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GENETIC DIFFERENTIATION AT NUCLEAR AND MITOCHONDRIAL LOCI AMONG LARGE WHITE-HEADED GULLS: SEX-BIASED INTERSPECIFIC GENE FLOW?

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Abstract.—We measured genetic differentiation among species of large white-headed gulls using mitochondrial (cytochrome *b* haplotypes) and nuclear (microsatellites) markers. Additional information was added using a previously published study of allozymes on the same species. Levels of differentiation among species at nuclear markers are much lower than would be expected for avian species and are not concordant with the level of differentiation in mitochondrial markers. This discrepancy is best explained by a combination of recent species origin and interspecific gene flow after speciation. The data also suggest that female-mediated gene flow is reduced compared to male-mediated gene flow, either due to behavioral bias or due to stronger counterselection of female hybrids in accordance with Haldane's rule for ZW species. Whatever the reasons for the low differentiation of the species' nuclear gene pools, the extensive similarity of their nuclear genome demonstrates that selection on a limited number of characters is an important factor in establishing and maintaining clear-cut phenotypic differences between these species and suggests that the number of loci involved in this process is quite low. This situation may not be exceptional in birds, indeed a number of studies have found similarly low level of differentiation in nuclear markers among congeneric bird species, although usually based on a single set of markers. Because hybridization is a widespread phenomenon in birds, many of these cases might be due to interspecific gene flow.

Key words.—Allozymes, birds, Haldane's rule, hybridization, microsatellites, mitochondrial DNA, speciation.

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The importance of reproductive isolation in speciation processes is still controversial. Whereas all biologists agree that some restriction to gene flow has to be acting for populations to retain their specificity in areas of sympatry, not all would accept that a substantial amount of gene flow can be maintained during and after the speciation process without resulting in the blending of these populations (for a short historical overview see Templeton 1998). According to Ehrlich and Raven (1969), the effects of selection can override the effects of gene flow, and selection can maintain species distinctness in spite of ongoing gene flow. Of course, this phenomenon is central to the models of sympatric speciation (e.g., Johnson and Gullberg 1998), but will also be essential in determining the outcome of allopatric differentiation when separated populations meet again after isolation, as can be seen in some hybrid zones that are maintained by a balance between gene flow and selection against hybrids (e.g., for a recent discussion see Kruuk et al. 1999).

In birds, allopatric speciation is believed to be the prevailing mode of speciation (Chesser and Zink 1994), and indeed none of the proposed cases of sympatric speciation concerns avian taxa (Berlocher 1998). In spite of this, selection can be expected to play an important role in maintaining many avian species. Hybrids between avian species are widespread: slightly more than 9% of all birds are known to have hybridized in nature (Grant and Grant 1992). Furthermore, bird species have generally low levels of genetic divergence compared with other vertebrates (Avise 1983; Evans 1987), which could partly explain that, although the fate of avian hybrids is difficult to monitor in nature, many are fertile and some even seem to experience little or no disadvantage compared to parents (Grant and Grant 1992; Grant and Grant 1998; Good et al. 2000). Premating isolation through mate choice thus appears crucial in maintaining many avian species. In birds, mate choice is at least partly dependent on sexual imprinting (Laland 1994; Grant and Grant 1997; Grant and Grant 1998). These observations led Gill (1998, p. 281) to propose that ''speciation in birds is as much a cultural phenomenon as it is a genetic phenomenon.'' Theoretical work by Laland (1994) confirms that sexual imprinting can act as a partial barrier to gene flow, preserving and accentuating genetic differences between the populations.

Gulls of the large white-headed group constitute an interesting model to study these questions. The large white-headed gulls (species related to the herring gull, *Larus argentatus*) have been recently shown to constitute a monophyletic group of closely related species (Crochet et al. 2000, 2002). Genetic distance, as measured by percent sequence divergence in mitochondrial DNA (mtDNA), is less than 1.5% between the most divergent species, indicating a recent origin of this group. Many of these species are known to hybridize in nature (Pierotti 1987), with highly variable frequency. In one of the best-studied examples, the western (*Larus occidentalis*) and

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glaucous-winged gulls (*L. glaucescens*) form a wide hybrid zone, in the center of which hybrids can outnumber parental species (Bell 1996). This is an extreme case, though, and most sympatric large gull species hybridize only occasionally. Experimental cross-fostering manipulations have demonstrated the importance of imprinting for mate choice in the sympatric *L. argentatus* and *L. fuscus* (lesser black-backed gull): gulls reared by adults of the other species were much more likely to form interspecific pair bonds as adults (Harris 1970), some of them producing hybrids that reached adulthood (Harris et al. 1978).

Snell (1991) examined allele frequencies at eight polymorphic enzymatic loci (of 34 assayed, 26 being monomorphic) in six undisputed species of large white-headed gulls, including many sympatric species pairs. His results showed that among-species differentiation accounted for a very limited part of the genetic structuring observed. The overall F_{ST}-value among all populations (0.108) was mainly due to geographical structuring, with diversity among taxa accounting for only 2.3% of the total genic variability. Nei's (1978) genetic distances were extremely low (0.000–0.009) in the study, with zero values observed both between conspecific and nonconspecific populations. These results are consistent with the hypothesis of an imperfect reproductive isolation between large white-headed gulls species, with ongoing interspecific gene flow counteracting the effect of genetic drift. However, two other nonexclusive hypotheses could similarly explain these observations: (1) very recent origin of the species, too recent for allele frequencies to diverge much; and (2) balancing selection maintaining similar allele frequencies in all populations.

To examine these different hypotheses, we employed mitochondrial (cytochrome *b* gene) and nuclear (microsatellites) DNA markers to evaluate genetic differentiation between five of the six species analyzed by Snell (1991). If the lack of differentiation detected with enzymatic markers result from selective constraints on the allozymes, DNA markers should result in stronger genetic differentiation between species. If, on the contrary, demographic or historic factors (gene flow, recent common ancestry) are responsible for the pattern observed with allozymes, nuclear DNA markers, mitochondrial DNA markers, and allozymes should provide concordant results.

Materials and Methods

Species Studied: Samples Origin and Information Collected

Snell's (1991) work was based on samples of the following large white-headed gull species: *L. hyperboreus*, *L. glaucoides*, *L. argentatus* (European and North American birds), *L. fuscus*, *L. marinus*, and *L. cachinnans* (western Mediterranean populations).

Systematics and evolution of these gulls are still largely unsettled, and the systematic hypotheses of del Hoyo et al. (1996) or Sibley and Monroe (1990) have been recently challenged. Concerning the populations analyzed by Snell (1991), new results (reviewed in Crochet et al. 2002) suggest that the North American form *smithsonianus* (species *L. argentatus*, locality 6 and 7 in Snell 1991) is not closely related to *L. argentatus* and more likely represents a valid species, while the populations referred by Snell to *Larus cachinnans* belong to the western Mediterranean taxon *michahellis*, which also constitutes a valid species, *L. michahellis*.

For the present study, we treat *L. hyperboreus*, *L. argentatus*, *L. fuscus*, *L. smithsonianus*, and *L. michahellis* as valid biological species, even if the proposed status for the North American *smithsonianus* must be considered tentative. These five species and *L. marinus* were all included in our analysis. We thus analyzed the same species as Snell (1991), except *L. glaucoides*, for which we could not get enough samples. Most samples were of breeding adults or nonflying chicks sampled on breeding colonies, but some were birds caught at refuse tips. There is usually no voucher specimen for the samples.

A 280-bp fragment of the cytochrome *b* gene was amplified in nine to 50 specimens of all species included in the analyses. In addition, specimens of *L. argentatus*, *L. smithsonianus*, *L. michahellis*, and *L. fuscus* have been typed for allelic length variation at five microsatellite loci. Collecting localities and size of the samples typed for each marker are given in Table 1.

DNA Extraction, Amplification, and Typing for Mitochondrial DNA and Microsatellite Variation

Samples consisted of muscle in ethanol, dried or ethanol preserved feather bases, or blood in buffer or in ethanol. DNA from muscles and feather bases was extracted by complete digestion in 5% Chelex 100 (BioRad, Hercules, CA) with 20 μl of proteinase K, followed by boiling for 10 min. Extractions from blood and dried wings were performed using QIAamp tissue extraction kit (Qiagen, Valencia, CA) following the supplier's procedure.

A short (~300-bp) segment of the cytochrome *b* gene was amplified and sequenced from a large number of individuals. Additional sequences from the control region (a 600-bp segment including parts of domain II and domain III; see Crochet and Desmarais 2000; Crochet et al. 2002) and cytochrome *b* (see Results) gene were obtained for most short cytochrome *b* haplotypes to improve estimation of phylogenetic relationships among the short cytochrome *b* haplotypes. The amplification primers and protocols for the short cytochrome *b* segment and the control region segment have been published previously (Crochet et al. 2000). Sequences have been deposited in the GenBank database (for accession numbers see Fig. 1; Crochet and Desmarais 2000; Crochet et al. 2002). For the additional cytochrome *b* sequences, amplification and sequencing primers were L14967 (5′-CAT CCA ACA TCT CTG CTT GAT GAAA-3′) and H15938 (5′-ATG AAG GGA TGT TCT ACT GGT TG-3′). L refers to light strands and H refers to heavy strands, and the numbers refer to the position of the 3′ nucleotide of the primer on the white leghorn chicken (*Gallus gallus*) mtDNA sequence (Desjardins and Morais 1990).

The five microsatellite loci (*HG 14, HG18, HG25, HG27*, and *HG16*) have been developed for the North American *L. smithsonianus* by one of us (J. Z. Chen). Primer sequences, amplification temperature, and the number and length of the observed alleles are given in Table 2. The polymerase chain reaction was performed in a 10-μl reaction containing 1× amplification buffer, 1 unit of *Taq* DNA polymerase, 2.5 mM

TABLE 1. Sample size per locality and per locus for microsatellites (*HG27, HG14, HG18, HG25*, and *HG16*) and cytochrome *b* haplotypes (mitochondrial DNA). Names of most collectors are given in Crochet et al. (2002). Additional specimens were collected by J.-M. Pons, B. Cadiou (SEPNB Bretagne vivante), A. Bermejo, and J. Cortes.

Species Locality number in Fig. 2/locality/tissue type	*H27*	*H14*	*H18*	*H25*	*H16*	mtDNA
argentatus	**34**	**34**	**36**	**32**	**23**	**52**
1/Béniguet Island, Brittany, France/feathers	16	16	18	14	6	34
2/Turku, Finland/feathers	10	10	10	10	9	10
3/Hornoya Island, Vardø, Finnmark, Norway/blood	8	8	8	8	8	8
fuscus	**31**	**36**	**37**	**24**	**23**	**49**
1/Béniguet Island, Brittany, France/feathers	12	13	14	8	6	25
4/Great Saltee Lake, Ireland/blood	5	6	6	3	4	6
5/Kuopio, Tuusniemi, Outokumpu, Kontiolahti, Vaasa, Oravainen, Kokkola, all Finland/blood	14	17	17	13	13	18
michahellis	**50**	**50**	**50**	**48**	**50**	**78**
6/Camargue, France/muscle	10	10	10	10	10	6
7/Essaouira, Morocco/embryos	10	10	10	10	10	10
8/Isla de Ons, Pontevedra, Spain/blood	10	10	10	10	10	6
9/Berlenga islands, Portugal/muscle	10	10	10	8	10	13
10/Casa di Colmata, Mira, N Adriatic Sea, Italia/blood	10	10	10	10	10	4
11/San Sebastian, Spain/blood	0	0	0	0	0	20
12/Selvagem islands, Portugal/blood	0	0	0	0	0	1
13/Gibraltar/blood	0	0	0	0	0	8
14/Isla de Vionta, A Coruña, Spain/blood	0	0	0	0	0	10
marinus	**8**	**8**	**8**	**6**	**0**	**18**
15/coast near Uppsala, Sweden/muscle	0	0	0	0	0	1
2/Turku, Finland/feathers	1	1	1	1	0	7
3/Hornoya Island, Vardø, Finnmark, Norway/blood	2	2	2	1	0	2
16/Helgoland Island, Germany/blood	1	1	1	1	0	1
1/Béniguet Island, Brittany, France/feathers	2	2	2	2	0	5
18/off Oregon Inlet, North Carolina, USA/muscle	1	1	1	1	0	1
19/Hamilton, Lake Ontario, Canada/feathers	1	1	1	0	0	1
smithsonianus	**9**	**9**	**9**	**9**	**9**	**9**
20/Manitoba, Canada/blood						
hyperboreus	**0**	**0**	**0**	**0**	**0**	**12**
21/Taimyr, Russia/muscle	0	0	0	0	0	2
22/Coats Island, NW Territory, Canada/blood	0	0	0	0	0	10

$MgCl_2$, 0.2 mM of each dNTP, 0.5 μM of forward primer, and 0.03 μM of γ^{32}P-ATP labeled reverse primer. The products were resolved on denaturing polyacrylamide gels, exposed for 12–220 h.

Data Analyses

Genetic differentiation between the populations (here the species) was evaluated using an F_{ST} approach for both the cytochrome *b* and microsatellites data. F_{ST}-values were estimated by the parameter θ (Weir and Cockerham 1984) using the Genetix 4.01 software (Belkhir et al. 2000, available at http://www.univ-montp2.fr/~genetix/genetix.htm). The significance of the θ-values was evaluated by comparing the observed values to the distribution of the values obtained from 1000 random permutations of the individuals between populations. The same method was used to test the significance of the F_{IS}-values, permuting this time alleles inside samples. Confidence intervals of θ-values were calculated using a bootstrap procedure over loci (1000 replications) using software TFPGA (Miller 2000, available at http://bioweb.usu.edu/mpmbio/tfpga.htm). Measures of population diversity (unbiased expected heterozygosity, mean number of alleles per locus) were computed using Genetix.

Another measure of population differentiation, R_{ST}, has been designed specifically for microsatellite data and is based not only on differences in allele frequencies but also in the number of repeat between alleles (Slatkin 1995). Gaggiotti et al. (1999) showed that, for a small number of scored loci and moderate sample size (50 or fewer), F_{ST} actually results in better estimates of gene flow than R_{ST}. We decided to use F_{ST} in this study because we only scored five microsatellite loci and most of our samples contained fewer than 50 individuals.

The cytochrome *b* data consists of haploid data that are treated by the Genetix software as diploid genotypes, which introduces a small bias in θ-values that was corrected by a factor of $(2n - 1)/(2n - 2)$, where n is the total number of individuals treated (Belkhir et al. 2000). Methods based on F_{ST} make use of differences in allele frequencies only. When applied to the mitochondrial DNA sequence data, they only take into account the differences in haplotype frequencies between populations, not the phylogenetic relationships among the haplotypes. Other methods are available to take account of this information, such as analysis of molecular variance (AMOVA, Excoffier et al. 1992). We also performed AMOVA using the program Arlequin (Schneider et al. 1997), but the results were not qualitatively different from those based on F_{ST} and will not be detailed here.

We reanalyzed the allozyme data of Snell (1991), using the same methods as above, for the species that we had sampled also (i.e., excluding *L. glaucoides*). The original data consisted of allelic frequency and sample sizes for eight en-

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                1 1111111112 2222222223 3333333334 4444444445 5555555556 6666666667 7777777778
     1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
MIC  CATTTGCCTA CTAACACAAA TCCTAACAGG ACTCCTGCTA GCTATGCATT ACACTGCAGA CACAACCCTA GCCTTCTCAT
MAR  .......... .......... .......... .......... .....A.... .......... .......... ..........
ARG  .......... .......... .......... ......A... .....A..C. ....C..... .......... ..........
70   .......... .......... .......... .......... .....A.... .......... .......... ..........
20   .......... .......... .......... .......... .....A..C. ....C..... .......... ..........
FUS2 ?????????? ????...... .......... .......... .....A.... ....C..... .......... ..........
FUS  .......... .......... .......... .......... .....A.... ....C..... .......... ..........
SMI  .......... .......... .......... .......... .....A.... ....C..... .......... ..........
HYP  .......... .......... .......... .......... .....A.... ....C..... .......... ..........
MIC2 .......... .......... .......... .......... .......... .......... .......... ..........
MAR2 .......... .......... .......... .......... ..C..A.... .......... .......... ..........

                      1 1111111111 1111111111 1111111111 1111111111 1111111111 1111111111
     8888888889 9999999990 0000000001 1111111112 2222222223 3333333334 4444444445 5555555556
     1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
MIC  CCGTCGCCCA CACATGTCGA AACGTACAAT ATGGCTGACT AATCCGAAAC CTCCACGCAA ACGGAGCATC ATTCTTCTTT
MAR  .......... .......... .......... .......... .......... .......... .......... ..........
ARG  .......... .......... .......... .......... .......... .......... .......... ..........
70   .......... .......... .......... .......... .......... .......... .......... ..........
20   .......... .......... .......... .......... .......... .......... .......... ..........
FUS2 .......... .......... .......... .......... .......... .......... .......... ..........
FUS  .......... .......... .......... .......... .......... .......... .......... ..........
SMI  .......... .......... .......... .......... .......... .......... .......G.. ..........
HYP  .......... .......... .......... .......... .......... .......... .......G.. ..........
MIC2 .......... .......... .......... .......... .......... .......... .......... ..........
MAR2 .......... .......... .......... .......... .......... .......... .......... ..........

     1111111111 1111111111 1111111111 1111111112 2222222222 2222222222 2222222222 2222222222
     6666666667 7777777778 8888888889 9999999990 0000000001 1111111112 2222222223 3333333334
     1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
MIC  ATTTGTATTT ACCTACACAT CGGACGAGGA TTCTACTATG GCTCATACCT CTATAAAGAA ACCTGAAATA CAGGAGTTAT
MAR  .......... .......... .......... .......... .......... .......... .......... ..........
ARG  .......... .......... .......... .......... .......... .......... .......... .......C..
70   .......... .......... .......... C......... .......... .......... .......... ..........
20   .......... .......... .......... .......... .......... .......... .......... .......C..
FUS2 .......... .......... .......... .......... .......T.. .......... .......... .......C..
FUS  .......... .......... .......... .......... .......... .......... .......... .......C..
SMI  .......... .......... .......... .......... .......... .......... .......... .......C..
HYP  .......... .......... .......... .......... .......... .......... .......... .......C..
MIC2 .......... .......... .......... .......... .......... .......... .......... ..........
MAR2 .......... .......... .......... .......... .......... .......... .......... ..........

     2222222222 2222222222 2222222222 2222222222 2222222222 2222222223 33333333  GenBank    Long cyt b
     4444444445 5555555556 6666666667 7777777778 8888888889 9999999990 00000000  accession  accession
     1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 12345678  number     number
MIC  TCTCCTCTTA ACCCTAATAG CAACTGCCTT CGTAGGATAT GTCTTGCCAT GAGGACAAAT ATCCTTCT  AF268493   AY321632
MAR  .......... .......... .......... .......... .......... .......... ........  AF268496   AY321628
ARG  .......... .......... .......... .......... .......... .....?.... ......??  AF268495   AY321631
70   .......... .......... .......... .......... .......... .......... ........  AF444253   -
20   .......... .......... .......... .......... .......... .......... ........  AF444254   -
FUS2 .......... .......... .......... ......G... .......... ?????????? ????????  AF444256   -
FUS  .......... .......... .......... ......G... .......... .......... ........  AF268494   AY321630
SMI  .......... .......... .......... .......... .......... .......... ...T....  AF444257   AY321629
HYP  .......... .......... .......... .......... .......... .......... ........  AF268500   AY321633
MIC2 ......T... .......... .......... .......... .......... .......... ........  AY321626   -
MAR2 .......... .......... .......... .......... .......... .......... ........  AY321627   -
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FIG. 1. Sequence (L-strand) of the short cytochrome *b* haplotypes found in this study. The first position corresponds to position 15009 of the chicken sequence in Desjardins and Morais (1990).

zymatic loci (*Est-1, Est-2, Gda, Gpd, Idh, Pgi, Pgd, Pgm*; see Snell 1991 for details). We reconstructed the individuals genotypes based on the frequency data and sample sizes given in the original publication, with the assumption that all populations were in Hardy-Weinberg equilibrium. This was not the case, as significant F_{IS}-values were detected for three loci by Snell. However, this has very little or no effect as far as F_{ST} is concerned because this statistic is precisely designed to distinguish within- and between-population departure from Hardy-Weinberg equilibrium.

Phylogenetic relationships among the haplotypes were examined using a composite sequence made of the available

TABLE 2. Primer sequences (F, forward; R, reverse), annealing temperature, number of alleles, and alleles size range for the five gull microsatellite loci.

Locus	Primer pairs (5′ → 3′)	Annealing temperature	Number of alleles	Size range (bp)
HG14	F: ATCGCTGCCAGGGCTGAGC R: TGTCTCGGGGAGTGTTTGCC	57°C	9	122–140
HG18	F: AGCCCACACCTCTGGCATTG R: TAGCAGCTGCCATACATCAG	58°C	11	110–134
HG25	F: TACCTCCGCTCTCCCCTCCA R: GGAGCAGCCGACAAAGCCTC	58°C	6	119–131
HG27	F: AGTGCAGGCAATAGTGTTGG R: GGATCTCTGGGCTCCTGGAG	55°C	5	111–117
HG16	F: TGATGCTTTGGCTGCAAATG R: GTCTTTGCCATATGGGTTCC	58°C	7	161–172

cytochrome *b* and control region segments. The resulting tree is a bootstrap (1000 replications) consensus tree (retaining only nodes supported by a bootstrap value above 50) obtained from a neighbor-joining tree based on Kimura two-parameter distance (pairwise deletion option for handling gap and missing data) using MEGA version 2.0 beta (Kumar et al. 2000, available at http://www.megasoftware.net/). The used outgroups were the common gull (*L. canus*,) one of the closest relatives of the large white-headed species, and the Herrmann's gull (*L. heermanni*), a basal representative of the large gulls (see Crochet et al. 2000). A minimum spanning network of the cytochrome *b* haplotypes based on the short cytochrome *b* sequences only was constructed using the program MINSPNET (Excoffier and Smouse 1994, available at http://www.cmpg.unibe.ch/services/software.htm) to visualize the number of mutations between haplotypes.

RESULTS

Haplotypes Sequencing and Analysis

A 280- to 300-bp segment from the cytochrome *b* (starting around position 15010 in the chicken mitochondrial DNA sequence; Desjardins and Morais 1990) was sequenced from 211 specimens (see Table 1). Eleven haplotypes were identified based on these short cytochrome *b* sequences (see Fig. 1). For practical reasons, the haplotypes were named after the taxon in which they are most frequent, although they are usually not diagnostic of this taxon. Missing information (due to ambiguous sequencing results) was rare and concerned regions with no variable site, except for the haplotype FUS2 (found in only one specimen). In this case, we supposed that the missing part of the sequence was identical to the widespread FUS haplotype (see Fig. 1). For the main haplotype typical of each species (MIC, MAR, ARG, FUS, SMI, and HYP) a longer (891-bp, including the short cytochrome *b* sequence) segment of the cytochrome *b* was also obtained (see Fig. 1 for GenBank accession numbers).

TABLE 3. Kimura two-parameter distances between the main cytochrome *b* haplotypes (based on the long 890-bp sequence).

	MIC	MAR	ARG	FUS	SMI	HYP
MIC						
MAR	0.003					
ARG	0.008	0.005				
FUS	0.008	0.005	0.005			
SMI	0.008	0.005	0.005	0.005		
HYP	0.008	0.005	0.005	0.005	0.002	

Sequence evolution was typical of mitochondrial genes, there was no stop codon in the amplification products, there was no ambiguous sequence, and the same sequence was obtained when using the two different primers pairs (for the short or long cytochrome *b* segments). Amplification of nuclear copies (numts) could thus not be suspected (for a more complete discussion of this problem see Crochet and Desmarais 2000). Divergence among the main haplotypes was low to very low (Kimura two-parameter distance between 0.2% and 0.8%; see Table 3, Fig. 2). Phylogenetic relationships among the haplotypes were poorly resolved, even among the haplotypes for which the longest sequences were available (Fig. 3).

Haplotype frequencies in each species are given in Table 4 and haplotype distribution in Figure 4. The overall θ-value based on the cytochrome *b* frequency data is 0.66. Table 5 gives the pairwise θ-values calculated for the cytochrome *b* data between the species. They vary from 0.45 between *L. marinus* and *L. argentatus* to 0.90 between *L. hyperboreus* and *L. smithsonianus* ($P < 0.001$ for all pairwise values). Intraspecific diversity explains that θ-values are less than one for comparisons between species that do not share any haplotype (Table 5).

Even if haplotype frequencies differ strongly between species, as indicated by the high and significant θ-values, no species can be identified by private haplotypes, and more than one haplotype was detected in nearly every species (Fig. 4). Some of these haplotypes were found in low frequency in one species only and differ from the most common haplotype in this species by a single substitution. This is the case with haplotypes MIC2, found in three specimens of *L. michahellis*, and FUS2, found in one *L. fuscus*. These two haplotypes most likely derive from the most common haplotypes in these species by a comparatively recent mutation. Nevertheless, haplotypes belonging to different lineages are also frequently found in one species. These haplotypes are typically present in one species in high frequency and in several other species in low frequency. Only in the isolated North American *L. smithsonianus* did we find a single haplotype.

This lineage sharing was often not symmetrical. For example, the Western European *L. fuscus* does not seem to carry

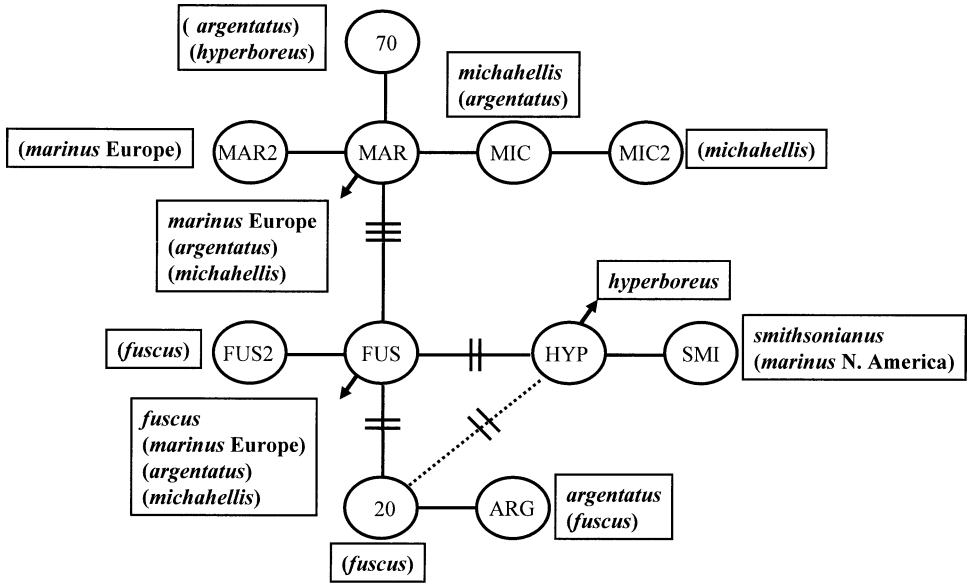

FIG. 2. Minimum spanning network of the short cytochrome *b* haplotypes (no additional control region or cytochrome *b* sequences used). Multiple substitutions between haplotypes are indicated by hash marks. Dotted lines indicate alternative connections between haplotypes. Boxes indicate species in which each haplotype has been found. Names in parentheses are for species in which frequency of a given haplotype is below 0.5.

any haplotype of the sympatric *L. marinus*, whereas *L. marinus* was found to carry the typical *L. fuscus* haplotypes. *Larus marinus* does not have the ARG haplotype that is the most common in the third sympatric species *L. argentatus*. In *L. argentatus*, we found both FUS and MAR haplotypes, typical of *L. fuscus* and *L. marinus*, respectively. Similarly, none of our many *L. michahellis* possessed the ARG haplotype, while we found the MIC haplotype in one *L. argentatus*.

One especially interesting situation concerns the North American gulls. We sequenced nine specimens of *L. smithsonianus* and two *L. marinus* from the North American populations of the species. Instead of the haplotypes found in European *L. marinus*, both North American *L. marinus* had the SMI haplotype, found in all our *L. smithsonianus* but never found in European gulls.

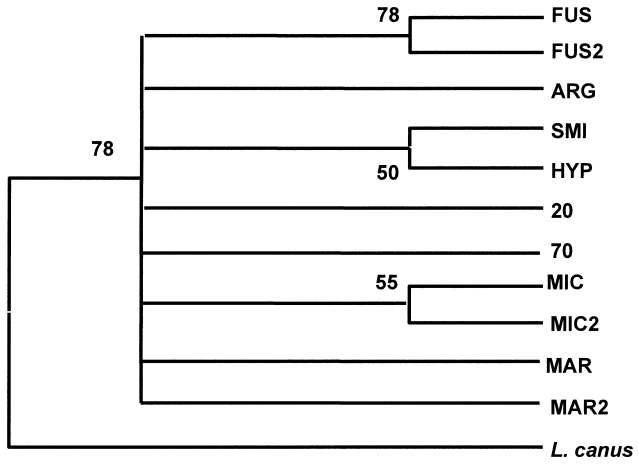

FIG. 3. Bootstrap consensus tree (Kimura two-parameter distance, neighbor-joining, pairwise deletion of missing data) of the large gull haplotypes. The tree was rooted with *Larus heermanni* (not shown). Tree based on long cytochrome *b* and control region sequences (~1540 bp in total) for haplotypes MIC, ARG, FUS, SMI, HYP, MAR; control region and about 450 bp of cytochrome *b* for haplotype 70; control region and short cytochrome *b* sequence for haplotype FUS2, *L. canus*, and *L. heermanni*; long cytochrome *b* segment only for haplotypes MAR2; and short cytochrome *b* segment only for haplotypes 20 and MIC2.

Intraspecific values of population differentiation overlapped slightly with interspecific values. Most comparisons of populations within species did not yield significant θ-values (results not shown) except for differentiation between the Essaouira population and other populations of *L. michahellis* ($\theta = 0.55$) and between the Scandinavian and Western European populations of *L. argentatus* (subspecies *argentatus* and *argenteus*, respectively, $\theta = 0.26$), both highly significant (permutations tests, $P < 0.001$). The results of the intraspecific comparisons are discussed in Crochet et al. (2002) and will thus not be detailed here.

Microsatellites Data

Among the species that we analyzed, no microsatellite data were available for *L. hyperboreus*, while the sample size for *L. marinus* is small. The number of individuals of each species typed for each locus is given in Table 1. Number of alleles per locus varied from five (locus HG27) to 11 (locus HG18). Genetic diversity within each species, as measured by expected heterozygosity and number of alleles averaged by loci, indicated that all species are similarly diverse (range of $H_{exp} = 0.511–0.716$, 3.00–5.75 alleles per locus). Some amount of heterozygote deficiency is to be expected within species due to the fact that samples for some species come from geographically distant localities with significant intraspecific differentiation between them (see below). When taking into account the geographic structure of the samples (grouping samples by locality within species), none of the

TABLE 4. Frequency of the microsatellite alleles or cytochrome *b* (CTB) haplotypes in the gull species (*Larus*).

Locus	Allele or haplotype	*argentatus*	*fuscus*	*michahellis*	*marinus*	*smithsonianus*	*hyperboreus*
CTB	FUS	0.019	0.837	0.013	0.056	0	0
	FUS2	0	0.020	0	0	0	0
	ARG	0.654	0.102	0	0	0	0
	MAR	0.192	0	0.077	0.778	0	0
	MAR2	0	0	0	0.056	0	0
	MIC	0.019	0	0.872	0	0	0
	MIC2	0	0	0.038	0	0	0
	HYP	0	0	0	0	0	0.917
	SMI	0	0	0	0.111	1.000	0
	20	0	0.041	0	0	0	0
	70	0.115	0	0	0	0	0.083
HG14	122	0	0	0	0	0.111	—
	126	0	0	0.140	0	0	—
	128	0.100	0.143	0.140	0	0.056	—
	130	0	0.071	0.040	0	0.111	—
	132	0.294	0.179	0.110	0.625	0.167	—
	134	0.412	0.429	0.360	0.312	0.222	—
	136	0.118	0.143	0.140	0	0	—
	138	0.073	0.036	0.070	0	0.278	—
	140	0	0	0	0.063	0.056	—
HG18	110	0.014	0	0	0	0	—
	114	0.139	0.162	0.190	0.062	0	—
	116	0	0.013	0	0.187	0.056	—
	118	0	0.054	0	0	0	—
	120	0.486	0.338	0.500	0.750	0.333	—
	122	0.056	0.122	0.010	0	0.111	—
	124	0.306	0.243	0.240	0	0.444	—
	126	0	0.027	0	0	0	—
	128	0	0	0.060	0	0.056	—
	130	0	0.027	0	0	0	—
	134	0	0.013	0	0	0	—
HG25	119	0	0.021	0	0	0	—
	123	0.172	0.375	0.562	0.167	0.167	—
	125	0.266	0.062	0.063	0.167	0.444	—
	127	0	0	0	0	0.056	—
	129	0.375	0.396	0.031	0.167	0.222	—
	131	0.187	0.146	0.349	0.500	0.111	—
HG27	111	0.015	0	0	0	0	—
	114	0	0.081	0.004	0	0	—
	115	0.059	0.290	0.026	0	0	—
	116	0.573	0.403	0.457	0.375	0.944	—
	117	0.353	0.226	0.504	0.625	0.056	—
HG16	161	0	0	0	—	0.056	—
	162	0.043	0	0.030	—	0	—
	166	0.043	0.022	0.080	—	0	—
	167	0	0.022	0	—	0	—
	168	0.783	0.413	0.590	—	0.722	—
	171	0	0.109	0	—	0	—
	172	0.130	0.435	0.300	—	0.222	—

loci showed any significant value of heterozygote deficiency for any species (as measured by F_{IS}-values).

There were no fixed allelic differences between the species at any locus, and the same alleles were often frequent in all or most species. Differences in allele frequency among species were moderate but significant. The overall θ-value (averaged over loci) is 0.069 ($P < 0.001$). It indicates that only 7% of the variance in allele frequency is explained by differences between species. Significant θ-values were detected at all five loci: HG14 ($\theta = 0.045$, $P < 0.001$), HG25 ($\theta = 0.129$, $P < 0.001$), HG18 ($\theta = 0.030$, $P = 0.008$), HG27 ($\theta = 0.083$, $P < 0.001$), and HG16 ($\theta = 0.056$, $P = 0.012$). Pairwise θ-values (averaged over loci) and their significance level are given in Table 6. They range from 0.045 (between *L. argentatus* and *L. smithsonianus*) to 0.210 (between *L. marinus* and *L. smithsonianus*). It should be noted, however, that these extreme values result from comparisons including at least one small sample and have very large confidence interval (95% confidence interval based on bootstrap over loci in TFPGA: 0.00–0.13 for the first comparison and 0.04–0.41 for the second).

As was the case with the cytochrome *b* data, these values overlap with the values of intraspecific comparisons. Comparisons between the subspecies *fuscus* (Finland samples) and *graellsii* (Ireland and Brittany samples) of *L. fuscus* yielded a θ-value of 0.020 ($P = 0.13$). Comparisons between Mediterranean (Camargue and Adriatic) and Atlantic (Portugal and northwestern Spain) populations of *L. michahellis* yielded

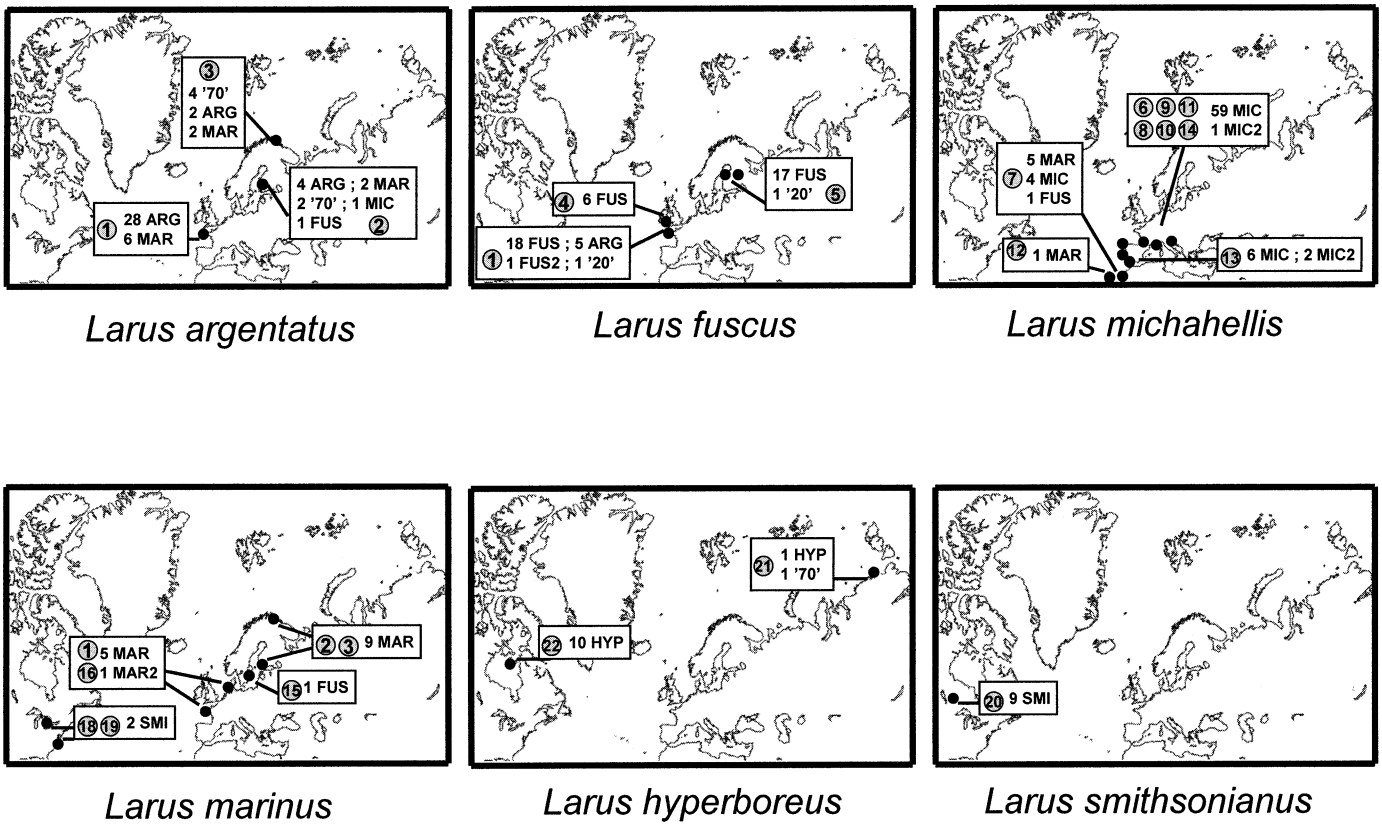

FIG. 4. Collecting localities for all species (small black dots) and haplotype distribution. Larger gray dots with number are locality numbers as in Table 1. For each locality or group of localities, the number of specimens carrying each haplotype is given inside the boxes.

a θ-value of 0.034 ($P = 0.01$). More differentiation was observed between the western subspecies *argenteus* (Brittany specimens) and the eastern subspecies *argentatus* (Finland and Norway specimens) of *L. argentatus* ($\theta = 0.105$, $P < 0.001$).

Allozymes Data

Details on the allozyme data such as variability or deviation from Hardy-Weinberg equilibrium are given in Snell (1991). The overall θ-value for the allozymes, calculated using the same species as for the microsatellites (i.e., excluding *L. hyperboreus*) and excluding locus *Pgd* (which is monomorphic when this species is excluded), is 0.041. Significant θ-values were detected at six out of the seven loci: *Est-1* ($\theta = 0.12$, $P < 0.002$), *Pgm* ($\theta = 0.09$, $P < 0.002$), *Est-2* ($\theta = 0.06$, $P < 0.002$), *Gda* ($\theta = 0.04$, $P = 0.002$), *Idh* ($\theta = 0.03$, $P = 0.01$), and *Gpd* ($\theta = 0.02$, $P = 0.006$). Differentiation at locus *Pgi* was not significant ($\theta = 0.006$, $P = 0.144$). Between-species pairwise θ-values are given in Table 7 with their significance level calculated as for the microsatellites data.

TABLE 5. Pairwise θ-values based on the cytochrome *b* haplotypes data among large gull species (*Larus*). Asterisks indicate species that have no haplotype in common. All values highly significant ($P < 0.001$, all permuted datasets give lower values than the observed value).

θ	*fuscus*	*marinus*	*michahellis*	*smithsonianus*	*hyperboreus*
argentatus	0.548	0.434	0.624	0.607*	0.574
fuscus		0.655	0.738	0.770*	0.740*
marinus			0.703	0.714	0.699*
michahellis				0.803*	0.778*
smithsonianus					0.905*

Comparison of the Various Markers

Although the overall F_{ST} is smaller for the allozymes (0.041) than for the microsatellites (0.069), the difference is not significant (Mann-Whitney *U*-test, four microsatellites loci, seven allozyme loci, $P = 0.51$). Given the low number of loci used and that the samples are not the same, the two types of nuclear markers give rather concordant measures of interspecific differentiation. On the contrary, the observed F_{ST}-value based on the cytochrome *b* data among our gull species ($\theta = 0.66$) is much larger. The reasons for this difference will be discussed below.

DISCUSSION

The amount of genetic similarity at nuclear markers displayed by the North Atlantic large white-headed gulls is surprisingly high. Both allozyme and microsatellite data suggest a weakly differentiated gene pool, with an amount of differentiation more typical of within-species population struc-

TABLE 6. Pairwise θ-values based on five microsatellite loci among large gull species (*Larus*) with minimum and maximum values from jackknife resampling over loci in parentheses (above diagonal) and associated significance values based on permutation tests (below diagonal). All *P*-values remain significant after sequential Bonferroni correction at $\alpha = 0.05$. Note that sample sizes for *L. marinus* and *L. smithsonianus* are <10 (see Table 1).

	argentatus	*smithsonianus*	*fuscus*	*michahellis*	*marinus*
argentatus	—	0.045 (0.02–0.06)	0.060 (0.004–0.07)	0.060 (0.02–0.08)	0.069 (0.05–0.08)
smithsonianus	0.017	—	0.094 (0.06–0.11)	0.115 (0.08–0.14)	0.210 (0.13–0.27)
fuscus	0.001	0.001	—	0.051 (0.03–0.06)	0.133 (0.12–0.15)
michahellis	<0.001	<0.001	0.001	—	0.099 (0.07–0.12)
marinus	0.020	<0.001	0.001	0.001	—

tures. This is highly unexpected given the undisputed species status of these taxa. Furthermore, the amount of differentiation measured with nuclear and mitochondrial markers differ substantially.

Several nonexclusive hypotheses can explain this situation. First, recent speciation events, associated with large effective population sizes, would result in still largely undifferentiated nuclear gene pools in the various species. Due to the smaller effective population size of mitochondrial DNA, haplotypes frequencies could diverge more quickly and result in larger values of differentiation measures. Second, peculiarities of the evolution of the markers could explain that nuclear allele frequencies remain similar over long periods of time, and/or selective sweeps could speed up the fixation of alternative allelic states at the mitochondrial locus. Finally, ongoing gene flow between the species could counterbalance the effects of drift and maintain similar allele frequencies in the species, explaining also the observed amount of mitochondrial lineage sharing. We will consider these hypotheses in the following discussion.

Recent Speciation without Subsequent Gene Flow

A recent origin of the species would result in low measures of population differentiation during periods of time depending on the effective population size of the species. This time can be evaluated using the formula of Nei and Chakravarti (1977), which gives the value of F_{ST} between a set of isolated subpopulations having split from a common origin as a function of t (number of generation since the separation) in a model without migration nor mutation. For nuclear genes, F_{ST} can be approximated by:

$$[(1 - 1/s)(1 - e^{-t/2N})]/[(1 - (1 - e^{-t/2N})/s], \qquad (1)$$

where s is the number of subpopulations and N their common effective size.

An estimate of species divergence times for large gulls can be obtained from mitochondrial sequences. Our own data on cytochrome *b* divergence (0.22–0.80% between the main haplotypes; see Table 3) suggest speciation between 110,000 and 400,000 years ago using the crude but conventional calibration of 2% divergence per million years. Of course, the estimation of divergence time given above is the time of divergence of the genes, not the species, which can be younger than that (for a discussion of gene versus population history see Edwards 1997). The difference between genes and populations divergence is due to the possibility of retaining genetic polymorphism (after gene divergence) within the ancestral population (prior to population divergence). Edwards (1997) suggested using the amount of current intrapopulation polymorphism to estimate population divergence time from gene divergence time. In the case of the large white-headed gulls, there is strong evidence that the intraspecific diversity in mtDNA is almost entirely due to introgression. When more than one haplotype is found in a species, the less frequent haplotype is identical to the common haplotype of another species, and geographic repartition of haplotypes are indicative of interspecific horizontal transfer (for a more thorough discussion of mitochondrial introgression see below and especially Crochet et al. 2002). Intraspecific diversity is consequently not the result of mutational and demographic processes within each species, which would need to be incorporated into the estimates of interspecific divergence time, but mostly of past hybridization. Taking into account this foreign diversity would thus give a flawed estimate of divergence time between the species. Instead, the lack of polymorphism within species other than due to introgression indicates that coalescence within species occurs in a short time and that gene and species divergence time are likely to be very similar.

Liebers et al. (2001) and Liebers and Helbig (2002) used the more variable left domain control region sequences in some of our species and applied Edwards's (1997) method to their data. Using the divergence data for control region sequences in Liebers et al. (2001) and the more accurate estimate of divergence rate in Liebers and Helbig (2002) provides an estimate of the basal divergence between gull species around 500,000 years ago, extremely similar to their estimate of the age of the ancestral population of the basal species (490,000 in the same paper). For the more recently derived *L. fuscus*, they estimated that the ancestral population lived approximately 165,000 years ago. All available information from mitochondrial data thus suggests that these large gull species originate from speciation events between 100,000 and 500,000 years ago, depending on the species.

TABLE 7. Pairwise θ-values based on seven enzymatic loci among large gull species (*Larus*; above diagonal) and associated significance values (below diagonal). All *P*-values remain significant after sequential Bonferroni correction at $\alpha = 0.05$.

θ	*smithsonianus*	*argentatus*	*michahellis*	*fuscus*	*marinus*
smithsonianus	—	0.034	0.033	0.088	0.072
argentatus	<0.002	—	0.029	0.051	0.036
michahellis	0.014	0.004	—	0.057	0.083
fuscus	<0.002	0.006	0.006	—	0.060
marinus	0.004	0.036	0.012	0.018	—

Based on equation (1), for $s = 6$, and based on an estimated generation time of 10 years for large gulls (using demographic data for *L. michahellis* in Defos du Rau 1995), it takes effective population sizes in the order of 100,000 individuals (63,000 to 230,000 for the microsatellites and 110,000 to 400,000 for the allozymes) to maintain the observed low level of nuclear differentiation for this amount of time. With effective population size around 10,000 birds, it takes 10,000 to 20,000 years only to reach the F_{ST}-values observed for allozymes and microsatellites, respectively, and the expected F_{ST}-value after 100,000 to 400,000 years would range from 0.35 to 0.84.

Estimates of current census population sizes for the European large gull species (see for instance Hagemeijer and Blair 1997) range from around 200,000 to 1,600,000 breeders. These current census sizes are very different from genetic effective population sizes because populations of all large gull species have increased markedly during the 20th century (e.g., Cramp and Simmons 1983). Several countries with large populations of *L. fuscus* have been colonized during the 20th century, and in all species and all countries for which we could find specific estimates of population sizes in the early 20th century, population sizes have been multiplied by 15 to 200 (Cramp and Simmons 1983; Risberg 1990; Lloyd et al. 1991; Yeatman-Berthelot and Jarry 1994). Census sizes must thus have been around 20,000 to 150,000 individuals a few generations ago, possibly fewer. Given that effective population sizes are usually substantially lower than census population sizes, effective population sizes have almost certainly been between 10,000 and 100,000 individuals for the species studied. These extrapolations even neglect bottlenecks during the last glacial maximum, which have been shown to affect genetic diversity in at least one species of large white-headed gulls (Liebers and Helbig 2002). Explaining the low differentiation of nuclear markers between the most distant gull species by lack of time to reach equilibrium only would thus require unrealistically large population sizes (long-term effective population size over 200,000 birds).

An even stronger argument against the scenario of recent speciation without subsequent gene flow is the incompatibility of the measures of genetic differentiation between gull species derived from mitochondrial and nuclear markers. In the absence of any migration or mutation, the amount of genetic differentiation between recently separated populations is expected to increase more rapidly for mitochondrial markers than for nuclear markers because of the smaller effective population size of mitochondrial genes compared to nuclear genes. Under the scenario of recent speciation and no gene flow, the expected nuclear F_{ST}-value corresponding to the level of differentiation between gull species obtained with mitochondrial markers (0.66) would be 0.20 according to the Nei and Chakravarti (1977) formula, much higher than the level observed in gulls (0.04 for allozymes and 0.07 for microsatellites). At no point between the population split and the time to reach equilibrium is the difference between nuclear and mitochondrial markers as large as observed. This again invalidates the recent speciation without subsequent gene flow hypothesis as the only explanation and indicates that another mechanism certainly participates in the low level of nuclear differentiation between gull species.

Constraints on Markers Evolution

Allozymes could be under balancing selection maintaining similar allele frequencies in the various species. This explanation is quite unlikely as it would require that this phenomenon applies to all seven loci and to populations that inhabit very different environments. Furthermore, it cannot hold for microsatellite loci, whose different allelic states are extremely unlikely to be under balancing selection.

A high mutation rate of the loci combined with constraints on allele sizes and/or large effective population sizes could explain the low level of differentiation observed for the microsatellite loci. The equilibrium values for *F*-statistics in a model with mutation and no migration is $(s - 1)/[s(4N\mu + 1) - 1]$ (e.g., Hedrick 1999), where s is the number of populations and μ the mutation rate. Microsatellites mutation rates around 10^{-3} to 10^{-4} (which seems reasonable for vertebrates, see Ciofi and Bruford 1999) combined with effective population sizes around 1000 to 10,000 individuals (see above) would produce the observed level of divergence for microsatellites. This explanation has been recently proposed for several vertebrate populations (Paetkau et al. 1997, 1998; Hedrick 1999; Balloux et al. 2000), but Estoup et al. (2002) recently showed that it is unlikely to affect many types of population genetics analyses realized by molecular ecologists.

Recently, de Knijff et al. (2001) analyzed 209 autosomal biallelic AFLP loci in large white-headed gulls and found a low level of interspecific differentiation, similar to what we found with microsatellites (among species percent genetic variation of 10.5 when using the same species as us). The fact that results similar to ours were found with a totally independent dataset containing many more marker loci, certainly evolving under very different constraints, excludes that the genetic similarity in nuclear markers among large gulls can be due only to the evolutionary processes germane to the markers.

Even if evolutionary constraints or selection are not responsible for the low level of nuclear differentiation, the higher value of differentiation for mitochondrial DNA compared with nuclear markers could be explained by selection on haplotypes. If different favorable mutations are favored in each species, the selective sweep resulting from such directional selection could lead to the rapid increase in frequency of one haplotype per species, resulting in stronger F_{ST}-value than expected under neutral evolution. Such events would not alter the amount of sequence divergence between the main haplotype of each species and the age of the species, and thus not invalidate our rejection of the recent speciation without gene flow hypothesis. Furthermore, directional selection for one type of mitochondrial DNA would reduce the time to complete lineage sorting among gulls and make even more likely that the instance of lineage sharing among gull species originate from introgression (see below and Crochet et al. 2002).

Interspecific Gene Flow

Some interspecific gene flow among large gulls, combined with a recent species origin, is thus the most likely explanation for the observed pattern of genetic differentiation

among species. Hybridization is known to occur among many large gull species, including all the species we analyzed (Gray 1958; Pierotti 1987).

Mitochondrial DNA data.—The extensive mitochondrial lineage sharing indicate that gene flow occurs among gull species. Alternative hypotheses cannot explain all instances of lineage sharing (for a thorough discussion of the mitochondrial data see Crochet et al. 2002), a conclusion also reached by Liebers et al. (2001) and Liebers and Helbig (2002).

Furthermore, some cases of lineage sharing are typical of hybridization events. One of these examples not discussed in Crochet et al. (2002) is found in *L. marinus*. Both North American specimens of *L. marinus* that we analyzed carry the *smithsonianus* haplotype, which has not been found anywhere outside North America. On the contrary, all European *L. marinus* have a *marinus* haplotype, which is also found in the European sympatric *L. argentatus*, except one Swedish individual carrying the common haplotype of the sympatric *L. fuscus*. Because *L. marinus* is widespread in Europe, while its range in North America is currently restricted to the western part of the continent, where it is expanding, one can suspect that this species is a comparatively recent colonist from Europe. The North American *marinus* must have acquired the *smithsonianus* haplotypes through hybridization, which is known to occur there in the wild (Pierotti 1987; D. L. Dittmann, pers. comm.; R. Curry, pers. comm.), after their colonization of North America.

Nuclear markers data.—The extensive similarity between large gulls nuclear markers cannot easily be explained by recent species origin or evolutionary constraints alone. Interspecific gene flow has to be introduced to explain the observed pattern.

If interspecific gene flow was the only evolutionary force that maintains the homogeneity of nuclear markers in large gulls, what level of gene flow would the population genetics models estimate? Using the crude but qualitatively correct relationship $F_{ST} = 1/(4Nm + 1)$, based on the assumptions of migration-drift equilibrium in the infinite-islands model, the estimated level of gene flow would be at most 11 migrants per generation (based on results from allozymes) between the sympatric *L. fuscus* and *L. argentatus*. This corresponds roughly to one hybrid successfully reproducing with each parental species every year. No precise data are available on hybridization frequency between these two species in natural conditions, but hybridization is clearly occasional (Harris 1970; Harris et al. 1978; pers. obs.), although it may have been more frequent in the past, at least locally during range expansions (Haffer 1982). The level of interspecific gene flow estimated using the molecular markers is thus realistic.

Nuclear versus mitochondrial gene flow.—If gene flow is partly responsible for the low level of differentiation between large gull species, the much higher level of differentiation among species detected with the cytochrome *b* than with the allozymes or microsatellites suggests that the amount of nuclear and mitochondrial gene flow differ substantially.

It should first be noted that a difference in F_{ST}-values between nuclear and mitochondrial markers is to be expected even when there is no sex bias in gene flow, due to the differences in effective population size between nuclear and mitochondrial genes: at equilibrium, $F_{ST(\text{mitochondrial})} = 4F_{ST(\text{nuclear})}/[1 + 3F_{ST(\text{nuclear})}]$ (see Crochet 2000). In our case, the observed mitochondrial F_{ST}-value among gull species ($\theta = 0.66$) would correspond to an expected nuclear F_{ST}-value of 0.33 at equilibrium and without sex bias in gene flow.

This expected value is almost an order of magnitude higher than the observed values for allozymes or microsatellites among the same species ($\theta = 0.04$ and $\theta = 0.07$, respectively) and suggests that differences in effective population size cannot explain the differences in F_{ST}-values between nuclear and mitochondrial markers. This discrepancy could be explained either by a stronger selection against alien mitochondrial haplotypes compared to nuclear loci (see above) or a higher male-mediated gene flow. The estimated amount of female-mediated gene flow between *L. argentatus* and *L. fuscus* (using the formula $F_{ST(\text{mitochondrial})} = 1/(1 + 2N_fm_f)$, where N_fm_f is the number of female migrants) would be close to 0.4 fertile female hybrid per generation. This is about 25 times less than the estimated gene flow mediated by both sexes.

Sex bias in gene flow could be due to behavioral effects or to different postzygotic isolation in male and female hybrids. Interspecific large gulls hybrids can be formed between sympatric species or between allopatric species when a vagrant gull mates and reproduces with a member of another species. Because males are the more philopatric sex in gulls (Greenwood and Harvey 1982), males should not be more prone to reproduce outside their normal range than females, and there should not be more alien nuclear genes than alien mtDNA. But in contact zones or areas of sympatry, female hybrids could pair preferentially with the same species as their mother, leading to a lack of mitochondrial gene flow compared to nuclear markers. This hypothesis can be evaluated by behavioral studies of hybrids mating behavior.

A strong disadvantage of female hybrids compared to male hybrids would explain the discrepancy in mitochondrial and nuclear amount of differentiation. This would be in accordance with the general observation in animals that when F_1 hybrids of one sex show reduced fitness compared to the other sex, that sex is nearly always the one with heterogametic sex chromosomes (Haldane's rule, for a summary on the importance of this phenomenon see Coyne et al. 1991). This would indicate that some postzygotic isolation is acting in (at least female) hybrids between most of the large gull species we analyzed, in spite of the apparent lack of hybrid disadvantage observed in the closely related *L. occidentalis* and *L. glaucescens* (Good et al. 2000). Stronger selection against female hybrids has also been suggested in a contact zone between two passerine species (*Phylloscopus collybita* and *P. ibericus* [called *P. brehmii* then]) that differ as little as our gulls species at nuclear loci but have substantially more in mtDNA divergence (Helbig et al. 2001).

Further research is needed to determine whether the apparently lower level of mitochondrial gene flow observed in gulls when compared to nuclear gene flow originates from selection against alien haplotypes or stronger disadvantage of female hybrids or can be explained by a similar level of gene flow between sexes in a situation where recent speciation creates a nonequilibrium. The latter hypothesis can be evaluated through a simulation approach. If the observed situation cannot be obtained without sex-biased gene flow, one

way to discriminate between the other hypotheses would be to use independent markers with the same inheritance bias that the mitochondrial DNA. Markers on the W chromosome, which is only transmitted by females and has the same effective population size as mtDNA, would be prime candidates.

The Role of Selection on Phenotypic Characters

Whereas neutral genes exhibit little differentiation, several diagnostic characters exist even between the species most similar in allozyme or microsatellite alleles frequency. For example, *L. argentatus* and *L. fuscus* differ in mantle color, leg color, voice, and displays (Cramp and Simmons 1983), and no overlap exists for any of these characters in the mixed colonies of Western Europe. Given the homogeneity of neutral markers, such traits can only be sorted out by selection. They cannot be too numerous in the genome, for a large number of loci under selection in the genome would act against the homogenizing action of gene flow on neutral markers through genetic linkage.

Mixed pairs are very rare among the sympatric European species, an illustration of the efficiency of prezygotic isolation. For this prezygotic isolation to be maintained, there must be some selection against hybrids. Because the nuclear genome of the large gulls is so similar, selection against hybrids is probably not a consequence of genome incompatibilities at autosomal loci for endogenous reasons. We hypothesize that part of these disadvantages may be due to sexual selection. Hybrids between *L. fuscus* and *L. argentatus* had intermediate leg and eye ring color (Harris et al. 1978). If, as it has been suggested by Pierotti (1987), bare-part coloration is important in mate recognition in large gulls, such hybrids could be disadvantaged when looking for a mate. The same disadvantage of hybrids with intermediate characters could apply to other traits involved in mate choice such as mantle color, voice, or behavior.

Conclusion: Hybridization and Speciation in Birds

Comparisons of the results from mitochondrial and nuclear markers as well as geographic arguments all indicate that interspecific gene flow is implicated in maintaining the low level of genetic differentiation between the recently diverged large white-headed gull species. The discordant patterns of mitochondrial and nuclear differentiation suggest a higher male-mediated gene flow, as a result of sex-differences in mating behavior or in postzygotic isolation, but further work is needed to exclude selective forces acting on mtDNA or a combined effect of recent speciation (nonequilibrium situation) and nonbiased gene flow. Whatever the reasons for the lack of differentiation at nuclear markers between gulls, the very different situation for phenotypic traits points to the importance of selection in generating (and possibly maintaining) species-specific differences in this group.

These results might seem surprising, as they imply that the amount of gene flow between gull species might be enough to prevent strong differentiation at nuclear markers. This phenomenon could still be less exceptional in birds than usually realized. It has long been known that the amount of genetic differentiation, as measured with enzymatic markers, is generally very low between bird species compared to other vertebrates (e.g., Barrowclough and Corbin 1978; Avise and Aquadro 1982; Kessler and Avise 1985; Evans 1987; Mindell et al. 1996). Nei's genetic distances between congeneric avian species are often smaller than 0.1, a value exceptionally low in other vertebrate groups (Avise 1983). Estimates of species divergence time derived from mtDNA suggests that this general pattern is best explained by a younger origin of most avian species compared with other vertebrates (Kessler and Avise 1985; Klicka and Zink 1997; Avise and Walker 1998), possibly combined with a slower rate of molecular evolution when compared to mammals (but apparently not to cold-blooded vertebrates; Martin and Palumbi 1993; Mindell et al. 1996). This may, however, not completely explain some observed cases of very high genetic similarity between species, where ongoing hybridization is an alternative explanation that deserves attention.

Two such cases concern North American species known to hybridize either along their contact zone (the essentially allopatric chickadees *Parus atricapillus* and *P. carolinensis*) or over a significant part of their range (the largely sympatric mallard, *Anas platyrhynchos*, and American black duck, *A. rubripes*). In both instances, genetic distance were extremely low between the species (Nei's genetic distance $D \leq 0.0019$) and in the case of the ducks, a number of comparisons between the two species yielded lower estimates of genetic distance than between conspecific populations (Ankney et al. 1986; Braun and Robbins 1986). This situation is very similar to what we observed in large gulls. Other cases of low genetic differentiation in birds measured by allozymes have been attributed to hybridization (*Sturnus vulgaris* and *S. unicolor*: de la Cruz-Cardiel et al. 1997; *Milvus milvus* and *M. migrans*: Schreiber et al. 2000; *Phylloscopus collybita* and *P. ibericus*: Helbig et al. 2001). In both cases, the species are known to hybridize in the wild. Strong support for the genetic consequences of hybridization in birds may be found in Darwin's ground finches of the Galapagos. In this group, ongoing hybridization is well documented (see introduction). Recent studies have shown a lack of clear differentiation at both nuclear and mitochondrial markers, as predicted by the high frequency of hybridization in this group (Freeland and Boag 1999). Gene flow among Galapagos finches is indeed so high that it has been suggested that most traditionally recognized species are in fact morphological variation of a few highly variable species (Zink 2002). Large white-headed gulls and the other examples discussed above suggest than bird species could be maintained even if speciation does not result in a complete reproductive isolation.

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