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Molecular and morphological patterns of introgression between two large white-headed gull species in a zone of recent secondary contact

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Abstract

Incomplete reproductive isolation promotes gene flow between diverging taxa. However, any gene encoding for traits involved in the reproductive barriers will be less prone to introgression than neutral markers. Comparing introgression rates among loci is thus informative of the number and functions of loci involved in the reproductive barriers. This study aimed at identifying possible mechanisms of restriction to gene flow across a zone of recent secondary contact between *Larus argentatus* and *Larus cachinnans* by comparing introgression patterns for nine microsatellite loci, a fragment of mitochondrial DNA and a set of phenotypic traits. The low linkage disequilibrium between neutral nuclear markers indicated introgression without any barrier to gene flow. However, asymmetric introgression of mitochondrial DNA suggested that interspecific crosses may be more successful in one direction. The introgression rate for phenotypic traits was variable and low compared to neutral molecular markers. This was particularly evident in colouration of bare parts: individuals with intermediate colouration were scarcer in sympatry than expected if the genomes recombined freely. We hypothesized that one of these variables, the orbital ring colour, may play a role in mate choice, acting as an incomplete premating barrier through assortative mating. This study emphasizes that multilocus approaches are useful to discriminate among possible mechanisms responsible for the maintenance of hybrid zones.

Keywords: cytochrome *b*, hybridization, introgression, *Larus*, microsatellites, phenotypic variance

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Speciation occurs when reproductive isolation arises between differentiated gene pools (Noor 2002). As long as premating isolation remains incomplete, hybridization can lead to introgression of genes between differentiated taxa unless postmating barriers are strong enough to prevent it (Borge *et al*. 2005; Mallet 2005). Introgression can be countered by selection against foreign alleles in the genome or by natural and sexual selection acting on these introduced genes. Thus, introgression rates can vary among loci: any gene encoding for traits involved in a reproductive barriers will be less prone to introgression than neutral loci (Barton & Hewitt 1981; Payseur *et al*. 2004). The introgression rate of a locus will also decrease the more it is linked to loci under selection (Barton & Hewitt 1985). If there is no selection against introgression, all loci behave as expected under the neutral diffusion model (Endler 1977). However, if there is strong selection acting on many loci distributed over the whole genome, all loci will form clines maintained by a selection–dispersal balance. Variable introgression rates among loci are therefore informative of the number and nature of loci involved in reproductive barriers.

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Furthermore, sex-biased introgression originating from sex-biased dispersal, behaviour-generating premating barriers or sex-specific postmating barriers, can also generate variable introgression rates of loci across the genome (Barton & Hewitt 1989). For example, sex-biased postmating barriers can be explained by Haldane's rule, which states that if there are sex-specific fitness differences in the F1 progeny, it is usually the heterogametic sex which is the least fit (Haldane 1922; Coyne *et al*. 1991). In birds, in which females are the heterogametic sex, the introgression rate of the maternal genome is therefore expected to be relatively lower, particularly in mitochondrial DNA (mtDNA) due to its maternal inheritance.

Hybridization and introgression is a frequently recognized phenomenon (e.g. Arnold 1997 and references therein). It is particularly common in birds (Grant & Grant 1992), apparently because postmating barriers evolve slower compared to other vertebrates (Prager & Wilson 1975; Price & Bouvier 2002; Fitzpatrick 2004). Detailed analyses of avian hybrid zones are thus crucial to our understanding of the mechanisms that maintain phenotypic differentiation among sympatric bird species despite incomplete reproductive isolation. In this study, we focus on two European gull species, the herring gull (*Larus argentatus*) and the Caspian gull (*Larus cachinnans*), hybridizing in a recent zone of secondary contact. We compared introgression rates among several categories of markers, including several supposedly neutral nuclear and mitochondrial loci as well as several phenotypic traits that are potentially involved in reproductive isolation, to understand if and how phenotypic divergence can be maintained despite gene flow.

Large white-headed gulls generally constitute an excellent model for studying the persistence of phenotypic differences between taxa with incomplete reproductive isolation. First, this group has a very recent origin (100 000–600 000 years ago, Crochet *et al*. 2003; Liebers *et al*. 2004). Second, genetic differentiation between taxa is surprisingly low at nuclear loci (F_{ST} between 5% and 10%, Crochet *et al*. 2003). Third, differentiation is usually higher in mtDNA, but most species still share polymorphisms. The discrepancy in differentiation levels between the nuclear and mitochondrial genome has been explained by a combination of recent species origin and interspecific gene flow after speciation (Crochet *et al*. 2003). Indeed, interspecific hybrids between large gull species have frequently been recorded (Pierotti 1987; Crochet *et al*. 2003; Olsen & Larsson 2004).

The breeding distribution of the herring gull is northern European, ranging from Iceland to the Kola Peninsula (Olsen & Larsson 2004). Its population size increased markedly during the 20th century, leading to a recent range expansion (Cramps & Simmons 1983; Pons & Migot 1995). The first breeding attempt in Poland was recorded in 1968, and since then, herring gulls have been expanding southwards. It is now a widespread breeder (Panov & Monzikov 1999; Neubauer *et al*. 2006). Caspian gulls have a southeastern Eurasian breeding distribution, ranging from the Black Sea and the Caspian Sea to eastern Kazakhstan (Olsen & Larsson 2004). This species has been expanding northwards, e.g. reaching Poland where the population size has increased exponentially since the first breeding events recorded in the late 1980s (Skorka *et al*. 2005). Today, the distribution ranges of both the herring and Caspian gull overlap in Poland and western Russia (Panov & Monzikov 1999). Hybridization is likely to occur in this zone of recent secondary contact, because mixed pairs and morphological hybrids have been observed (Neubauer and Zagalska-Neubauer, unpublished observations). However, morphologically intermediate individuals indicate hybridization but not necessarily introgression. Due to the close similarity and extensive variability in all phenotypic characters of these two species, molecular markers are needed to understand patterns of introgression across this zone of secondary contact. Furthermore, comparing rates of introgression for phenotypic characters and neutral molecular markers allows a more efficient identification of any barrier to gene flow and better understanding of its mechanisms.

The aim of this study is thus to identify possible mechanisms of restrictions to gene flow between hybridizing herring and Caspian gulls by comparing introgression patterns between supposedly neutral molecular markers, including nine nuclear microsatellite loci and a fragment of the mitochondrial cytochrome *b* gene, and a set of phenotypic variables describing the colour of bare parts and plumage as well as morphometry. The first step was to verify with molecular markers that suspected cases of hybridization actually lead to genetic introgression. Since we cannot directly compare introgression rates of morphological traits and molecular markers, we sequentially addressed the following questions. (i) Is there any evidence of barriers to introgression in the neutral genomes? (ii) Does sex biased introgression lead to different introgression rates of the mitochondrial genome compared to neutral nuclear markers? (iii) Is there any evidence that introgression rates are reduced in any of the phenotypic traits compared to neutral molecular markers, suggesting that these traits are involved in reproductive isolation? For this purpose, we developed a new method based on phenotypic variance that allows comparing qualitatively the introgression rates of various traits.

Materials and methods

Study site and sample collection

Samples for genetic analyses were collected in seven populations along a transect spanning over the distribution range of both *Larus argentatus* and *Larus cachinnans*,

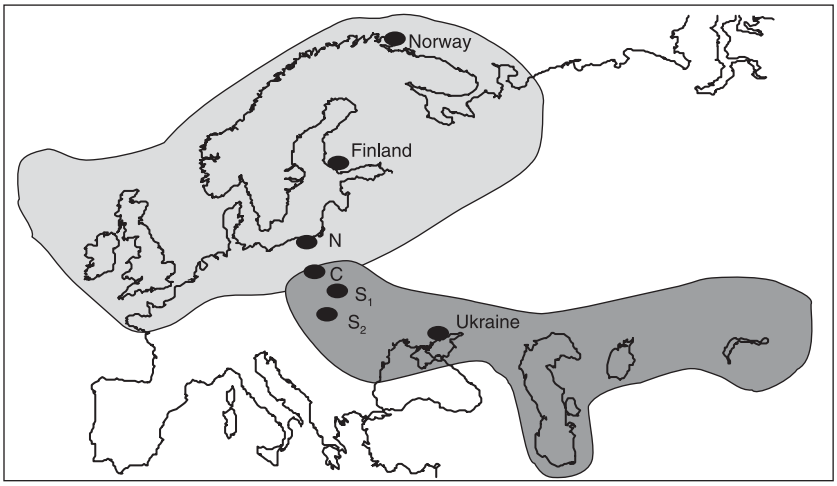

Fig. 1 Breeding distributions of the herring gull, *Larus argentatus* (light grey), and Caspian gull, *Larus cachinnans* (dark grey) (from Olsen & Larsson 2004) depicting sampling sites. Norway (Vardø); Finland (Turku); Ukraine (Molochnyy Lyman) and four sampling sites in Poland: N, northern Poland (Gdynia); C, central Poland (Włocławek); S_1, southern Poland 1 (Zastow); S_2, southern Poland 2 (Tarnow).

Table 1 Summary statistics of molecular markers and phenotypic variables studied in seven populations across the zone of secondary contact between *Larus argentatus* and *Larus cachinnans*: sample size (n_{micro}), number of alleles ($n_{alleles}$), expected heterozygosity (H_E with standard error in brackets) and global F_{IS} for the nine microsatellite loci; sample size (n_{cytb}), nucleotide (π) and haplotype diversity (H) for cytochrome *b* sequences; sample size for morphological measurements (n_{morpho}). The significant F_{IS} values in Finland and Ukraine were both attributable to a large heterozygote deficiency at one single locus (HG27 and HG14, respectively)

		Microsatellites				mtDNA			Phenotype
		n_{micro}	$n_{alleles}$	H_E	F_{IS}	n_{cytb}	π	H	n_{morpho}
Allopatric *argentatus*	Norway	8	3.56	0.45 (0.30)	−0.12	8	0.008 (0.005)	3	
	Finland	30	5.33	0.55 (0.27)	0.17**	10	0.010 (0.006)	5	
	northern Poland	20	4.67	0.50 (0.29)	0.11*	7	0.005 (0.003)	4	32
Sympatry	central Poland	106	6.89	0.62 (0.24)	0.11**	23	0.006 (0.004)	6	177
Allopatric *cachinnans*	southern Poland 1	37	6.11	0.54 (0.29)	−0.005	7	0.002 (0.001)	3	
	southern Poland 2	20	5.22	0.55 (0.28)	0.10*	6	0.001 (0.001)	2	45
	Ukraine	32	5.44	0.56 (0.27)	0.11**	9	0.002 (0.002)	2	57

including their zone of secondary contact (see Fig. 1). Populations sampled in Norway (Vardø), Finland (Turku) and northern Poland (Gdynia) belong to *L. argentatus* only (referred to as 'allopatric *argentatus*'), while populations sampled in southern Poland (Zastow and Tarnow) and Ukraine (Molochnyy Lyman) belong to *L. cachinnans* only (referred to as 'allopatric *cachinnans*'). In the population from central Poland (Włocławek) both species occur (referred to as 'sympatric'). Sample sizes are described in Table 1. Samples including blood stored in EDTA buffer, growing feather quills or muscle in ethanol were collected during the breeding seasons from 1998 to 2004. They were mainly taken from adult gulls trapped on the nest, except in Finland where adult and subadult birds were caught on waste deposits. In the sympatric colony, nests were sampled randomly irrespective of parents' identity. To increase sample sizes in some of the colonies, samples were additionally taken from chicks or freshly dead birds that were not directly related to the already sampled individuals.

Morphological measurements

Twenty-three quantitative or semiquantitative variables concerning morphometry, plumage melanism and colouration of bare parts (see Table S1, Supplementary material) were measured on adults in four populations (including northern, central and southern Poland 2 along with Ukraine). The seven morphometric variables were measured with a calliper to the nearest millimetre: tarsus, toe, wing, head and bill length, bill depth at gonys and minimum bill depth. Seven quantitative (number of primaries with black, length of white on the ninth and 10th primary, length of black on the seventh, eighth, ninth and 10th primary) and four semiquantitative variables (colour of the tongue on the inner web of the 10th primary, type of black pattern on the sixth, ninth and 10th primary) described the pattern of the primary feathers. Five semiquantitative variables concerned bare parts colouration (tarsus, toe, web and eye-ring colour and dark spots in the iris). The nine

semiquantitative traits (ranked categories) will be treated as quantitative variables in subsequent analyses (Siegel & Castellan 1988). Photograph vouchers were taken for most individuals (available from authors upon request).

Measurements were taken by nine different observers. However, analyses of variance (ANOVA) carried out in SAS version 8 (SAS Institute 2000) did not show any significant observer effect in any of the three populations in which there was more than one observer. We estimated repeatability by testing for correlation between measurements in all birds measured twice ($n = 30$, data only available for the most frequent observer GN). Repeatability was very high for most traits (from 82% to 100%). Only eye-ring colour had a lower repeatability (75%), probably due to variability in individual physiological state. However, differences between measurements were always smaller than the mean difference between eye-ring colour in *L. argentatus* and *L. cachinnans*.

Genetic analyses

Total genomic DNA from blood samples was extracted using the DNeasy Tissue Kit (QIAGEN) following the recommended procedure. Feathers and muscle tissue were digested in 10% Chelex 100 (Biorad) with 5 μL of proteinase K followed by two 15-min boiling steps following the procedure described in Walsh *et al*. (1991).

Nine microsatellite loci were amplified in 253 individuals: five loci (HG27, HG25, HG18, HG14, HG16) were developed for the American herring gull *Larus smithsonianus* (Crochet *et al*. 2003) and four (K31, K32, K67, K71) for the black-legged kittiwake *Rissa tridactyla* (Tirard *et al*. 2002). For HG- loci, the polymerase chain reaction (PCR) was performed in 10 μL reaction volumes containing 2 μL DNA template of variable concentration, 10 mM Tris-HCl, 50 mM KCl, 2 mM $MgCl_2$, 2 mM of each dNTP, 0.4 μM of each primer and 0.1 U *Taq* DNA polymerase (Eurogentec). All HG-loci were amplified with an annealing temperature of 58 °C. K- loci were amplified following the protocol in Tirard *et al*. (2002). PCR products were visualized using an ABI PRISM 310 Genetic analyser (Applied Biosystems).

An approximately 500 bp long fragment of the cytochrome *b* gene was amplified in a subset of 70 individuals from each of the seven populations (Table 1) using the primers L14967 and H15503 or H15938, following procedures described in Pons *et al*. (2004). All amplification products were sequenced on both strands.

All individuals with available DNA sample were sexed using the universal molecular sexing method described in Griffiths *et al*. (1998). We also sexed birds in the field, based on measurements of tarsus, bill and wing length. As there was no discrepancy between these methods ($n = 149$), we determined the sex of further individuals based on morphological measures only.

Genetic differentiation between L. argentatus *and* L. cachinnans

We estimated the genetic differentiation between allopatric *argentatus* and allopatric *cachinnans* populations, both in nuclear and mitochondrial markers. For microsatellites, F_{ST} data were calculated in GENETIX (Belkhir *et al*. 1998), testing for significance by 1000 permutations of individuals among populations. For cytochrome *b* sequences, Φ_{ST} was calculated using an analysis of molecular variance (Excoffier *et al*. 1992) in ARLEQUIN (Schneider *et al*. 2000) with Kimura 2-parameter distance and random permutations of 1000 replicates.

Detection of hybridization

Admixture between two differentiated populations can increase the genetic diversity and create deviations from Hardy–Weinberg proportions. We estimated the number of alleles, the expected heterozygosity (H_E) and the heterozygote deficiency (F_{IS}) in all populations across the transect using GENETIX (Belkhir *et al*. 1998). Significance of the F_{IS} values tested by permutation of alleles within populations. We also estimated the nucleotide and haplotype diversity on cytochrome *b* sequences. We performed a multiple correspondence analysis (MCA) on individuals from the seven populations using the genotype at the nine microsatellite loci in ADE-4 (Thioulouse *et al*. 1997). MCA is particularly well-suited for the analysis of hybrid zones as it generally displays the differentiation between species on the first axis F1 (Guinand 1996; Parisseaux 1997).

To detect individuals with mixed ancestry, microsatellite genotypes of individuals from all seven populations were assigned to either *L. argentatus* or *L. cachinnans* using the Bayesian assignment method (Pritchard *et al*. 2000) implemented in STRUCTURE (http://pritch.bsd.uchicago.edu/software.html). Analyses were performed using the admixture model and without any prior information on the parental populations to avoid an artificial increase of intermediates in nonparental populations. The number of populations was set to $K = 2$ (for two species). We used a burn-in period of 500 000 iterations to reach the stationary distribution followed by another set of 500 000 iterations. We reiterated the analysis and checked for convergence and the absence of autocorrelation between iterations using the CODA package (Cowles & Carlin 1995) of the software R (R-Development-Core-Team 2004). Following recommendations by Pritchard *et al*. (2000), individuals were assigned to either *L. argentatus* or *L. cachinnans* if their assignation probability was higher than 90%. Others were classified as 'intermediate'. We estimated the power of assignment as the success of assigning *L. argentatus* and *L. cachinnans* individuals originating from allopatric populations to their own species, with prior information on

populations. It reached 89% (the remaining 11% of birds were classified as intermediates).

Additionally, we performed a principal component analysis (PCA) on a subset of morphological variables measured in individuals from four populations (northern Poland, central Poland, southern Poland 2 and Ukraine) in ADE-4. These included six morphological variables (bill length, bill depth at gonys, number of primaries with black, length of black on the seventh primary, iris and eye-ring colour, see Table S1), chosen to maximize sample size. As there are sex-specific differences in these traits (Zagalska-Neubauer and Neubauer, unpublished data), we analysed both sexes separately (n = 126 males and 174 females). Those individuals whose PC1 scores fell outside the 95% boundaries of PC1 distributions in allopatric *argentatus* and allopatric *cachinnans* populations (northern Poland and Ukraine, respectively) were defined as morphological intermediates.

Finally, we compared the proportions of intermediate individuals determined by these two methods in allopatric and sympatric populations, assuming that a higher proportion of intermediates in sympatry would indicate hybridization.

Introgression of the neutral nuclear genome? Linkage disequilibrium analyses

Within a hybrid zone, strong associations between loci, chromosomes and morphological traits originating from parental populations (linkage disequilibrium) are frequent (Goodman *et al*. 1999; Barton 2000). Peripheral parental populations supply parental gene combinations through migration, while recombination and segregation in hybrids break them down. An absence of linkage disequilibrium thus indicates extensive introgression resulting in complete recombination of the parental genomes. Multilocus within-genome disequilibrium $K_{1,1}$ (analogous to an average heterozygote deficiency) and between-genome disequilibrium $K_{0,2}$ (analogous to an average linkage disequilibrium) were estimated in MATHEMATICA (http://helios.bto.ed.ac.uk/evolgen/barton/index.html), using a method for combination of data across loci developed by Barton (2000). As recommended, multiple alleles were pooled into two species-specific compound alleles to reduce the noise created by within-species diversity, which is not relevant to the analysis of disequilibria within the hybrid zone. For all microsatellite loci, each allele was assigned to a species-specific compound allele according to its coordinates on F1 axis (Daguin *et al*. 2001). Likelihoods for different nested models were compared using likelihood ratio tests (Barton 2000). We estimated $K_{1,1}$, $K_{0,2}$ and their 95% confidence intervals from the best model. We selected the five most differentiated loci (HG18; HG14; HG16; K32; K67) because calculations including all nine loci would have been unmanageable.

For comparison with the values observed, we estimated the expected linkage disequilibrium under a model of neutral diffusion, using the subsequent equations. Genetic linkage disequilibrium between two loci (D) depends on the width of the clines in alleles frequency (w assuming concordant clines for both loci), dispersal distance per generation (σ) and recombination rate between loci (r) (Barton 1982):

$$D = \frac{\sigma^2}{rw^2} \qquad \text{(eqn 1)}$$

This relation is independent of density and robust for weak to moderate selection (Barton & Gale 1993). Moreover, under the neutral diffusion model, assuming linear and homogeneous habitat, the width of the cline (w) only depends on the dispersal rate (σ) and the time since contact measured in number of generations (T) (Barton & Hewitt 1985):

$$w^2 = 6.28\sigma^2 T \qquad \text{(eqn 2)}$$

By combining equations 1 and 2, the relationship between linkage disequilibrium and time since contact assuming neutral diffusion depends only on recombination rate and is independent of dispersal:

$$D = \frac{1}{6.28rT} \qquad \text{(eqn 3)}$$

The microsatellite loci used in this study are assumed to be unlinked, as no significant linkage disequilibrium could be detected between any pair of loci in allopatric populations (results not shown). The recombination rate of these loci is therefore assumed to be 0.5. The expected linkage disequilibrium was then calculated for a variable number of generations using equation 3, comparing these to the observed value.

Mitochondrial introgression

We investigated mitochondrial introgression by examining patterns of association between microsatellites or phenotype assignments and mitochondrial haplotypes. No haplotype was shared between *argentatus* and *cachinnans* in allopatry (see Results). We thus assigned all haplotypes found in allopatric *argentatus* populations (respectively allopatric *cachinnans*) as *L. argentatus* haplotypes (respectively *L. cachinnans*). Associations between microsatellite genotypes, haplotypes and morphotypes were tested using Fisher exact tests in SAS version 8 (SAS-Institute 2000). We also tested if there was an excess of any of the haplotypes in hybrids (using binomial probabilities).

Introgression rate for phenotypic traits

To investigate the introgression level of phenotypic traits, we summarized the morphological variables using four

discriminant analyses to maximize the differentiation between parental phenotypes with the program ADE-4 (Thioulouse *et al*. 1997), within each of the following groups (Table S1): colouration of bare parts (five variables, $n = 236$), plumage melanism features (11 variables, $n = 203$) and male and female morphometry (seven variables, $n = 99$ for females and 109 for males). Allopatric birds from northern Poland and Ukraine were used as reference populations (because they are the most distant populations available) and birds from central Poland and southern Poland 2 as supplementary individuals. We treated southern Poland 2 as a potentially admixed population to avoid biasing the allopatric sample.

To describe introgression of morphological traits, we developed an approach analogous to the analysis of linkage disequilibrium based on the variance of phenotypes. We assumed that the phenotypic trait is encoded by k loci and that variance is totally additive. Using a quantitative genetic model of admixture, the expected phenotypic variance in an admixed population (V_{G_0}) at the first generation of admixture (before reproduction) is

$$V_{G_0} = pV_1 + (1 - p)V_2 + p(1 - p)(\Delta\mu)^2 \quad \text{(eqn 4)}$$

where V_1 and V_2 are the phenotypic variances of parental population 1 and 2, respectively; p is the admixture coefficient that represents the proportion of individuals originating from parental population 1; $\Delta\mu$ is the difference between phenotypic means of parental population 1 (μ_1) and 2 (μ_2). If reproductive isolation is complete, the phenotypic variance will remain the same (V_{G_0}). On the contrary, if the genomes of population 1 and 2 have completely recombined after a sufficient number of generations of panmixis, assuming each of the k loci equally contributing to the difference in phenotypic means, the expected variance V_{mix} is

$$V_{mix} = pV_1 + (1 - p)V_2 + p(1 - p)\frac{(\Delta\mu)^2}{k} \quad \text{(eqn 5)}$$

If the phenotype considered is encoded by a large number of loci (i.e. large values of k), then

$$V_{mix} \approx pV_1 + (1 - p)V_2 \quad \text{(eqn 6)}$$

We defined Ukraine as population 1 (μ_1; V_1; $p = 1$) and northern Poland as population 2 (μ_2; V_2; $p = 0$). We treated central Poland (μ_{CP}; V_{CP}; p_{CP} such as $\mu_{CP} = p_{CP}\mu_1 + (1 - p_{CP})\mu_2$) and southern Poland 2 (μ_{SP}; V_{SP}; p_{SP}) as two potentially admixed populations. We used the position of individuals on each of the four discriminant axis as synthetic variables resembling colouration of bare parts, plumage melanism and male and female morphometry. We estimated the four phenotypic means and variances with their confidence intervals for each of the above populations. Estimates of the admixture coefficients for central Poland (p_{CP}) and southern Poland 2 (p_{SP}) were deduced from means estimates ($\mu_{CP} = p_{CP}\mu_1 + (1 - p_{CP})\mu_2$). These values were then compared to a graph viewing the expected phenotypic variances both under the hypothesis of complete reproductive isolation (V_{G_0}, equation 4) and complete recombination (V_{mix}, equation 6) as a function of the admixture coefficient (p). If the observed phenotypic variance is not significantly different from V_{G_0}, the trait has a bimodal distribution, indicating high dispersal, very recent admixture or some degree of reproductive isolation between parental populations. On the contrary, if the observed variance is not significantly different from V_{mix}, phenotypes are totally recombined due to extensive introgression in the absence of barriers preventing gene flow. The degree of introgression can thus be assessed as the position of population variance relative to both curves.

Results

Hybridization between two poorly differentiated taxa across a narrow contact

The nine microsatellite loci displayed between three and 17 alleles. None of these loci was diagnostic for either *Larus argentatus* or *Larus cachinnans* and 70% of alleles were shared between species (see Table S2, Supplementary material). The mean number of alleles and the expected heterozygosity were both maximal in sympatry (Table 1). Differentiation between allopatric populations was significant but low ($F_{ST} = 0.108$ [0.051; 0.169], $P < 0.001$). The MCA performed on the multilocus microsatellite genotype showed that 6.3% of the total genetic variance was explained by the first axis, separating the two species ($n = 166$ individuals from the seven populations). We found no significant difference between the distribution of F1 scores in Finland–Norway–northern Poland (post hoc Tukey's tests, $P > 0.98$) or southern Poland 1 and 2–Ukraine ($P > 0.99$), which confirmed that allopatric *argentatus* and allopatric *cachinnans* were valid groups. The distributions of the F1 scores for allopatric *argentatus* and allopatric *cachinnans* overlapped (Fig. 2). In sympatry (central Poland), a large number of intermediate individuals made the distribution of F1 scores roughly unimodal (Fig. 2). More than 38% of individuals in the sympatric population were classified as intermediate by the assignment test using microsatellite genotype (Table 2). It was significantly higher than the proportion of intermediate individuals in allopatric populations ($\chi^2 = 7.156$, d.f. = 1, $P = 0.007$).

The cytochrome *b* sequences were 490–629 bp long and defined eight haplotypes (GenBank Accession nos EF513623–EF513630). Seven of these have been described in earlier studies and are named after the taxon in which they were most frequent (Crochet *et al*. 2003; Liebers *et al*. 2004). L20

Table 2 Total number of individuals from allopatric *argentatus*, allopatric *cachinnans* and sympatric populations assigned to *Larus argentatus*, intermediate or *Larus cachinnans* using the three different data sets: microsatellites, mitochondrial DNA haplotype or phenotype. The individuals are not necessarily the same for each assignment method

		Microsatellites	mtDNA	Phenotype
Allopatric *argentatus*	*L. argentatus*	36	25	25
	intermediate	17	—	4
	L. cachinnans	1	0	—
Sympatry	*L. argentatus*	44	15	66
	intermediate	41	—	58
	L. cachinnans	21	8	53
Allopatric *cachinnans*	*L. argentatus*	1	1	—
	intermediate	14	—	11
	L. cachinnans	74	21	45

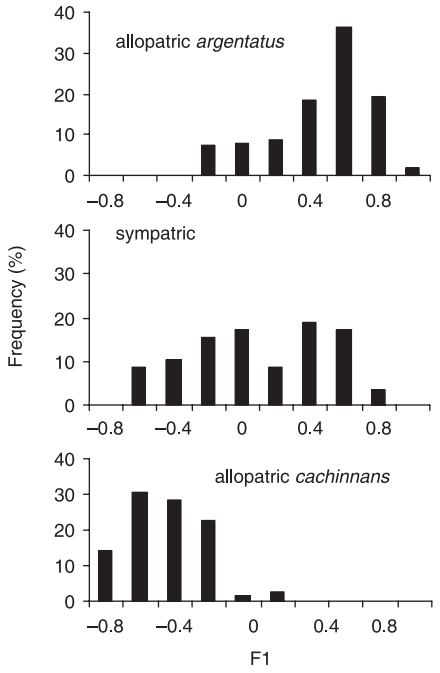

Fig. 2 Frequency distribution of F1 scores from multiple correspondence analysis based on nine microsatellite loci typed in seven populations grouped as allopatric *argentatus*, sympatric *argentatus*/*cachinnans* and allopatric *cachinnans* (see Methods).

corresponds to a fragment of a haplotype earlier described in one individual of *Larus smithsonianus* from Alaska (Liebers *et al*. 2004). CACH was a new haplotype prevalent in all allopatric *cachinnans* populations. It had a maximum divergence of only 0.8% from the five haplotypes found in *L. argentatus* (ARG, OMI, FUSC, MICH and MAR). The haplotype diversity was maximal in sympatry (Table 1). Allopatric *argentatus* and *cachinnans* populations did not share any haplotype (apart from one omissus haplotype in southern Poland 1, Fig. 3) and were significantly differentiated ($\Phi_{ST} = 0.278$, $P < 0.0001$, analysis performed on the 490 bp long fragment sequenced in all individuals).

The PCA on morphological variables confirmed that allopatric populations (northern Poland vs. Ukraine) differed in their phenotype, as the distribution of PC1 scores explaining 51.3% of the total variance for females and 40.7% for males did not overlap. The proportion of intermediate individuals in sympatry (32.8%, Table 2) was significantly higher than in allopatric populations (test on assignments from PC1 scores, northern Poland + Ukraine vs. central Poland: $\chi^2 = 5.802$, d.f. = 1, $P = 0.016$).

Introgression of the neutral genome: microsatellite linkage disequilibrium analyses

When a different diagnostic allele is fixed in each allopatric population, the maximum linkage disequilibrium in sympatry is 0.25. In our secondary contact, allopatric populations were polymorphic: the mean frequency of the *cachinnans*′ synthetic allele over the five microsatellite loci was 0.18 in allopatric *argentatus* (Finland) and 0.66 in allopatric *cachinnans* (Ukraine). The maximum linkage disequilibrium expected in sympatry with complete reproductive isolation was thus 0.057.

The estimates of average multilocus heterozygote deficiency ($K_{1,1}$) and average linkage disequilibrium ($K_{0,2}$) plotted against the mean frequency of the synthetic *cachinnans* allele together with the maximum values expected if reproductive isolation was complete are shown in Fig. 4. There was no significant heterozygote deficiency in any of the populations, neither in allopatry or sympatry (Fig. 4a). Linkage disequilibrium was significant in sympatry only (central Poland, $K_{0,2} = 0.041$; Fig. 4b) but lower than expected if reproductive isolation was complete. This low linkage disequilibrium indicated that F1 hybrids (detected by their intermediate phenotype) successfully reproduce, resulting in introgression and recombination of the parental genomes.

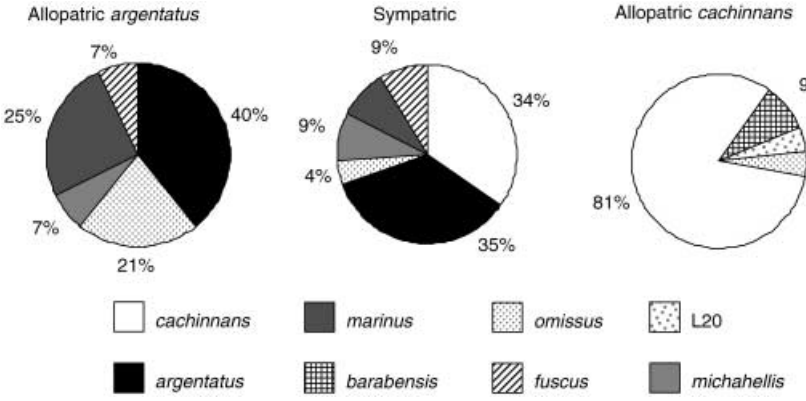

Fig. 3 Mitochondrial haplotype frequencies in seven populations grouped as allopatric *argentatus*, sympatric *argentatus*/*cachinnans* and allopatric *cachinnans*. All haplotypes except CACH have been described in earlier studies (Crochet *et al*. 2003; Liebers *et al*. 2004).

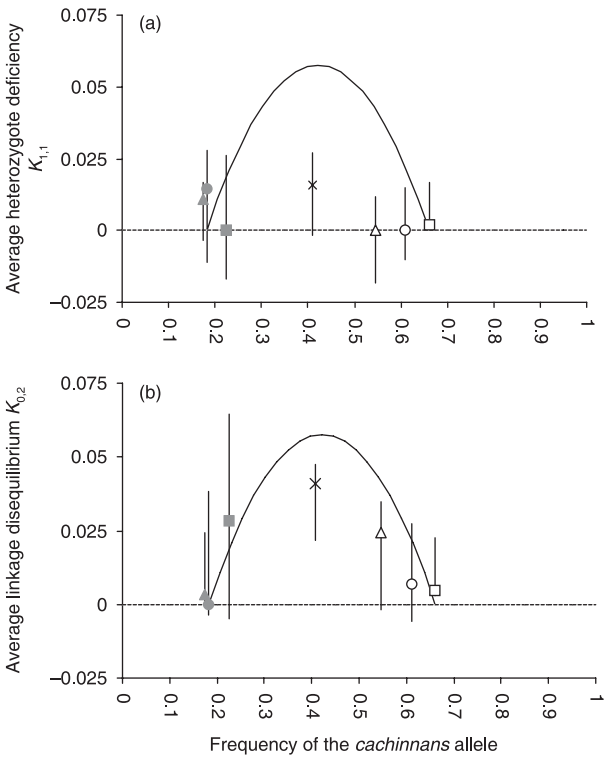

Fig. 4 Multi-locus average heterozygote deficiency $K_{1,1}$ (a) and average linkage disequilibrium $K_{0,2}$ (b) and their 95% confidence intervals based on five loci in relation to the mean frequency of the synthetic *cachinnans* allele for populations of allopatric *argentatus* (grey symbols; Norway, squares; Finland, circles; northern Poland, triangles), sympatric *argentatus*/*cachinnans* (crosses) and allopatric *cachinnans* (open symbols; southern Poland 1, triangles; southern Poland 2, circles; Ukraine, squares) and their respective theoretical maximum values expected if there is complete reproductive isolation (black curves) (see methods).

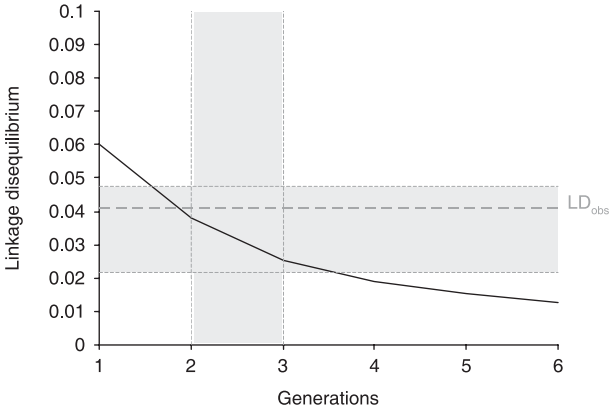

Fig. 5 Expected decay of linkage disequilibrium with time in number of generations after contact (generation 1 corresponds to F1). The recombination rate *c* was set to 0.5. The grey zones represent the current situation in sympatry (central Poland): 2–3 generation-old contact between *Larus argentatus* and *Larus cachinnans* and observed linkage disequilibrium $LD_{obs} = 0.041$ [0.022; 0.048].

The expected decay of linkage disequilibrium with time in a panmictic population (neutral diffusion, equation 3) is shown in Fig. 5, starting with a linkage disequilibrium value of 0.057 at the first generation of admixture (see above). Heterospecific pairs of *L. argentatus* and *L. cachinnans* started to be reported in our sympatric population (central Poland) about 25 years ago (P. Chylarecki, personal communication). This is equivalent to two to three generations (see Migot 1992 for generation time in large gulls), as presented by the grey vertical shading in Fig. 5. The linkage disequilibrium in this population ($K_{0,2} = 0.041$ [0.022; 0.048], see above) is shown by the horizontal grey shading. Comparing the observed linkage disequilibrium with the expected after two to three generations of neutral diffusion shows no significant difference.

Introgression of the mitochondrial genome

In sympatry (central Poland), there was a significant cytonuclear association, i.e. between *L. argentatus* (respectively *L. cachinnans*) nuclear genotype (microsatellites

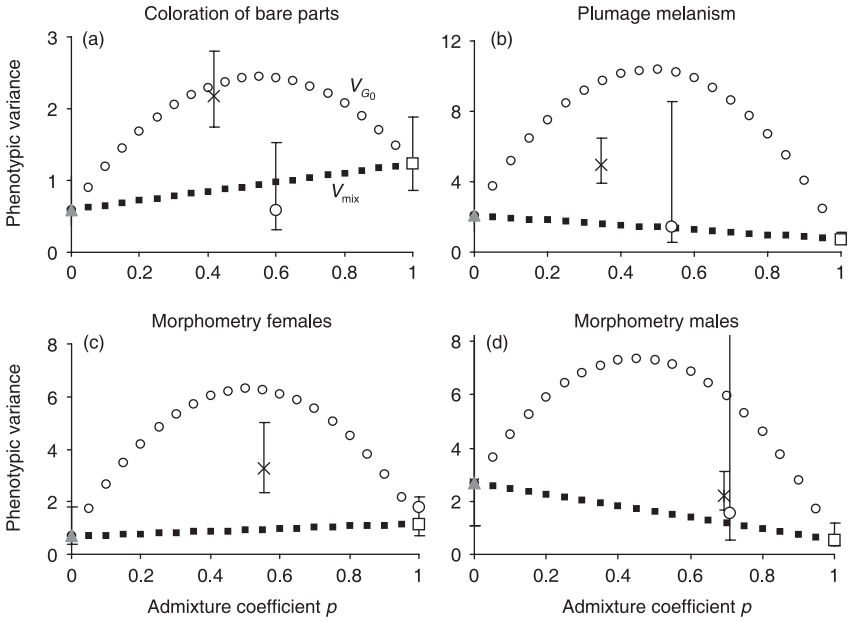

Fig. 6 Phenotypic variances expected for a situation of complete reproductive isolation (V_{G_0}: ■ ■ ■) and free recombination (V_{mix}: ○○○) and observed variances and their 95% confidence intervals for colouration of bare parts, plumage melanism, and female and male morphometry in populations of allopatric *argentatus* (grey symbols; northern Poland, triangles), sympatric *argentatus*/*cachinnans* (crosses) and allopatric *cachinnans* (open symbols; southern Poland 2, circles; Ukraine, squares).

(a)

Genotype / Haplotype	*L. argentatus* Obs.	*L. argentatus* Exp.	Intermediate Obs.	Intermediate Exp.	*L. cachinnans* Obs.	*L. cachinnans* Exp.
L. argentatus	7	*5.6*	4	*4.4*	2	*3.4*
L. cachinnans	0	*2.8*	1	*2.2*	6	*1.6*

(b)

Morphotype / Haplotype	*L. argentatus* Obs.	*L. argentatus* Exp.	Intermediate Obs.	Intermediate Exp.	*L. cachinnans* Obs.	*L. cachinnans* Exp.
L. argentatus	4	*6.2*	7	*3.4*	4	*5.5*
L. cachinnans	1	*3.2*	0	*1.8*	7	*2.8*

Table 3 Association between individuals with *Larus argentatus* and *Larus cachinnans* mitochondrial haplotypes and their microsatellite genotypes (a) or morphotypes (b) in a sympatric population (central Poland). Individual genotypes and morphotypes were assigned to *L. argentatus*, intermediate or *L. cachinnans* using assignment tests and PCA, respectively. Fisher exact tests were used to test for significant difference from random association (in italics). Obs., observed; Exp., expected

or morphotype) and mitochondrial haplotype (Table 3, microsatellite-haplotype: Fisher exact test: $n = 20$, $P = 0.0045$; morphotype-haplotype: $n = 23$, $P = 0.0113$). Intermediates, however, had *L. argentatus* haplotype more often than expected if mating was not sex-biased: this was significant for phenotype (Table 3b), as the probability to have seven *L. argentatus* haplotypes in the intergrades is 0.008; but not significant for microsatellites (Tables 3a, $P = 0.156$).

Introgression in phenotypic traits: Phenotypic variance analyses

Observed phenotypic variances for colouration of bare parts, plumage melanism and female and male morphometry are plotted against the admixture coefficient p in Fig. 6. Thereby, it was possible to compare these values to the variance expected under the model of free recombination (V_{mix}) and if reproductive isolation was complete (V_{G_0}). In sympatry (central Poland), the observed variances for plumage and morphometry (both sexes) lied between the expected values for complete reproductive isolation (V_{G_0}) or free recombination (V_{mix}), suggesting introgression in line with the genetic linkage disequilibrium. The variance of colouration of bare parts was very high and not significantly different from the model of complete reproductive isolation (V_{G_0}). This indicated a bimodal distribution of bare parts colouration in sympatry, with a deficit of individuals with intermediate phenotypes. This difference in introgression level between traits shows that some traits are involved in barriers to gene flow.

Discussion

Evidence for introgression

There have been several lines of evidence that hybridization occurs between *L. argentatus* and *L. cachinnans*, leading to introgression between the genomes. First, the excess of individuals with intermediate phenotypes or genotypes in sympatry demonstrated that these species hybridized in the mixed colony of Włocławek in central Poland. Then, both genetic linkage disequilibrium (Fig. 4b) and phenotypic variance (Fig. 6) were significantly lower than expected if reproductive isolation was complete, further indicating genetic introgression through successful reproduction of F1 hybrids. Empirical data thus confirmed that the reproductive isolation between *L. argentatus* and *L. cachinnans* is at best incomplete.

This result fits well with the low genetic divergence observed between *L. argentatus* and *L. cachinnans* for nuclear loci (F_{ST} = 10.8%) and mitochondrial haplotypes (maximum divergence 0.8%), indicating that speciation occurred very recently (see also Liebers *et al*. 2004). Low levels of genetic divergence like this would not be expected to result in strong intrinsic postzygotic barriers. These develop first with increasing levels of genetic divergence, as mutations, selection and genetic drift accumulates (Orr 1997; Coyne & Orr 2004). A link between intrinsic postzygotic barriers and genetic divergence has indeed been observed in a variety of taxa (see Coyne & Orr 2004 for a review). Moreover, post–mating barriers evolve particularly slowly in birds (Prager & Wilson 1975; Price & Bouvier 2002; Fitzpatrick 2004). Both species, *L. argentatus* and *L. cachinnans*, have been shown to be similar in their breeding phenology, habitat choice and food resources in sympatry (Neubauer *et al*. 2006), making strong ecological selection against hybrids unlikely (MacCallum *et al*. 1998). Low levels of postmating isolation are probably a general phenomenon among large gulls. For example, egg-switching experiments between *L. argentatus* and *L. fuscus* resulted in the formation of many hybrids the following generation, many of which survived and reproduced without any obvious disadvantages (Harris *et al*. 1978).

Is there any barrier to neutral gene flow?

Linkage disequilibrium (LD) is highly informative to assess the potential future of a secondary contact. When reproductive isolation is complete, LD in sympatry is maximal because there is no recombination; its value is then only limited by the initial difference in allele frequency. In our case, the significant linkage disequilibrium between neutral nuclear markers in sympatry (Fig. 4) did not differ from the LD expected after two to three generations of neutral diffusion (Fig. 5). It can thus be accounted for by dispersal and recent contact alone, without any reduction of gene flow at neutral loci.

In the absence of strong barriers to neutral gene flow, the gradient in allele frequency is expected to fade away with time under the effect of dispersal. Dispersal has been shown to be substantial in large gulls, e.g. in *L. argentatus* 35% of birds started to breed outside their natal colony in Britain (Coulson 1991), and dispersal distances up to 250 km have been documented (Cramps & Simmons 1983). These findings support the idea that gene flow across the zone of secondary contact may be substantial. Merging of parental genomes across zones of secondary contact has recently been documented in different taxa, including two evolutionary units of the golden-striped salamander *Chioglossa lusitanica* in Portugal (Sequeira *et al*. 2005), chromosomal races of common shrew *Sorex araneus* (Andersson *et al*. 2004), and American black ducks *Anas rubripes* and mallards *A. platyrhynchos* (Mank *et al*. 2004).

Asymmetric gene flow in mtDNA?

The higher-than-expected association of *L. argentatus* mtDNA with intermediate nuclear genotype (Table 3) highlighted asymmetric introgression of the mitochondrial genome. Because mtDNA is maternally transmitted, it might result either from a lower success of crosses involving females *L. cachinnans* and males *L. argentatus* than the reverse crosses or from an asymmetry in pair formation (females *L. cachinnans* avoiding interspecific crossings more than female *L. argentatus*). Sequencing more individuals and studying pair formation in the field is necessary to better understand the issue of asymmetric introgression. Whatever is the mechanism creating this asymmetry, a partial barrier to gene flow must be involved. This asymmetry should then lead to a lower introgression of mtDNA compared to nuclear DNA.

Sex-biased introgression has been described in several hybrid zones, for example in flycatchers, in which female collared flycatchers mate more frequently with male pied flycatchers than the reverse (Veen *et al*. 2001). Other examples of sex-biased introgression include two species of char *Salvelinus malma* and *S. confluentus* (Redenbach & Taylor 2003) or hare *Lepus europeus* and *L. timidus* (Thulin *et al*. 1997). However, the heterogeneity of introgression rates can be reverse, i.e. introgression rates in mtDNA are higher than in the nuclear genome (Arnold 1997). This indicates that sex-biased introgression, explained for example by Haldane's rule or mate choice behaviour, is probably widespread and contributes to the heterogeneity of introgression rates within the genomes.

Crochet *et al*. (2003) suggested that sex-biased gene flow, as expected under Haldane's rule, could explain the discrepancy between mitochondrial and nuclear differentiation among large gull species. Helbig *et al*. (2001) also suspected the influence of Haldane's rule in a warbler hybrid zone.

The complete lineage sorting between *L. argentatus* and *L. cachinnans* allopatric populations on cytochrome *b* contrasts here with the large amount of shared polymorphism in microsatellites. This could result from stronger drift on mtDNA whose effective size is four times lower than for nuclear DNA. It is possible to correct for effective size differences between markers, but under the assumption of a finite island model and migration/drift equilibrium (Gay *et al*. 2004), which is very unlikely to be reached in our zone of recent secondary contact. Moreover, differences in intraspecific diversity (Hedrick 1999) between nuclear and mtDNA also make the effects of asymmetric introgression on the differentiation difficult to apprehend. A proper testing of sex-biased introgression would require sampling more populations along the transect, comparing the slopes of the clines in mitochondrial vs. nuclear allele frequencies.

Selection on the colouration of bare parts reduces introgression

We supposed that exogenous selection or incomplete prezygotic barriers may reduce introgression for some phenotypic traits in the contact zone of *L. argentatus* and *L. cachinnans*. Thus, selection is a potential factor important to maintain phenotypic differences.

Analyses of phenotypic variance illustrated that introgression rates of phenotypic traits were variable. Do any of the assumptions underlying our analysis limit its accuracy? First, we neglected environmental variance and assumed that phenotypic variance was fully additive. However, even if some of the differentiation between allopatric populations was due to environmental variance, omitting environmental variance would only decrease the variance expected under complete reproductive isolation (V_{G_0}) and not affect phenotypic variance in sympatry (i.e. in the same environment), which is conservative. Second, we assumed that a large number of genes encoded the phenotypic traits investigated (high value of *k*). If only few genes are involved (low value of *k*), then the expected variance after many generations of free recombination (V_{mix}) would be higher (see equation (5)). This limitation is particularly relevant for the colouration of bare parts. However, as this is a synthetic trait summarizing both the amount of carotenoïds (eye-ring and leg colour) and melanin (dark sports in the iris), we are confident that it is encoded by more than two genes.

The high variance of colouration of bare parts indicated a bimodal distribution of bare parts colouration in sympatry, caused by a deficit of individuals with intermediate phenotypes. This suggests the existence of premating barriers, for example assortative mate choice with regard to the colouration of bare parts (Jiggins & Mallet 2000). It has been previously suggested that the colouration of bare parts, especially bill and foot colouration, may be involved in mate choice in seabirds (Pierotti 1987). However, there was no clear difference either in bill or in foot colouration between taxa in our study. Instead, iris and eye-ring colour were the most divergent traits with highest weight in the discriminant analysis. Experiments performed by Smith (1966) suggested that the contrast between the fleshy eye-ring and iris and the white head may be involved in isolating mechanisms in Arctic gulls. Birds with light coloured irises and eye-rings (like *L. argentatus*) were reproductively isolated from birds with dark irizes and eye-rings (like *L. cachinnans*). Although Smith's experiments are controversial and should be replicated (Pierotti 1987), they support our hypothesis that iris and eye-ring colour may play a role in mate choice in large gulls. Further experimental testing is needed to confirm that traits like eye-ring and iris colour are involved in mate choice and contribute to assortative mating. Introgression of these phenotypic traits seems nevertheless slower compared to neutral markers, which diffuse without any apparent barrier.

Conclusion

The diffusion-like behaviour of neutral markers across the contact zone between *Larus argentatus* and *L. cachinnans* suggested that there are no or few genes involved in reproductive isolation. Comparison with a series of phenotypic traits identified a few characters that show reduced introgression compared to neutral markers. Incomplete reproductive barriers could act as selective filters that prevent the introgression of foreign alleles with differential effectiveness depending on the locus involved and the modes of inheritance. A similar variability in introgression rate at the genome scale was observed in a wide diversity of hybrid zones, from birds (*Manacus candei* and *M. vitellinus*, Brumfield *et al*. 2001), to Lepidoptera (*Papilio machaon* and *P. hospiton*, Cianchi *et al*. 2003) or cottonwoods (*Populus fremontii* and *P. angustifolia*, Martinsen *et al*. 2001). More generally, as soon as reproductive isolation is incomplete (i.e. viable and fertile F1 hybrids), neutral diffusion is expected for most markers as long as they are not linked to a locus counter-selected in hybrids or intergrades. Hence, the fewer loci involved in reproductive isolation (either because the level of isolation is low or because there are few large effect genes), the more neutral diffusion is expected in a large portion of the genome. In the zone of contact we studied, the only loci experiencing a reduction of gene flow and thus potentially involved in reproductive isolation encoded phenotypic traits that could play a role in mate choice. This result is in agreement with the hypothesis that premating isolation (and especially mate choice) could be a major component of reproductive isolation between bird species (Gill 1998). This study highlights the usefulness of a multilocus approach to study hybrid zones and discriminate among possible mechanisms responsible for their maintenance (Marshall & Sites 2001).

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Laurène Gay's PhD focused on hybridization and its consequences on genetic structure in the large headed gulls. Her supervisor was Pierre-André Crochet, permanent researcher at CEFE-CNRS. Grzegorz Neubauer's PhD focused on breeding biology and hybridization of gulls from the *L. argentatus-L. cachinnans-L. michahellis* complex. This study was completed as part of these two PhDs. Magdalena Zagalska-Neubauer is a researcher from the Institute for Ornithology in Gdansk, Poland. Jean-Marc Pons is a researcher at the Muséum National d'Histoire Naturelle in Paris, France and Patrice David is a population biologist from CEFE-CNRS. All coauthors are mainly interested in the evolutionary strengths driving population or community divergence and on the mechanisms of speciation.

Supplementary material

The following supplementary material is available for this article:

Table S1 Phenotypic traits measured and description of units or categories. Pictures of head and wing tip pattern were taken for most individuals (available upon request).

Table S2 Allele frequency for each of the seven populations and the nine microsatellite loci.

This material is available as part of the online article from:
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