuals carrying the SMITHSONIANUS mtDNA with an essentially MARINUS nuclear background) in a very small population of MARINUS (thus making initial frequency of SMITHSONIANUS mitochondria larger) could probably result in a large frequency of SMITHSONIANUS mtDNA in an otherwise 'pure' MARINUS population. Hudson and Turelli (2003) have recently shown that the differential effect of drift on mitochondrial and nuclear genomes is even stronger than that suggested by their difference in effective population size, which could arguably make this scenario more likely.

Sex-biased gene flow is unlikely to explain strong cases of mito-nuclear discordance, for the same reason as above, unless sex-biases in dispersal are extreme. If females disperse very little but males of species B move readily to the range of species A, they could theoretically 'swamp' the nuclear genome of species A while species A mitochondrial would remain *in situ*. Such extreme sex-biases in dispersal are rare in birds (outside of Anseriformes), and both SMITHSONIANUS and MARINUS co-occur in North America, therefore this scenario does not account for our results. A related hypotheses has been posited by Currat *et al.* (2008) (see also Excoffier *et al.*, 2009): in case of range expansion where one species invades the distribution of another species, these authors have shown by simulation that genetic introgression largely occurs from the resident into the invading species and that introgression is stronger when intraspecific gene flow is reduced. These results led them to propose that reduced gene flow of mitochondrial DNA relative to nuclear DNA (a combination of smaller effective size for mtDNA and, when relevant, sex-biased dispersal, see Birky *et al.*, 1983; Crochet, 2000) could generate a higher level of introgression for uniparentally inherited markers. The fact that introgression was detected from the resident SMITHSONIANUS to the more recent colonizer (MARINUS, see Sternkopf *et al.*, 2010) fits this scenario well. However, we argue this scenario should be evaluated by explicit modeling of nuclear and mitochondrial introgression under the same demographic parameters before it can be regarded as fully supported. In our species, where females should have a greater tendency for dispersal than males (Greenwood, 1980), it is unclear whether mitochondrial gene flow would remain reduced compared with nuclear gene flow or if female-biased dispersal would be sufficient to counter the effect of smaller effective size of the mitochondria and equilibrate levels of nuclear and mitochondrial gene flow between species.

An alternative hypothesis to explain strong discordance in the level of mitochondrial and nuclear introgression invokes stronger negative selection of nuclear alleles in the genome of an alien species compared with mtDNA. As noted by several authors, 'mitochondrial alleles might be expected to introgress farther, on average, than nuclear loci if their persistence in a foreign gene pool is less constrained by linkage to selected loci than are the alleles of nuclear genes' (Funk and Omland, 2003). In large white-headed gulls, including SMITHSONIANUS and MARINUS, evidence of selection on the nuclear genome is lacking, and what data available suggest is that species cohesion is largely maintained by selection on a small number of loci likely influencing phenotype, with most neutral markers experiencing weak barriers to gene flow (Gay *et al.*, 2009). Therefore, direct selection against alien alleles is unlikely to explain the limited signal of introgression at putatively neutral microsatellite markers based on the hypothesis by Gay *et al.* (2009), as gene flow at most neutral nuclear markers should not be directly counter selected.

Positive selection of alien mitochondria in the nuclear background of another species is increasingly proposed as a possible explanation for strong mito-nuclear biases in introgression (see Toews and Brelsford, 2012). It has been hypothesized that high mtDNA introgression rate, sometimes leading to the complete replacement of the specific mtDNA lineages in the introgressed species, may happen if selection favours some mtDNA variants that are better adapted to local conditions (see Ballard and Whitlock, 2004 for a review). Our failure to reject the selective neutrality of SMITHSONIANUS haplotypes found in introgressed *L. marinus* populations does not necessarily contradict this hypothesis: these tests detect the loss of diversity due to the increase in frequency of a given haplotype through selective processes and are sensitive to demographic processes. In the case of locally adapted mtDNA, the entire population of mitochondria would have a selective advantage, and there is no reason to expect a significant loss of genetic diversity unless the initial number of hybridization events was very low. The selective pressure generally invoked to explain differences in the fitness of mtDNA variants is most often related to external temperatures (*Drosophila*: Matsuura *et al.*, 1993; mammals: Ballard and Whitlock, 2004; Fink *et al.*, 2004; Ropiquet and Hassanin, 2006; Melo-Ferreira *et al.*, 2009; fish: Wilson and Bernatchez, 1998; reptiles: Heulin *et al.*, 2011). Indeed, MARINUS in western Europe and northeastern North America experience very different climatic niches (much colder winter in North America, Seager *et al.*, 2002) and therefore may explain the pattern of introgression observed here.

CONCLUSION

It has been proposed that nuclear introgression should be higher than mtDNA introgression in birds, because markers associated with the dispersing sex (usually females in birds) should be less prone to introgression (Petit and Excoffier, 2009). Indeed, all case studies of birds listed in Petit and Excoffier (2009) support this prediction. Females being the heterogametic sex in birds, Haldane's rule would also predict that mitochondrial haplotypes show patterns of reduced introgression compared with nuclear alleles (Carling and Brumfield, 2008). However, several cases of interspecific gene flow documented in birds depicted the reverse pattern; no nuclear introgression is detected while the maternally transmitted mtDNA exhibits high levels of introgression (Tejedor *et al.*, 2007; Bellemain *et al.*, 2008; Irwin *et al.*, 2009). This is the same pattern we observed between the North American *L. marinus* and the sympatric *L. smithsonianus*. Fitness data (clutch volume, hatching success) suggest that Haldane's rule does not apply to *L. argentatus* and *L. cachinnans*, two closely related gull species hybridizing in Europe (Neubauer *et al.*, 2009). Similarly to MARINUS and SMITHSONIANUS, these two species of recent origin exhibit a low level of genetic divergence. In contrast with other organisms, birds are often able to produce fertile hybrids even at relatively high levels of genetic distance (Price and Bouvier, 2002). This characteristic may explain why in several hybridizing bird species maternally

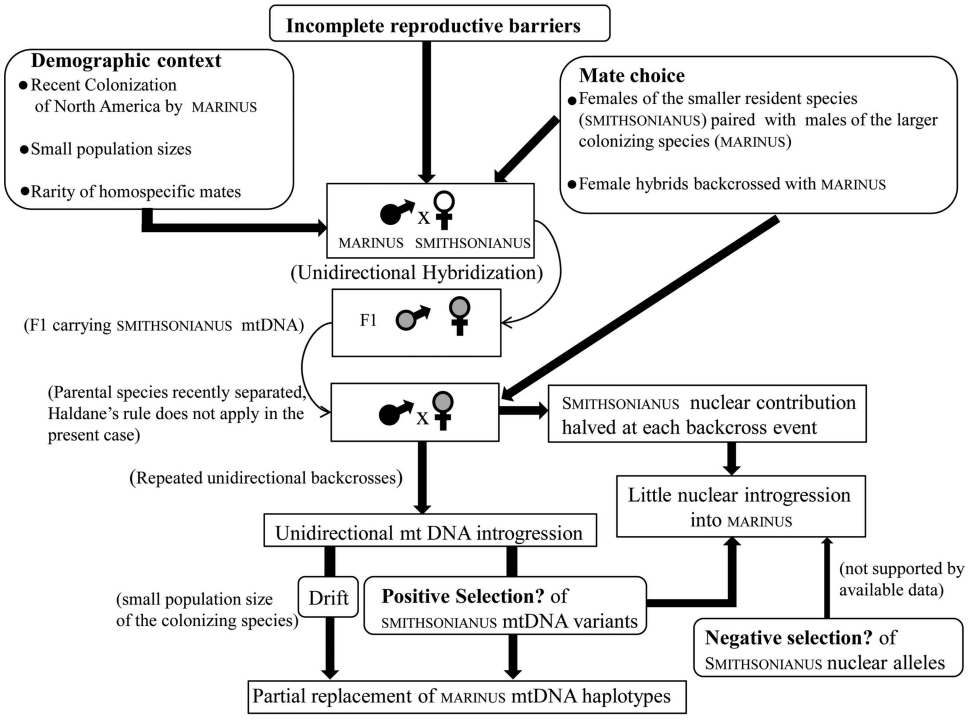

Figure 4 Schematic representation of demographic and selective mechanisms that may explain mito-nuclear and asymmetric introgression patterns resulting from past hybridization events between *L. marinus* and *L. smithsonianus*. Among alternative neutral explanations, only drift in small populations and asymmetric mate choice in an invasive context are consistent with our data. We were unable to demonstrate selection as the main cause of mito-nuclear introgression discordance but cannot dismiss the possible role of selection on mtDNA in the observed pattern. As it is generally difficult to assess the role of selection in patterns of introgression observed in natural populations, we advocate that evaluating alternative neutral hypotheses may help to indirectly support or reject hypotheses based on selective processes.

inherited mtDNA shows higher levels of introgression than autosomal markers in contrast with Haldane's rule predictions.

Additionally, studies proposing direct selection on mitochondrial variants driven by local climatic adaptation are increasingly reported in various organisms (see above), including birds (Cheviron and Brumfield, 2009; Ribeiro *et al.*, 2011). This led Rheindt and Edwards (2011) to suggest that adaptive introgression is an underappreciated process shaping patterns of mtDNA diversity within, and differentiation between, avian species. As we have discussed above, we were unable to support selection as the cause of discordant mito-nuclear introgression in our species, but we could dismiss most of the alternative scenario invoking neutral, demographic processes. Only drift in small populations and biased introgression as a result of unequal gene flow in an invasion context remain as a possible demographic explanation (see Figure 4).

It will often be difficult to demonstrate that selection was the causal mechanism of mtDNA introgression unless there are obvious signs of selective sweeps. In our case, we have been unable to discriminate between several alternative scenarios, including drift in a small population, effects of different levels of gene flow or positive selection of introgressed mitochondrial variants. However, we suggest that researchers carefully evaluate the alternative demographic neutral scenarios that have been proposed to explain strongly discordant patterns of mitochondrial and nuclear introgression and that several verbal models advanced in previous studies need validation via more formal approaches before being considered as valid explanations. Possible future developments include explicit modelling of mitochondrial and nuclear introgression in individual-based simulations under the various demographic scenarios discussed above, with nuclear markers either linked to loci under divergent selection ('speciation loci') or not. Another much needed study is how selection on the whole mitochondrial diversity of one species into the nuclear background of the other species (as opposed to the classical effects of selection on mutational variants of mtDNA) affects patterns of diversity in the population of selected mitochondria. If most of the suggested neutral scenarios are unable to explain extensive mitochondrial introgression without substantial nuclear gene flow, we feel this will provide increased support for hypotheses invoking selective processes as the mechanisms promoting patterns of discordant rates of mito-nuclear introgression.

DATA ARCHIVING

Mitochondrial haplotypes were deposited in Genbank (accession numbers KF422942–KF422973). Microsatellite data were deposited in DRYAD (doi:10.5061/dryad.f789n).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on Heredity website (http://www.nature.com/hdy)

APPENDIX 1: INFORMATION ON SPECIMENS INCLUDED IN THE STUDY; LOCALITIES, COLLECTORS, INSTITUTIONS AND GENETIC MARKERS AVAILABLE FOR EACH SPECIMEN

Specimen number	*Tissue number*	*Species*	*Group*	*Localities*	*Collector, Institution*	*HVR-I*	*Cyt b*	*Microsatellites*
Mari490	PAC 547	*L. marinus*	*mA*	Staten Island, New York, USA	AMNH	H19	H1	Yes
Mari491	PRS 337	*L. marinus*	*mA*	JFK Airport, New York, USA	AMNH	H19	H1	Yes
Mari493	PRS 1317	*L. marinus*	*mA*	JFK Airport, New York, USA	AMNH	H19	H1	Yes
Mari492	PRS 319	*L. marinus*	*mA*	JFK Airport, New York, USA	AMNH	H19	H1	Yes
Mari494	PEP S-51	*L. marinus*	*mA*	North Carolina, USA	AMNH	H21	H1	Yes
Mari6162	6162	*L. marinus*	*mA*	Wachreague Inlet, Virginia, USA	ANSP	H20	x	Yes
Mari438	13396	*L. marinus*	*mA*	Louisiana, USA	Donna Dittmann, LSUMZ	H19	H1	Yes
Mari442	6749	*L. marinus*	*mA*	Louisiana, USA	Donna Dittmann, LSUMZ	H19	H1	Yes
Mari435	16342	*L. marinus*	*mA*	Maine, USA	Donna Dittmann, LSUMZ	H21	H1	Yes
Mari249	MNHN249	*L. marinus*	*mA*	Iles Sainte-Marie, Quebec	Gilles Chapdelaine, Jean-François Rail, SCF	H19	H1	Yes
Mari255	MNHN255	*L. marinus*	*mA*	Iles Sainte-Marie, Quebec	Gilles Chapdelaine, Jean-François Rail, SCF	H23	H1	Yes
Mari16342	16342	*L. marinus*	*mA*	Maine, USA	LSUMZ	H21	x	Yes
Mari503	MNHN503	*L. marinus*	*mA*	Smithville, Missouri, USA	Mark Robbins, KNHM	H29	H2	Yes
Mari28	USNM638661	*L. marinus*	*mA*	Maryland, USA	NMNH	H19	x	Yes
Mari30	USNM638663	*L. marinus*	*mA*	Maryland, USA	NMNH	H19	x	Yes
Mari23	USNM638693	*L. marinus*	*mA*	Maryland, USA	NMNH	H22	x	Yes
Mari08	USMN641193	*L. marinus*	*mA*	JFK Airport, New York, USA	NMNH	H19	x	Yes
Mari01	USMN641190	*L. marinus*	*mA*	JFK Airport, New York, USA	NMNH	H20	x	Yes
Mari02	USMN641184	*L. marinus*	*mA*	JFK Airport, New York, USA	NMNH	H20	x	Yes
Mari05	USMN641188	*L. marinus*	*mA*	JFK Airport, New York, USA	NMNH	H20	x	Yes
Mari09	USMN641187	*L. marinus*	*mA*	JFK Airport, New York, USA	NMNH	H22	x	Yes
Mari9359	DOT9359	*L. marinus*	*mAint*	JFK Airport, New York, USA	AMNH	H10	x	Yes
Mari9360	DOT9360	*L. marinus*	*mAint*	JFK Airport, New York, USA	AMNH	H3	x	Yes
Mari10092	DOT10092	*L. marinus*	*mAint*	JFK Airport, New York, USA	AMNH	H3	x	Yes
Mari6148	6148	*L. marinus*	*mAint*	Wachreague Inlet, Virginia, USA	ANSP	H10	x	x
Mari6140	6140	*L. marinus*	*mAint*	Wachreague Inlet, Virginia, USA	ANSP	H7	x	Yes
Mari51712	51712	*L. marinus*	*mAint*	Wantry Island, New York, USA	Cornell Laboratory of Ornithology	H6	x	x
Mari444	16341	*L. marinus*	*mAint*	Maine, USA	Donna Dittmann, LSUMZ	H1	H5	Yes
Mari434	8664	*L. marinus*	*mAint*	North Carolina, USA	Donna Dittmann, LSUMZ	H1	H5	Yes

Appendix 1 (Continued)

Specimen number	*Tissue number*	*Species*	*Group*	*Localities*	*Collector, Institution*	*HVR-I*	*Cyt b*	*Microsatellites*
Mari250	MNHN250	*L. marinus*	*mAint*	Iles Sainte-Marie, Quebec, Canada	Gilles Chapdelaine, Jean-François Rail, SCF	H1	H5	Yes
Mari251	MNHN251	*L. marinus*	*mAint*	Iles Sainte-Marie, Quebec, Canada	Gilles Chapdelaine, Jean-François Rail, SCF	H1	H5	Yes
Mari252	MNHN252	*L. marinus*	*mAint*	Iles Sainte-Marie, Quebec, Canada	Gilles Chapdelaine, Jean-François Rail, SCF	H1	H5	Yes
Mari254	MNHN254	*L. marinus*	*mAint*	Iles Sainte-Marie, Quebec, Canada	Gilles Chapdelaine, Jean-François Rail, SCF	H1	H5	Yes
Mari256	MNHN256	*L. marinus*	*mAint*	Iles Sainte-Marie, Quebec, Canada	Gilles Chapdelaine, Jean-François Rail, SCF	H1	H5	Yes
Mari257	MNHN257	*L. marinus*	*mAint*	Iles Sainte-Marie, Quebec, Canada	Gilles Chapdelaine, Jean-François Rail, SCF	H1	H5	Yes
Mari258	MNHN258	*L. marinus*	*mAint*	Iles Sainte-Marie, Quebec, Canada	Gilles Chapdelaine, Jean-François Rail, SCF	H1	H5	Yes
Mari260	MNHN260	*L. marinus*	*mAint*	Iles Sainte-Marie, Quebec, Canada	Gilles Chapdelaine, Jean-François Rail, SCF	H1	H5	Yes
Mari253	MNHN253	*L. marinus*	*mAint*	Iles Sainte-Marie, Quebec, Canada	Gilles Chapdelaine, Jean-François Rail, SCF	H18	H5	Yes
Mari259	MNHN259	*L. marinus*	*mAint*	Iles Sainte-Marie, Quebec, Canada	Gilles Chapdelaine, Jean-François Rail, SCF	H18	H5	Yes
Mari16341	16341	*L. marinus*	*mAint*	Maine, USA	LSUMZ	H1	x	Yes
Mari2723	KU 2723	*L. marinus*	*mAint*	Massachusetts, USA	Mark Robbins, KNHM	H18	x	Yes
Mari2724	KU 2724	*L. marinus*	*mAint*	Massachusetts, USA	Mark Robbins, KNHM	H4	x	x
Mari502	MNHN502	*L. marinus*	*mAint*	Smithville, Missouri, USA	Mark Robbins, KNHM	H18	H5	x
Mari29	USNM638662	*L. marinus*	*mAint*	Maryland, USA	NMNH	H1	x	Yes
Mari15	USNM638660	*L. marinus*	*mAint*	Maryland, USA	NMNH	H18	x	Yes
Mari22	USNM638692	*L. marinus*	*mAint*	Maryland, USA	NMNH	H18	x	Yes
Mari03	USNM84115	*L. marinus*	*mAint*	JFK Airport, New York, USA	NMNH	H1	x	Yes
Mari06	USNM641192	*L. marinus*	*mAint*	JFK Airport, New York, USA	NMNH	H18	x	Yes
Mari04	USNM641183	*L. marinus*	*mAint*	JFK Airport, New York, USA	NMNH	H2	x	Yes
Mari172	MNHN172	*L. marinus*	mE	Brittany, France	Bernard Cadiou, SEPNB	H20	H1	Yes
Mari174	MNHN174	*L. marinus*	*mE*	Brittany, France	Bernard Cadiou, SEPNB	H20	H1	Yes
Mari173	MNHN173	*L. marinus*	*mE*	Brittany, France	Bernard Cadiou, SEPNB	x	x	Yes
Mari153	AJ276949 ($n=3$)	*L. marinus*	*mE*	Faeroe Islands, Denmark	GenBank cf Liebers *et al*., 2004	H19	H1	x
Mari155	AJ508306	*L. marinus*	*mE*	Faeroe Islands, Denmark	GenBank cf Liebers *et al*., 2004	H24	H1	x
Mari152	AJ508305	*L. marinus*	*mE*	Faeroe Islands, Denmark	GenBank cf Liebers *et al*., 2004	H25	H1	x
Mari150	AJ508304	*L. marinus*	*mE*	Faeroe Islands, Denmark	GenBank cf Liebers *et al*., 2004	H26	H1	x
Mari149	AJ276948 ($n=3$)	*L. marinus*	*mE*	Faeroe Islands, Denmark	GenBank cf Liebers *et al*., 2004	H27	H1	x
Mari381	MNHN381	*L. marinus*	*mE*	Ouessant, Brittany, France	Pierre-André Crochet	H20	H1	Yes
Mari384	MNHN384	*L. marinus*	*mE*	Denmark	Pierre-André Crochet	H30	H1	Yes
Mari199	MNHN199	*L. marinus*	*mE*	Finland	Visa Rauste, Annika Forsten	H20	H1	Yes
Mari200	MNHN200	*L. marinus*	*mE*	Finland	Visa Rauste, Annika Forsten	H20	H1	x
Mari195	MNHN195	*L. marinus*	*mE*	Finland	Visa Rauste, Annika Forsten	H28	H1	x
Mari196	MNHN196	*L. marinus*	*mE*	Finland	Visa Rauste, Annika Forsten	H31	H1	Yes
Mari198	MNHN198	*L. marinus*	*mE*	Finland	Visa Rauste, Annika Forsten	H31	H1	Yes
Mari197	MNHN197	*L. marinus*	*mE*	Finland	Visa Rauste, Annika Forsten	H32	H1	Yes
Mari385	MNHN385	*L. marinus*	*mE*	Lommer, Sweden	Pierre-André Crochet	H19	H1	Yes
Mari383	MNHN383	*L. marinus*	*mE*	Spillebeng, Malmi, Sweden	Pierre-André Crochet	H20	H1	Yes
Mari382	MNHN382	*L. marinus*	*mE*	Spillebeng, Sweden	Pierre-André Crochet	H19	H1	Yes
Mari386	MNHN386	*L. marinus*	*mE*	Spillebeng, Sweden	Pierre-André Crochet	H19	H1	Yes
Mari194	MNHN194	*L. marinus*	*mE*	Sweden	Pierre-André Crochet	H17	H3	x
smi497	DOT10247	*L. smithsonianus*	*s*	New York, USA	AMNH	H1	H5	Yes
Smi501	PRS 205	*L. smithsonianus*	*s*	North Branch, New Jersey, USA	AMNH	H1	H5	Yes
Smi10245	DOT10245	*L. smithsonianus*	*s*	JFK Airport, New York, USA	AMNH	H1	x	Yes
Smi10247	DOT10247	*L. smithsonianus*	*s*	JFK Airport, New York, USA	AMNH	H1	x	Yes
Smi10248	DOT10248	*L. smithsonianus*	*s*	JFK Airport, New York, USA	AMNH	H1	x	Yes
Smi10250	DOT10250	*L. smithsonianus*	*s*	JFK Airport, New York, USA	AMNH	H1	x	Yes

Appendix 1 (Continued)

Specimen number	*Tissue number*	*Species*	*Group*	*Localities*	*Collector, Institution*	*HVR-I*	*Cyt b*	*Microsatellites*
Smi10252	DOT10252	*L. smithsonianus*	*s*	JFK Airport, New York, USA	AMNH	H1	x	Yes
Smi498	PRS 1618	*L. smithsonianus*	*s*	JFK Airport, New York, USA	AMNH	H1	x	Yes
Smi7269	DCP 100	*L. smithsonianus*	*s*	Jake's Landing, New Jersey, USA	ANSP	H2	x	Yes
Smi1	AJ277127 ($n=7$)	*L. smithsonianus*	*s*	Lake Huron, Ontario, Canada	GenBank cf (Liebers *et al*., 2004)	H1	H5	x
Smi2	AJ276946	*L. smithsonianus*	*s*	Lake Huron, Ontario, Canada	GenBank cf (Liebers *et al*., 2004)	H11	x	x
Smi3	AJ276938 ($n=3$)	*L. smithsonianus*	*s*	Lake Huron, Ontario, Canada	GenBank cf (Liebers *et al*., 2004)	H2	H4	x
Smi505	24405	*L. smithsonianus*	*s*	Riverside Co, California, USA	Donna Dittmann, LSUMZ	x	x	Yes
Smi437	5885	*L. smithsonianus*	*s*	California	Donna Dittmann, LSUMZ	H1	H5	Yes
Smi436	26270	*L. smithsonianus*	*s*	Louisiana, USA	Donna Dittmann, LSUMZ	H1	H5	Yes
Smi440	22548	*L. smithsonianus*	*s*	Louisiana, USA	Donna Dittmann, LSUMZ	H1	H5	Yes
Smi441	32863	*L. smithsonianus*	*s*	Louisiana, USA	Donna Dittmann, LSUMZ	H1	H5	Yes
Smi443	1438	*L. smithsonianus*	*s*	Louisiana, USA	Donna Dittmann, LSUMZ	H1	H5	Yes
Smi445	1439	*L. smithsonianus*	*s*	Louisiana, USA	Donna Dittmann, LSUMZ	H1	H5	Yes
Smi446	1598	*L. smithsonianus*	*s*	Louisiana, USA	Donna Dittmann, LSUMZ	H11	H5	Yes
Smi439	1597	*L. smithsonianus*	*s*	Louisiana, USA	Donna Dittmann, LSUMZ	H5	H5	Yes
Smi504	24404	*L. smithsonianus*	*s*	Riverside Co, California, USA	Donna Dittmann, LSUMZ	x	x	Yes
Smi261	MNHN261	*L. smithsonianus*	*s*	Iles Sainte-Marie, Quebec, Canada	Gilles Chapdelaine, Jean-François Rail, SCF	H1	H5	x
Smi262	MNHN262	*L. smithsonianus*	*s*	Iles Sainte-Marie, Quebec, Canada	Gilles Chapdelaine, Jean-François Rail, SCF	H1	H5	Yes
Smi263	MNHN263	*L. smithsonianus*	*s*	Iles Sainte-Marie, Quebec, Canada	Gilles Chapdelaine, Jean-François Rail, SCF	H1	H5	Yes
Smi270	MNHN270	*L. smithsonianus*	*s*	Iles Sainte-Marie, Quebec, Canada	Gilles Chapdelaine, Jean-François Rail, SCF	H1	H5	Yes
Smi271	MNHN271	*L. smithsonianus*	*s*	Iles Sainte-Marie, Quebec, Canada	Gilles Chapdelaine, Jean-François Rail, SCF	H1	H5	x
Smi272	MNHN272	*L. smithsonianus*	*s*	Iles Sainte-Marie, Quebec, Canada	Gilles Chapdelaine, Jean-François Rail, SCF	H1	H5	Yes
Smi275	MNHN275	*L. smithsonianus*	*s*	Iles Sainte-Marie, Quebec, Canada	Gilles Chapdelaine, Jean-François Rail, SCF	H1	H5	Yes
Smi276	MNHN276	*L. smithsonianus*	*s*	Iles Sainte-Marie, Quebec, Canada	Gilles Chapdelaine, Jean-François Rail, SCF	H1	H5	Yes
Smi264	MNHN264	*L. smithsonianus*	*s*	Iles Sainte-Marie, Quebec, Canada	Gilles Chapdelaine, Jean-François Rail, SCF	H1	H5	Yes
Smi266	MNHN266	*L. smithsonianus*	*s*	Iles Sainte-Marie, Quebec, Canada	Gilles Chapdelaine, Jean-François Rail, SCF	H12	H5	Yes
Smi279	MNHN279	*L. smithsonianus*	*s*	Iles Sainte-Marie, Quebec, Canada	Gilles Chapdelaine, Jean-François Rail, SCF	H12	H5	Yes
Smi280	MNHN280	*L. smithsonianus*	*s*	Iles Sainte-Marie, Quebec, Canada	Gilles Chapdelaine, Jean-François Rail, SCF	H12	H5	Yes
Smi268	MNHN268	*L. smithsonianus*	*s*	Iles Sainte-Marie, Quebec, Canada	Gilles Chapdelaine, Jean-François Rail, SCF	H13	H5	Yes
Smi274	MNHN274	*L. smithsonianus*	*s*	Iles Sainte-Marie, Quebec, Canada	Gilles Chapdelaine, Jean-François Rail, SCF	H14	H5	Yes
Smi267	MNHN267	*L. smithsonianus*	*s*	Iles Sainte-Marie, Quebec, Canada	Gilles Chapdelaine, Jean-François Rail, SCF	H2	H5	Yes
Smi273	MNHN273	*L. smithsonianus*	*s*	Iles Sainte-Marie, Quebec, Canada	Gilles Chapdelaine, Jean-François Rail, SCF	H2	H5	Yes
Smi277	MNHN277	*L. smithsonianus*	*s*	Iles Sainte-Marie, Quebec, Canada	Gilles Chapdelaine, Jean-François Rail, SCF	H2	H5	Yes
Smi269	MNHN269	*L. smithsonianus*	*s*	Iles Sainte-Marie, Quebec, Canada	Gilles Chapdelaine, Jean-François Rail, SCF	H8	H5	Yes
Smi265	MNHN265	*L. smithsonianus*	*s*	Iles Sainte-Marie, Quebec, Canada	Gilles Chapdelaine, Jean-François Rail, SCF	H9	H5	Yes
Smi278	MNHN278	*L. smithsonianus*	*s*	Iles Sainte-Marie, Quebec, Canada	Gilles Chapdelaine, Jean-François Rail, SCF	H9	H5	Yes

Appendix 1 (Continued)

Specimen number	*Tissue number*	*Species*	*Group*	*Localities*	*Collector, Institution*	*HVR-I*	*Cyt b*	*Microsatellites*
Smi16	USNM638657	*L. smithsonianus*	*s*	Maryland, USA	NMHN	H1	x	Yes
Smi17	USNM638658	*L. smithsonianus*	*s*	Maryland, USA	NMHN	H12	x	Yes
Sm24	USNM638685	*L. smithsonianus*	*s*	Maryland, USA	NMHN	H15	x	Yes
Smi18	USNM638659	*L. smithsonianus*	*s*	Maryland, USA	NMHN	H16	x	Yes
Smi11	USNM641186	*L. smithsonianus*	*s*	JFK Airport, New York, USA	NMHN	H1	x	Yes
Smi26	USNM638687	*L. smithsonianus*	*s*	Maryland, USA	NMHN	x	x	Yes
Smi19	USNM638688	*L. smithsonianus*	*s*	Maryland, USA	NMHN	x	x	Yes
Smi447	UAMX2122, 1598	*L.smithsonianus*	*s*	Alaska, USA	University of Alaska Museum	x	x	Yes
Smi448	UAMX2123	*L.smithsonianus*	*s*	Alaska, USA	University of Alaska Museum	x	x	Yes
Smi449	UAMX2124	*L.smithsonianus*	*s*	Alaska, USA	University of Alaska Museum	x	x	Yes
Smi450	UAMX2126	*L.smithsonianus*	*s*	Alaska, USA	University of Alaska Museum	x	x	Yes
Smi451	UAMX2128	*L.smithsonianus*	*s*	Alaska, USA	University of Alaska Museum	x	x	Yes

Abbreviations: AMNH, American Museum of Natural History; ANSP, Academy of Natural Sciences of Philadelphia; KNHM, Kansas Natural History Museum; LSUMZ, Louisiana State University Museum of Zoology; NMNH, National Museum of Natural History, Smithsonian Institution; SCF, Service Canadien de la Faune; SEPNB, Société d'Etudes et de Protection de la Nature en Bretagne.