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Genetic differentiation and phylogeography of gulls in the *Larus cachinnans–fuscus* group (Aves: Charadriiformes)

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Abstract

We studied mitochondrial genetic differentiation among nine taxa of large gulls of the *Larus cachinnans–fuscus* group, which form part of the circumpolar Herring Gull complex. Our primary interest was to see if there were unrecognized gene flow barriers, to what extent mitochondrial genetic population structure conformed to current taxonomic boundaries, and what it might reveal about possible differences in population history. Sequences (430 nucleotides) of the hypervariable control region I (HVR-I) were obtained from 580 individuals and proved highly informative within this recently diverged group of birds. Contrary to current classification, a basal split was revealed between an Atlantic–Mediterranean clade (*atlantis*, *michahellis*, *armenicus*) and a NW Palearctic–Central Asian clade (*cachinnans*, *barabensis*, *mongolicus*, *fuscus*-group). There was almost no mitochondrial gene flow between these two groups, although they are in geographical contact in two areas (eastern North Atlantic, Black Sea). Within each of the two major groups, there was strong phylogeographic structure with gene flow barriers between some neighbouring taxa (e.g. *cachinanns* vs. *barabensis*), but also a case of poor genetic differentiation between phenotypically distinct forms (*barabensis* vs. *heuglini*). At the subspecies level, current taxonomy corresponded well to molecular genetic structure: over 80% of the molecular genetic variance was partitioned among six (groups of) taxa. This is in sharp contrast to previous studies using allozymes and amplified fragment length polymorphism (AFLP) markers, which seemed to indicate extensive nuclear gene flow. Within-taxon haplotype phylogenies and mismatch distributions revealed contrasting demographic histories: *cachinnans* (Ponto–Caspian region) and *atlantis* (NE Atlantic) represent ancient lineages with large long-term population sizes, inland forms stem from very recent colonization events (*barabensis*, *mongolicus*) or passed through a population bottleneck (*armenicus*).

Keywords: AMOVA, historical demography, HVR-I, mitochondrial control region, phylogeography, Yellow-legged Gulls

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Introduction

Gulls of the *Larus argentatus–cachinnans–fuscus* complex (Charadriiformes: Laridae) are a classic textbook example of recent speciation in birds. The group comprises approximately 30 taxa (currently ranked as species or subspecies), which differ primarily in the darkness of their dorsal plumage (mantle colour varying from pale grey to black), wing-tip pattern, leg and iris colour as well as in their stereotyped 'long-call' display (Cramp & Simmons 1982; Haffer 1982; Burger & Gochfeld 1996). Mayr (1940, 1963) proposed the ring species model to explain the evolution of this group. According to this hypothesis, an ancestral light-mantled gull population inhabiting the Aralo–Caspian region expanded northwards and subsequently spread west and east along the coast of northern Eurasia as well as westward into the Mediterranean and eastward into Central Asia. Along the North Eurasian coasts a west–east cline in mantle colour

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developed, western birds having a dark mantle (*graellsii*, *fuscus*, *heuglini*), those further east having a progressively lighter mantle (*taimyrensis*, *birulai*, *vegae*). From eastern Asia, according to Mayr's model, light-mantled gulls colonized North America, spreading eastward across the entire continent, and later reinvaded Europe via the North Atlantic, thus closing the circumpolar ring. While neighbouring populations all along the ring are supposed to interbreed, a reproductive barrier exists in NW Europe, where secondary contact was established between the two ends of the ring. This gene flow barrier is obvious in the sympatric coexistence in NW Europe of light-mantled Herring Gulls (*Larus argentatus*) and dark-mantled Lesser Black-backed Gulls (*Larus fuscus*). In line with Mayr's hypothesis, traditional taxonomy recognized a basic split between the dark-mantled Lesser Black-backed Gull (with ssp. *fuscus*, *intermedius*, *graellsii*, *heuglini*, *taimyrensis*) and light-mantled Herring Gull (all other taxa; Peters 1934).

While the reproductive relationships among sympatric forms in NW Europe are quite clear, less attention has been paid to a second, more southernly distributed chain of taxa that originally spread east and west from the Aralo–Caspian region. This chain is composed — from west to east — of the forms *atlantis* (eastern North Atlantic Ocean), *michahellis* (Mediterranean Sea), *armenicus* (inland lakes of Anatolia, Armenia, Iran), *cachinnans* (Black, Caspian, Aral Seas), *barabensis* (western Siberia) and *mongolicus* (central Asian steppe region). According to Mayr (1963), they form a chain of interbreeding populations, the eastern end of which (*mongolicus*) is supposed to be the result of mixing between *cachinnans* and *vegae* (from NE Siberia). In recent handbooks (Haffer 1982; Burger & Gochfeld 1996; Snow & Perrins 1998), the southern taxa were assigned to two species separate from *L. argentatus* and *L. fuscus*, namely Armenian Gull *L. armenicus* (monotypic) and Yellow-legged Gull *L. cachinnans* (with ssp. *atlantis*, *michahellis*, *cachinnans*, *barabensis*, *mongolicus*). This group and its relationships with the northern, dark-mantled *L. fuscus* group was the subject of the present study.

Previous molecular work revealed little genetic differentiation within the *argentatus–fuscus–cachinnans* complex. Two allozyme studies found 90% of the variation within colonies with no fixed allelic differences between taxa (Johnson 1985; Snell 1991). Amplified fragment length polymorphism (AFLP) markers (de Knijff *et al*. 2001) yielded a somewhat higher (15.8%) among-taxon (or taxon-group) component of variance, but most of the molecular variance was again within populations. Random amplified polymorphic DNA (RAPD) profiles indicated bidirectional introgression between *argentatus* and *cachinnans* along the Volga river system in Russia (Panov & Monzikov 1999). Mitochondrial DNA (mtDNA) sequence divergence between *argentatus*, *fuscus* and *michahellis* was minimal, both at the cytochrome *b* gene (Wink *et al*. 1994; Heidrich *et al*. 1996) and in domain II and III of the control region (Crochet *et al*. 2000; Crochet & Desmarais 2000). None of these mtDNA studies were based on sample sizes large enough to investigate differentiation between taxa (cf. Helbig 1994).

Available genetic data thus indicate a very recent origin of gulls of the *argentatus–fuscus–cachinnans* complex, in which phenotypic divergence was apparently accompanied by very little genetic differentiation. So far, however, no study on gulls has made use of the most variable part of the mitochondrial control region [hypervariable control region I (HVR-I) = domain I in Baker & Marshall 1997], which has been shown to be highly informative at the population level in closely related avian taxa (e.g. Edwards 1993; Wenink *et al*. 1996; Marshall & Baker 1997; Barrowclough *et al*. 1999; Avise 2000) and many other organisms including humans (e.g. von Haeseler *et al*. 1996).

Here we use an extensive set of mitochondrial HVR-I sequences to investigate the genetic differentiation and population structure of nine gull taxa, which include all forms of Yellow-legged Gull (*cachinnans* group) and three forms of Lesser Black-backed Gull (*fuscus* group) with which the former come into geographical contact. This is the case: (i) in the eastern North Atlantic, where northern, dark-mantled *graellsii* (breeding on Iceland, Faeroes, British Isles, NW France) meet southern paler-mantled *atlantis* (Azores, Madeira, Canary Islands) and *michahellis* along the French Atlantic coast (Yésou 1991); and (ii) along the Ob river depression in western Siberia, where the range of southern *barabensis* (*cachinnans* group) approaches that of inland-breeding, northern *heuglini* (*fuscus* group). *L. argentatus s. str.* and two other subspecies of *L. fuscus* (*intermedius*, *fuscus*) were not included here, but will be treated in separate analyses.

Our primary interest was: (i) to see if there might be unrecognized gene flow barriers; (ii) to what extent mitochondrial genetic population structure conformed to current taxonomic boundaries; and (iii) what it might reveal about possible differences in population history. We expect this group of gulls to serve as a suitable model for the many other complexes of closely related forms of birds, which were traditionally classified as polytypic species, but often consist of distinct taxa separated by quite pronounced gene flow barriers [e.g. *Phylloscopus* warblers (Helbig *et al*. 1996; Irwin *et al*. 2001)].

Materials and Methods

Sampling

Samples of blood or flight muscle were collected from 580 individuals at 34 localities (Fig. 1), comprising all

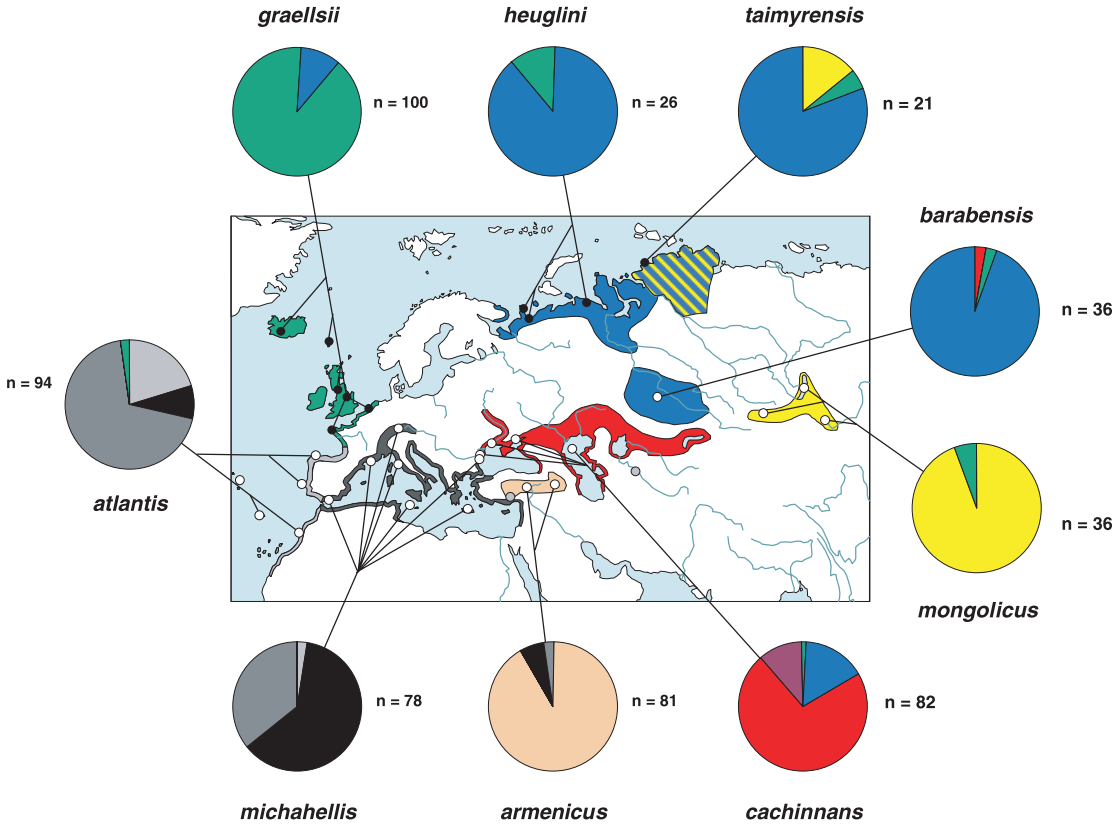


Fig. 1 Breeding ranges, sampling locations (map) and haplotype composition (pie charts) of gull taxa investigated. Darkness of symbols on the map corresponds to paleness of the mantle in the respective taxa, which ranges from pale grey (white dots) to dark grey (black), 'mixed' colonies are shown by grey dots. Colours in the pie charts correspond to those in the haplotype phylogeny (Fig. 3).

described taxa in the *Larus cachinnans* group (*atlantis*, *michahellis*, *armenicus*, *cachinnans*, *barabensis*, *mongolicus*) and three taxa of the *L. fuscus* group (*graellsii*, *heuglini*, *taimyrensis*). Material was collected exclusively from breeding colonies (see Table 1), either from incubating adults caught with walk-in traps on the nest or from chicks (one per nest). Phenotypic characters of adults (colour of mantle, legs, bill, wing-tip pattern, standard measurements) were recorded at all colonies. Representative voucher specimens and aliquots of all samples investigated in this study have been deposited in the Zoological Museum Greifswald. In phylogenetic analyses, *L. canus* (from the Netherlands) was used as the outgroup taxon.

DNA extraction, long-fragment polymerase chain reaction, DNA sequencing

Blood and tissue samples were preserved in APS-buffer (Arctander 1988) or 95% ethanol, respectively, and stored at –20 °C. For each specimen, total cellular DNA was isolated using a salting-out procedure (Miller *et al*. 1988). We amplified a 2500–3000 bp fraction of the mitochondrial genome, which included the entire control region, the ND6 gene and a part of the 12S rRNA gene. From this, 430 nucleotides of the HVR-I region were sequenced directly. Primer sequences were as follows (H = heavy strand; L = light strand; numbers give the position of the 3′-end in *Gallus gallus* mitochondrial genome, cf. Desjardin & Morais 1990): amplification primers: UUL (L-16076) AAA ACA TTG GTC TTG TAA ACC; cyt-H (L-15722) CAC ATC AAA CCA GAA TGA TAC TTC CTA TT; primers cyt-D and DDL see Helbig & Seibold (1999); sequencing primers: HLB (H-519) GGC CCT GAC ATA GGA ACC AGA GG; H419 (H-419) GGG TTG CTG ATT TCA CGT GA (designed for this study).

Each 50 µL polymerase chain reaction (PCR) contained 50 µg of total DNA, 350 µM of each dNTP, 0.5 pmol of each primer, 2 units DNA polymerase (Expand™ Long template PCR System, Boehringer Mannheim) and a final concentration of 2.25 mM $MgCl_2$. After initial denaturation (2 min at 94 °C), 30 cycles were run in a Perkin Elmer thermocycler (GeneAmp 2400) as follows: 10 s denaturation at 94 °C, 30 s annealing at 50–55 °C, primer extension initially 2.5 min at 68 °C. After the first 10 cycles, the annealing temperature was raised to 55–57 °C and the extension time was increased by 20 s each cycle. Seven µL of the reaction

Table 1 List of taxa investigated, breeding colonies, sample sizes (*n*) and grouping of colonies into populations

cachinnans group: pale-grey mantle, southern Palearctic			
Taxon	Population	Breeding colony	*n*
atlantis	atlAZO	Azores	20
	atlMDR	Madeira	20
		Morocco	7
	atlBER	Berlenga, Portugal	17
	atlGAL	Galicia, NE Spain	30
michahellis	micWeM	Gibraltar	11
		Camargue, France	18
		Alsac, France	12
		NW Italy	7
	micEaM	Malta	15
		Crete	9
		Constanta, Romania	6
armenicus	armTUZ	Tuz Gölü	51
	armVAN	Van Gülü	30
	armBEY*	Beysehir Gölü*	16
cachinnans	cacBLS	Istria, Romania	7
		Odessa, Ukraine	9
	cacUKR	Asov Sea, Ukraine	23
	cacCSS	Caspian Sea, Russia	43
barabensis	barKAZ	Tengiz, Kazakhstan	36
	barUBK*	Tuzkan, Uzbekistan*	10
mongolicus	monEAS	Eastern Mongolia	11
	monBAI	Lake Baikal, Russia	12
	monWES	Western Mongolia	13
fuscus group: dark-grey mantle, northwestern Palearctic			
Taxon	Population	Breeding colony	*n*
graellsii	graNAT	Iceland	9
		Faeroe Islands	35
	graUKD	Northern England	20
		Central England	6
	graEUR	Finistère, France	5
		Rotterdam, Netherlands	25
heuglini	heuKAN	Western Sibiria	9
		Kanin Peninsula	14
		Petchora Delta	3
taimyrensis	taiPJA	Taimyr Peninsula	21

*Colony consisting of two different phenotypes.

mixture were used as template for sequencing. Excess amplification primers and nucleotides were digested with Exonuclease I (10 units) and Shrimp Alkaline Phosphatase (2 units; PCR product presequencing kit, Amersham). Cycle sequencing reactions were performed with AmpliCycle™ Sequencing Kit (Perkin Elmer) and [α^{33}P]-labelled dATP according to the manufacturer's specifications. Reaction products were electrophoresed on 6% polyacrylamide gels.

We took special care to ensure the mitochondrial origin of sequences and to avoid (co)amplification of possible nuclear copies of mtDNA: *Long-template PCR*. Most mitochondrial-like sequences in the nuclear genome are short (Blanchard & Schmidt 1996), so we performed long-template PCR amplifications making (co)amplification of nuclear copies less likely. (ii) *Amplifications with different primer combinations*: In addition to the standard amplification with primers UUL-DDL (product length 2515 bp), we amplified the control region with primers cyt-H-DDL (length 2869 bp) in 23 individuals and with primers cyt-D-DDL (length 3022 bp) in 21 individuals. Thus, sequences of up to three different amplifications from the same target DNA were compared. (iii) *CsCl purification*: mtDNA out of 14 muscle samples was purified by ultracentrifugation in CsCl gradients and sequences were compared to those derived from total DNA of the same 14 individuals (for amplifications with up to three different primer combinations each).

Sequence analysis, phylogeny reconstruction

Sequences were aligned by eye using ESEE (Cabot & Beckenbach 1989). Genetic distances between haplotypes were computed in MEGA (Kumar *et al*. 1993) based on the Kimura 2-parameter model (K 2-p; Kimura 1980) with gamma correction. Single nucleotide indels (two positions) were treated like transversions. Rate heterogeneity between sites was taken into account by assuming gamma-distributed rates. The α-parameter was estimated from the sequence matrix by a Hidden Markov Model without correlation (Felsenstein & Churchill 1996) using PUZZLE (version 4.0.2; Strimmer & von Haeseler 1996). The resulting distance matrices were used to construct haplotype phylogenies using the Kitsch algorithm (PHYLIP 3.5c; Felsenstein 1993). Support values for internal branches of the haplotype phylogeny were calculated by likelihood mapping using the quartet puzzling algorithm (Strimmer & von Haeseler 1997) with the Hasegawa–Kishino–Yano (HKY) substitution model (Hasegawa *et al*. 1985) and 10 000 quartets per branch (PUZZLE 4.0.2).

To illustrate geographical partitioning of haplotypes within subsets of taxa, uncorrected median-joining networks (Bandelt *et al*. 1999) were computed using the program NETWORK version 2.0 (Röhl 1998). For reasons of clarity, in the very diverse Atlantic–Mediterranean group we included only haplotypes occurring at least twice in the total sample.

Population genetic analyses

For the purpose of this analysis, we pooled individuals of the same taxon from geographically close locations,

yielding a total of 22 'populations' (Table 1), each with a minimum sample size of 10. At two sites, Lake Beysehir, SW Turkey, and Tuzkan, Uzbekistan, two distinct phenotypes were breeding in mixed colonies, *michahellis* and *armenicus* at the former, *cachinnans* and *barabensis* at the latter. Here it was not possible to assign chicks to one or the other taxon (no adults were caught), therefore sequences were excluded from the population genetic analyses. To characterize and compare mitochondrial genetic diversity between taxa, numbers of haplotypes (HT) and polymorphic sites (S), nucleotide diversity (π) with variance V(π) and mean number of pairwise differences (d) were derived for all taxa using the program ARLEQUIN version 1.1 (Schneider *et al*. 1998). We tested for differences in nucleotide diversity (π) between taxa using the formula $t = \pi_1 - \pi_2/[V(\pi_1)^2 + V(\pi_2)^2]^{1/2}$ (Nei 1987). *T*-values were compared with percentage points of the standard normal distribution (Kirkwood 1988).

To assess to what extent we had sampled the mitochondrial genetic diversity adequately, we calculated for each taxon an index of sample saturation (SAT) defined as SAT = N_{obs}/N_{sat}, where N_{obs} is the number of individuals sequenced in a given taxon and N_{sat} is the number at which the addition of a further 10 individuals would be expected to recover less than one additonal haplotype (see Helgason *et al*. 2000). Values of SAT > 1 indicate adequate sampling, i.e. a disproportionate increase in sample size would be needed to recover new haplotypes.

Mitochondrial genetic differentiation between populations and taxa was assessed by calculating pairwise Φ_{ST} values and testing their significance by running 10 000 permutations in the program ARLEQUIN. Estimates of gene flow (number of female migrants per generation [N_m]) were computed assuming that the mutation rate is negligible compared to the migration rate between two populations. The transition/transversion (ts/tv) parameter κ was estimated using the maximum likelihood method implemented in PUZZLE. Hierarchical analyses of molecular variance (AMOVA; Excoffier *et al*. 1992) were performed to study the partitioning of genetic variance within and among populations and taxa. To find the smallest possible number of taxon groups explaining a maximum of between-group genetic variance (i.e. to capture as much of the geographical structure as possible), we assigned the 32 phenotypically pure colonies to two, three, six or nine groups (see 'Results') and estimated the partitioning of genetic variance for each hypothesis using ARLEQUIN. We computed frequency distributions of pairwise sequence differences ('mismatch distributions') to infer historical demographic patterns within each taxon (Slatkin & Hudson 1991; Rogers & Harpending 1992) and to calculate per cent sequence divergence between taxa.

Results

Characterization of sequence variation

A total of 580 mitochondrial HVR-I sequences (430 nucleotides each) representing nine gull taxa were obtained. The full length sequence of HT 01 (Appendix I; this appendix is available from the Molecular Ecology web site, URL http://www.blackwell-science.com/products/journals/suppmat/mec/mec1370/mec1370sm.htm) and that of the outgroup taxon *Larus canus* were deposited in the EMBL nucleotide data bank (accession nos AJ277127, AJ310427). Based on two lines of evidence we are confident that all sequences are of mitochondrial origin: (i) separate amplifications with three different primer combinations yielded identical sequences for each of the 44 individuals for which such comparisons were made. (ii) Sequences derived from CsCl-purified mtDNA were identical to those derived from total DNA of the same individuals ($n = 14$).

Sequence alignment required the insertion of 1-bp gaps at two positions (no. 125 in *michahellis*-type sequences, no. 236 in *cachinnans*-type sequences; cf. Appendix I). A total of 44 sites (10.5%) were variable, 34 (8.1%) of which were parsimony informative. Transversions occurred at only four positions, the ts/tv ratio was estimated to be 17.1 (using PUZZLE 4.0.2). Substitution rates varied strongly among sites resulting in an overall α value of 0.04. Base composition was biased with a deficiency of guanine (G = 15.0%, T = 27.2%, A = 27.0%, C = 30.8%). Overall, 90 haplotypes were detected, of which 26 were shared between at least two colonies. The number of haplotypes found per taxon ranged from three (*mongolicus*) to 25 (*cachinnans*; Table 2). The frequency of each haplotype per population is given in Appendix I.

The index of sample saturation (Table 2) indicates that most taxa were sampled adequately (values > 1), except *heuglini* and *taimyrensis*, for which sample sizes were smallest, and *cachinnans*, which is by far the most genetically diverse taxon investigated (see below).

Geographical structure of genetic variation

The haplotype composition of the nine phenotypically distinct taxa showed obvious geographical structure (Fig. 1) with pairwise Φ_{ST} values ranging from 0.032 to 0.928 (Table 3). There was significant genetic differentiation between all pairs of taxa except between *barabensis* and *heuglini*. In a hierarchical AMOVA, we investigated how the overall genetic variation was distributed among groups of populations (Table 4). Model A: the traditional division into two species, the southern *L. cachinnans* (pale grey mantle) and the northern *L. fuscus* (dark grey mantle; cf. Mayr 1963), accounted for only 26.8% of the overall

Table 2 Genetic diversity indices of the gull taxa based on HVR-I sequences (430 nucleotides) of the control region. For taxa in which obvious introgression was observed, values given in *italics* are based on samples excluding introgressed haplotypes. Number of individuals (*n*); number of haplotypes (HT); saturation index (SAT); nucleotide diversity ($\pi \times 10^{-3}$) with variance V(π); number of segregating sites (S); mean number of pairwise nucleotide differences (d); expansion coefficient (S/d); maximum Kimura 2-parameter (K 2-p) distance with gamma correction ($\alpha = 0.04$) between haplotypes

Taxon	*n*	HT	SAT	$\pi \pm V(\pi)$	S	d	S/d	K 2-p
atlantis	94	16	1.57	6.07 ± 3.6	24	2.55	9.4	0.095
	92	*15*	*1.84*	*5.29 ± 3.3*	*18*	*2.22*	*8.1*	*0.048*
michahellis	78	17	1.11	4.07 ± 2.7	13	1.71	7.6	0.027
armenicus	81	10	2.03	4.79 ± 3.0	14	2.01	7.0	0.057
	74	*8*	*2.47*	*1.71 ± 1.5*	*7*	*0.72*	*9.7*	*0.012*
cachinnans	82	25	0.68	9.91 ± 5.5*	22	4.16	5.3	0.128
barabensis	36	7	1.20	1.44 ± 1.3	9	0.61	14.8	0.019
	35	*6*	*1.17*	*0.81 ± 0.9*	*5*	*0.34*	*14.7*	*0.005*
mongolicus	36	3	1.80	1.42 ± 1.3	6	0.60	10.1	0.021
	34	*2*	*1.70*	*0.14 ± 0.4*	*1*	*0.06*	*16.7*	*0.002*
graellsii	100	14	2.50	1.75 ± 1.5	13	0.73	17.8	0.012
heuglini	26	7	0.65	1.88 ± 1.6	6	0.79	7.6	0.009
taimyrensis	21	10	0.30	4.40 ± 2.9	10	1.85	5.4	0.021

*Value is significantly greater than in all other taxa except *atlantis* and *taimyrensis* ($P < 0.05$, test after Nei 1987).

variance. Further subdividing *L. cachinnans*, under Model B, into an Atlantic–Mediterranean group (*atlantis*, *michahellis*, *armenicus*) and a West-central Asian group (*cachinnans*, *barabensis*, *mongolicus*) increased the among-groups variance component to 61.5%. Boundaries between all nine taxa (Model D) explained 81.3% of the variance. An equally large proportion (82.1%) can be accounted for by only six groups (Model C), if poorly differentiated taxa (Φ_{ST} values below 0.2; Table 3) are merged (*atlantis–michahellis* and *barabensis–heuglini–taimyrensis*, respectively). In conclusion, the AMOVA showed that the overall genetic variation has a strong geographical structure and that this structure is well reflected in the current taxonomic subdivisions, which are based on phenotypic characters.

Levels of gene flow in contact zones

An aspect of particular interest was the question to what extent, if any, gene flow between geographically neighbouring taxa might be restricted. This is expressed quantitatively in the Φ_{ST} values of the AMOVA (Table 3) and is illustrated by the relative frequencies of haplotypes in each taxon (Fig. 1). At one extreme, a strong restriction of mitochondrial gene flow was detected: (i) in the eastern North Atlantic between *graellsii* and *atlantis/michahellis*; and (ii) on the western coast of the Black Sea between *michahellis* and *cachinnans*. In the latter case, no evidence of introgression was found in fairly large samples ($n > 80$) from the entire range of each taxon. Note that these forms have, until now, been regarded as conspecific. In case (i), two *graellsii* haplotypes were detected in *atlantis* breeding colonies (off Portugal and Morocco) indicating that introgression does occur at a very low level. A third contact area with restricted gene flow but higher levels of (unidirectional) introgression is in southern Turkey between *michahellis* (Mediterranean Sea) and *armenicus* (Anatolian plateau). The westernmost colony of phenotypically pure *armenicus* at Tuz Gölü contained 14% *michahellis* haplotypes (Liebers & Helbig 1999).

Gene flow between *cachinnans* and *barabensis* appeared to be asymmetrical. Although they were significantly differentiated, a considerable proportion (15.8%) of haplotypes typical of *barabensis* (blue colour in Fig. 1) was found in the *cachinnans* population, while only 2.8% *cachinnans* haplotypes were found in *barabensis*. Unexpectedly, *barabensis* and *heuglini* turned out not to be differentiated at

Table 3 Pairwise sequence divergence and differentiation between taxa. Below diagonal: Φ_{ST} values based on AMOVA (ARLEQUIN, Schneider *et al*. 1998; settings: transition/transversion = 17 : 1; Kimura 2-parameter distance, $\alpha = 0.04$). Above diagonal, per cent sequence divergence (median of all pairwise comparisons)

Taxon	*atlantis*	*michahellis*	*armenicus*	*cachinnans*	*barabensis*	*mongolicus*	*graellsii*	*heuglini*	*taimyrensis*
atlantis	—	0.46	1.86	3.02	2.09	2.56	2.33	2.09	2.09
michahellis	0.125	—	1.86	3.02	2.09	2.56	2.33	2.09	2.09
armenicus	0.777	0.812	—	3.48	2.79	2.56	2.79	2.79	2.79
cachinnans	0.808	0.839	0.867	—	1.16	1.86	1.40	1.16	1.16
barabensis	0.831	0.901	0.896	0.308	—	0.93	0.23	0.23	0.23
mongolicus	0.879	0.925	0.878	0.646	0.863	—	1.16	0.93	0.93
graellsii	0.881	0.926	0.928	0.533	0.532	0.874	—	0.23	0.48
heuglini	0.817	0.892	0.887	0.323	(0.032)	0.850	0.469	—	0.23
taimyrensis	0.796	0.867	0.872	0.340	0.147	0.715	0.501	0.090	—

Φ_{ST} value in parentheses, not significant at $P < 0.01$ (10 000 permutations).

Table 4 Analysis of molecular variance (ARLEQUIN, Schneider *et al*. 1998; settings: transition/transversion = 17 : 1, Kimura 2-parameter distance, $\alpha = 0.04$) in gulls: four models (A to D) representing different groupings of taxa (*gra*, *graellsii*; *heu*, *heuglini*; *tai*, *taimyrensis*; *atl*, *atlantis*; *mic*, *michahellis*; *arm*, *armenicus*; *cac*, *cachinnans*; *bar*, *barabensis*; *mon*, *mongolicus*) are investigated

Model		Taxa in groups	Variance component Φ-statistics	% variance explained
(A)	2 groups: *fuscus* vs. *cachinnans* group	(1) *gra*, *heu*, *tai*	AG: $\Phi_{CT} = 0.268$	26.8
		(2) *atl*, *mic*, *arm*, *cac*, *bar*, *mon*	AP: $\Phi_{SC} = 0.797$	58.3
			WP: $\Phi_{ST} = 0.851$	14.9
(B)	3 groups: one northern, two southern	(1) *gra*, *heu*, *tai*	AG: $\Phi_{CT} = 0.615$	61.5
		(2) *atl*, *mic*, *arm*	AP: $\Phi_{SC} = 0.633$	24.4
		(3) *cac*, *bar*, *mon*	WP: $\Phi_{ST} = 0.859$	14.1
(C)	6 groups	(1) *atl*, *mic* (2) *arm*	AG: $\Phi_{CT} = 0.821$	82.1
		(3) *cac* (4) *mon* (5) *gra*	AP: $\Phi_{SC} = 0.144$	2.6
		(6) *bar*, *heu*, *tai*	WP: $\Phi_{ST} = 0.847$	15.3
(D)	9 groups: subspecies boundaries	one taxon per group	AG: $\Phi_{CT} = 0.813$	81.3
			AP: $\Phi_{SC} = 0.130$	2.4
			WP: $\Phi_{ST} = 0.837$	16.3

Variance components: AG, among groups; AP, among populations within groups; WP, within populations.
All Φ values are significant at $P < 0.001$ (10 000 random permutations of sequences among populations).

the mitochondrial genetic level (Table 3), although they clearly differ in mantle colour and, according to current knowledge, their ranges do not come into contact. The degree of gene flow restriction was not obviously related to topographical barriers: no such barriers separate *michahellis* from *cachinnans* on the Black Sea coast (no gene flow) or *graellsii* from *atlantis*/*michahellis* on the Atlantic coast and islands (very little introgression). On the other hand, *michahellis* and *armenicus* (more extensive introgression) are separated by the Taurus mountains (up to 3000 m high), inhospitable terrain for large gulls which, however, has not prevented *michahellis* from wandering into *armenicus* territory at least occasionally.

Population history and haplotype phylogeny within taxa

To investigate possible differences in the demographic history, we analysed the following parameters in each of the nine taxa:

1 Nucleotide diversity and expansion coefficient (Table 2).
2 Branching pattern (Fig. 2) and deepest divergence (maximum K 2-p distance, Table 2) of the haplotype tree.
3 Mismatch distribution (Fig. 2); a unimodal distribution indicates recent exponential population growth, while a multimodal distribution characterizes a large population that has been of relatively constant size over time (Rogers & Harpending 1992).

In *atlantis*, *armenicus*, *barabensis* and *mongolicus* a low proportion of phylogenetically distantly related haplotypes was found which, in all probability, reflect recent introgression (highlighted by grey shading in Fig. 2). To assess the impact of such introgression on the population genetic parameters, we calculated them separately with and without introgressed haplotypes in the four taxa concerned (Table 2).

With regard to genetic diversity and haplotype divergence patterns, two extremes can be distinguished: (i) taxa with a shallow branching pattern of their haplotype tree corresponding to a strongly left-skewed, unimodal mismatch distribution and limited genetic diversity (*graellsii*, *heuglini*, *barabensis*, *armenicus*, *mongolicus*); and (ii) taxa with a multimodal mismatch distribution, a correspondingly deep branching pattern of the haplotype phylogeny and high genetic diversity (*atlantis*, *cachinnans*). Two taxa had a fairly broad, but unimodal, mismatch distribution and intermediate depth of branching pattern (*taimyrensis*, *michahellis*). Pairwise comparisons showed that *cachinnans* had significantly higher nucleotide diversity than all other taxa except *atlantis* and *taimyrensis*.

Historical changes in population size can be assessed by the 'expansion coefficient' S/d, i.e. the ratio of variable sequence positions (S) relative to the mean number of pairwise nucleotide differences between haplotypes (d) within a taxon. Large values indicate recent population expansion, whereas the ratio will be small in populations that have been constant in size (von Haeseler *et al*. 1996). S/d ratios ranged from 5.3 to 17.8 with highest values in *graellsii*, *mongolicus* and *barabensis* (Table 2). The latter three also exhibit a shallow haplotype phylogeny (Fig. 2), together strongly indicating recent population expansion. Among the other taxa, the S/d ratio varied little (5.3–7.6), suggesting that their populations have been relatively constant in

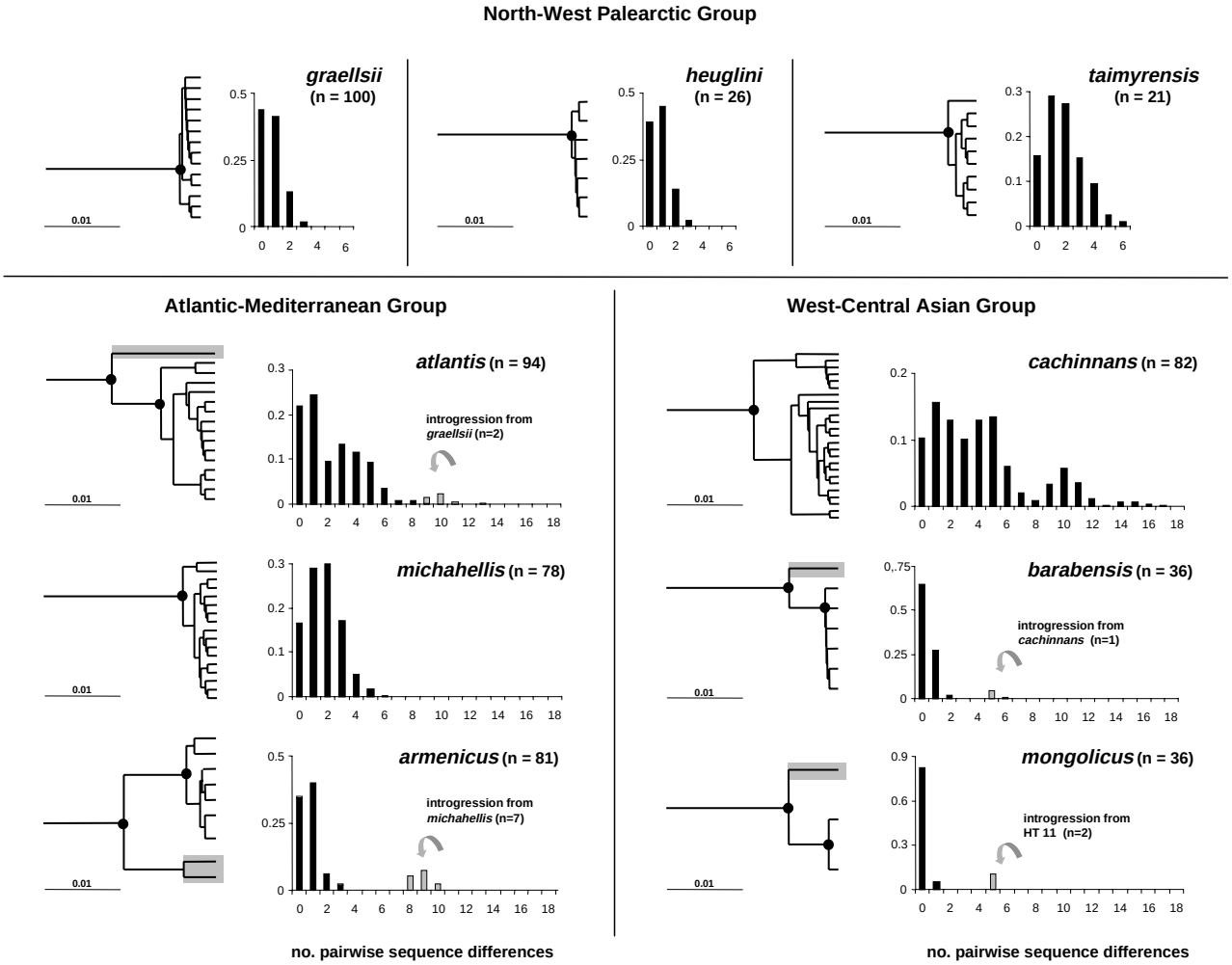


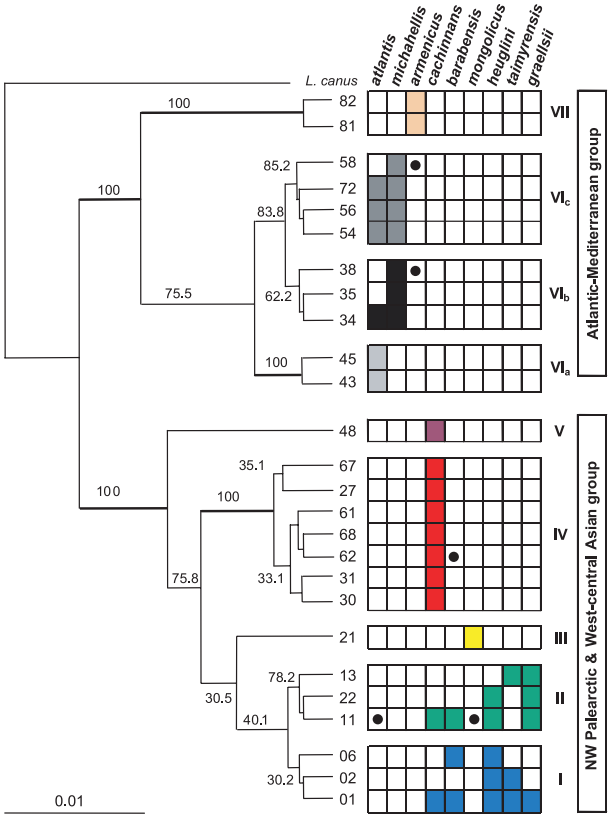
Fig. 2 Comparative illustration of mitochondrial phylogenetic structure within nine gull taxa. For each taxon we show on the left the haplotype phylogeny (Kitsch tree, Kimura 2-parameter distances) and on the right the relative frequencies of pairwise sequence differences (mismatch distribution). Haplotypes suspected to be the result of introgression are shown in grey. Haplotype trees were rooted with the sequence of *Larus canus* (branch not shown) and are drawn to the same scale to illustrate the relative depths of branching patterns.

size over long periods of time. The Armenian Gull (*armenicus*), although representing a phylogenetically relatively old lineage (see below), had an intermediate S/d value of 9.5, if introgressed haplotypes were disregarded. This indicates that the population went through a bottleneck relatively recently.

mtDNA haplotype phylogeny

Of the total of 90 haplotypes, a large fraction (71%) was confined to single breeding colonies (or colonies in close proximity). We restrict our analysis of the mtDNA phylogeny to those 26 haplotypes that were shared between at least two populations (see Appendix I), because these contain most information about relationships of mtDNA lineages and taxa. The molecular clock hypothesis was not rejected in a likelihood ratio test (PUZZLE), i.e. rates of molecular evolution did not differ significantly between lineages. Maximum parsimony (PAUP 3.1.1.; Swofford 1993) and maximum likelihood distances obtained by quartet puzzling (PUZZLE) yielded tree topologies (not shown) that were very similar to the Kitsch tree in Fig. 3.

The haplotype phylogeny (Fig. 3) reveals a strongly supported basal split into two clades, one occurring exclusively in the Atlantic–Mediterranean group of gulls (*atlantis*, *michahellis*, *armenicus*), the other being largely restricted to a NW Palearctic–Central Asian group. None of the nine taxa were represented by a monophyletic clade of haplotypes relative to all other forms. In some cases this was due to obvious recent introgression, such as the two *michahellis* haplotypes (nos 38, 58) found in *armenicus*, one *cachinnans* haplotype (62) found in *barabensis* and one clade II haplotype (11) found in *atlantis* and *mongolicus* (marked by dots in grid of Fig. 3). In other cases the lack of reciprocal



monophyly may be due to incomplete lineage sorting between very recently diverged forms (*heuglini*, *taimyrensis*, *barabensis*) and/or ongoing gene flow (e.g. *atlantis*, *michahellis*).

A prominent feature is the paraphyletic status of *cachinnans* relative to the other five taxa in its group. While the occurrence of clade I and II haplotypes in *cachinnans* could be interpreted as the result of extensive (recent?) introgression via *barabensis*, a more ancient and quite divergent mtDNA lineage (represented by HT 48 in Fig. 3, but actually consisting of at least six haplotypes, see cluster V in Fig. 4b) was restricted to *cachinnans*. This pattern of 'deep' paraphyly suggests that *cachinnans* is ancestral to the other forms in the NW Palearctic–Central Asian group.

The Atlantic–Mediterranean clade exhibits a somewhat clearer phylogenetic structure: haplotypes found in *atlantis*

Fig. 3 Haplotype phylogeny and occurrence of haplotypes across taxa (colours and dots in grid cells). The Kitsch-tree on the left was constructed from Kimura 2-parameter distances and includes only haplotypes shared between at least two populations ($n = 26$; sequences are identified by haplotype numbers, cf. Appendix I). Support values for internal branches were derived by likelihood mapping (PUZZLE 4.0.2, Strimmer & von Haeseler 1997). Major haplotype clades are colour-coded and identified by roman numerals. Occurrences of haplotypes resulting from obvious recent introgression are indicated by dots in the grid cell diagram.

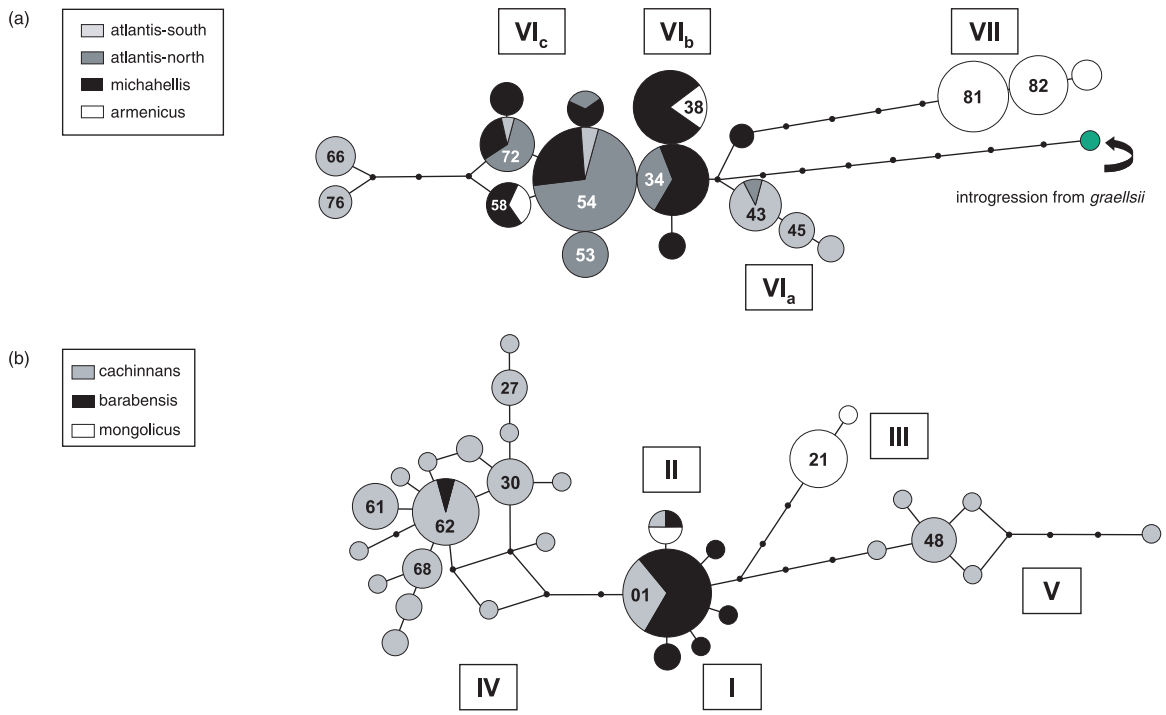


Fig. 4 Median-joining networks showing relationships among mtDNA haplotypes (Network 2.0; Röhl 1998). (a) Haplotypes found at least twice in the *atlantis–michahellis–armenicus* group ($n = 235$ individuals); (b) all haplotypes of the *cachinnans–barabensis–mongolicus* group ($n = 154$ individuals). Branch lengths are proportional to the number of inferred substitutions. Diameters of circles are proportional to frequencies of the respective haplotypes. Roman numerals refer to clades in the haplotype phylogeny (Fig. 3).

Table 5 Degree of differentiation between populations of (a) the Atlantic–Mediterranean group and (b) the west-central Asian group of Yellow-legged gulls, based on AMOVA (ARLEQUIN, Schneider *et al*. 1998; settings: transition/transversion = 17 : 1; Kimura 2-parameter distance, $\alpha = 0.04$; $P = 0.01$; 10 000 permutations). Above diagonal, inferred number of migrants per generation (N_m); below diagonal, Φ_{ST} values (all are significant at $P < 0.01$, except values in parentheses)

(a)

	atlantis		*michahellis*		*armenicus*			
Population	atlAZO	atlMDR	atlBER	atlGAL	micWeM	micEaM	armTUZ	armVan
atlAZO	—	1.19	2.52	51.08	1.79	0.91	0.16	0.02
atlMDR	0.297	—	1.16	1.10	1.25	1.32	0.24	0.12
atlBER	0.166	0.301	—	2.59	1.32	1.01	0.17	0.05
atlGAL	(0.010)	0.312	0.162	—	3.06	1.20	0.15	0.03
micWeM	0.218	0.286	0.274	0.140	—	8.01	0.16	0.05
micEaM	0.354	0.274	0.331	0.294	(0.059)	—	0.18	0.05
armTUZ	0.763	0.677	0.744	0.773	0.763	0.735	—	8.12
armVAN	0.953	0.805	0.906	0.941	0.904	0.902	(0.058)	—

(b)

	cachinnans		*barabensis*		*mongolicus*		
Population	cacBLS	cacUKR	cacCSS	barKAZ	monEAS	monBAI	monWES
cacBLS	—	5.32	56.33	0.93	0.27	0.26	0.40
cacUKR	(0.086)	—	15.92	0.37	0.17	0.16	0.23
cacCSS	(0.009)	(0.030)	—	1.03	0.30	0.29	0.40
barKAZ	0.350	0.573	0.327	—	0.05	0.05	0.14
monEAS	0.648	0.748	0.628	0.907	—	inf.	9.53
monBAI	0.658	0.754	0.633	0.909	(0.000)	—	8.01
monWES	0.553	0.682	0.556	0.780	(0.050)	(0.059)	—

inf., infinite number of migrants.

and *michahellis*, some of which are shared between them, together make up a well-supported monophyletic group (clades VIa-c in Fig. 3). *Armenicus* was found to be their sister taxon and, notwithstanding its shallow current branching pattern (see Fig. 2), represents a fairly ancient haplotype lineage (clade VII) with some, apparently recent, introgression from *michahellis*.

Population genetic structure of 'Yellow-legged Gulls'

We have shown that the six gull taxa of the southern Palearctic fall into two major groups, between which no mitochondrial gene flow was detected: (i) Atlantic–Mediterranean group: *atlantis*, *michahellis*, *armenicus*; and (ii) West-central Asian group: *cachinnans*, *barabensis*, *mongolicus*. We will look at the population genetic structure within each of these groups in some more detail.

Atlantic–Mediterranean group. A median-joining network (Fig. 4a) shows that mitochondrial haplotypes occurring in the *michahellis* population are a subset, or are recently derived from, those found in *atlantis*. *Michahellis* also has a more shallow haplotype phylogeny and lower nucleotide diversity than *atlantis*. Within the *michahellis* range there is no significant geographical substructure, whereas there is significant differentiation between most of the five *atlantis* colonies analysed (Table 5a). Among gulls of the Atlantic islands and mainland coasts we found an unexpected north–south pattern of differentiation: the Azores population was similar to that of the Iberian Atlantic coast (1350 km away), but was significantly differentiated from the Madeiran (900 km distant) and mainland Moroccan breeding birds (Table 5a). Since most *michahellis* haplotypes are either identical or closely related to those of 'northern' (Azores, mainland Iberia) rather than 'southern' (Madeira, Morocco) *atlantis* populations, it seems that the latter contributed few colonizers to the Mediterranean.

West-central Asian group. The median-joining network (Fig. 4b) highlights two striking features: (i) haplotypes of *cachinnans* fall into three distinct clusters (I, IV and V) which are not each other's closest relatives; and (ii) the complex haplotype network of *cachinnans* contrasts with a very uniform composition of *barabensis* and *mongolicus* (cf. their shallow haplotype phylogeny, Fig. 2). The *cachinnans* population of the Black/Azov Sea is not differentiated from that of the Caspian Sea (Table 5b), although the two marine basins are separated by a land bridge 500 km

wide. Similarly, populations of *mongolicus* from isolated wetlands/lakes up to 2000 km apart are not differentiated. Given such genetic uniformity within taxa over large distances, it is remarkable that there is significant differentiation across the narrow range disjunction between *cachinnans* (Caspian Sea) and *barabensis* (Table 5b).

Discussion

Genetic markers and geographical population structure

Following Mayr's (1940, 1963) proposal of the ring species model, gulls of the *argentatus–cachinnans–fuscus* complex have long been regarded as a chain of interbreeding geographical forms. This view was supported by studies of allozyme variation (Johnson 1985; Snell 1991) and AFLP markers (de Knijff *et al*. 2001), which revealed little differentiation between taxa, a finding that was interpreted as indicating extensive autosomal gene flow. However, variation in allozymes and anonymous AFLP markers may be too conservative to reflect the very recent pattern of differentiation in gulls. mtDNA with its smaller effective population size and more rapid evolution compared to allozymes is expected to better reflect very recent genetic differentiation (Avise 2000). This proved to be particularly true of control region HVR-I sequences, which in gulls, as in other birds, evolve much faster than domains II and III of the control region (Crochet & Desmarais 2000) and were thus more informative about the phylogeography of this young radiation.

Some 82% of the total molecular variance was partitioned among six taxa or groups of taxa. This structure was well-reflected in the traditional subspecies boundaries, while the current species delimitation (*Larus cachinnans* vs. *L. fuscus*) poorly represents the genetic differentiation. In particular, the strongest barrier to mitochondrial gene flow we identified, that between *michahellis* and *cachinnans*, was between two taxa which have so far been regarded as subspecies either of *L. cachinnans* (Burger & Gochfeld 1996) or even of *L. argentatus* (Cramp & Simmons 1982). Compared to other seabird studies, in which the among-population variance component was generally lower, gulls exhibit a strong phylogeographic structure. For instance, in the Black Guillemot *Cepphus grylle*, only 24.9% of the molecular variance was between the subspecies (Kidd & Friesen 1998), while in the Sooty Tern *Sterna fuscata* 38% was between ocean basins (Avise *et al*. 2000). In the Dunlin *Calidris alpina*, a migratory shorebird, 76.3% of the variance was partitioned among five geographical regions (Wenink *et al*. 1996).

Possible causes of phylogeographic structure

The fact that gulls show such strong geographical structure in their molecular variance suggests either strong natal philopatry or the existence of gene flow barriers. Current differentiation could be due to the persistence of a range disjunction (e.g. *armenicus* vs. *cachinnans*, *barabensis* vs. *mongolicus*) or to the fact that contact is very recent so that gene flow has not yet eroded pre-existing differentiation. An AFLP study found high inbreeding coefficients and reduced heterozygocity indicative of high breeding site fidelity (de Knijff *et al*. 2001). However, gulls have a high colonization potential (see below), so site fidelity alone is unlikely to account for the strong geographical structure. Rather, intrinsic reproductive barriers must be involved, at least in the cases of *michahellis* vs. *cachinnans* and *graellsii* vs. *atlantis/michahellis*. These taxa breed in close proximity or even locally sympatrically. On the Atlantic coast of France and Galicia (Spain), Lesser Black-backed Gulls (*graellsii*) have been known to breed sympatrically with Yellow-legged Gulls (*michahellis*) for more than a decade (Yésou 1991; Paterson 1997). Although mixed pairs and adult hybrids have been observed, our data indicate that mitochondrial gene flow is rare.

On the western coast of the Black Sea, *cachinnans* and *michahellis* have been found breeding only 50 km apart (Klein & Buchheim 1997) in quite different habitats: while *michahellis* bred on buildings in a large town, *cachinnans* colonies were located on sand banks in coastal lagoons. Different habitat preferences may, in this case, be part of the isolating mechanisms opposing gene flow. Differences in wing-tip patterns, vocalizations and display postures (distinct spread-wing display in *cachinnans*; Klein & Gruber 1997) may be important in mate choice and thus contribute to reproductive isolation. These obvious phenotypic differences are reflected in a relatively large sequence divergence between *michahellis* and *cachinnans* (median 3.02%). The combined evidence strongly suggests that intrinsic isolation mechanisms are operating between these forms.

The same arguments apply to the case of *cachinnans* vs. *armenicus*: phenotypic differences between them are at least as pronounced (*armenicus*, in addition, being smaller overall than *michahellis* and *cachinnans*; Liebers & Helbig 1999), the genetic divergence (median 3.48%) is the largest found between any two taxa we investigated. In this case current lack of gene flow may just reflect the range disjunction (at least 300 km between *armenicus* breeding at Lake Sevan, Armenia, and *cachinnans* on the Caspian Sea coast). On the other hand, gulls are good flyers and have colonized even the most remote lake systems in central Asia (*mongolicus*). So one would not expect a gap of a few hundred kilometers to present any challenge to gull dispersal and gene flow.

Phylogenetic relationships

Earlier attempts based on phenotypic characters (Stegmann 1934; Johansen 1960; Chu 1998) allozymes

(Johnson 1985; Snell 1991), AFLP markers (de Knijff *et al*. 2001) and some mtDNA sequences (Heidrich *et al*. 1996; Crochet *et al*. 2000) achieved only poor resolution of the phylogenetic relationships, suggesting that these large gulls derive from a relatively rapid radiation. HVR-I sequences, however, yielded surprisingly clear phylogenetic information, which greatly improves our understanding of relationships in this group of birds. Interestingly, the deepest split in the mitochondrial phylogeny did not separate northern dark-mantled (*heuglini*, *taimyrensis*, *graellsii*) from southern light-mantled taxa (all others), which current taxonomy regards as two different species (Lesser Black-backed Gull vs. Yellow-legged Gull). Instead, the basal split was within the southern taxa, separating the Atlantic–Mediterranean *atlantis*/*michahellis* from the Aralo–Caspian *cachinnans*/*barabensis*, all of which were so far regarded as the same species (*L. cachinnans*).

The Armenian Gull (*armenicus*), which had been split off as separate species by Haffer (1982), but whose phylogenetic affinities were unclear, was firmly placed in the Atlantic–Mediterranean clade. It branched off the *atlantis*/*michahellis* lineage rather basally, indicating that *armenicus* is a relict of an early colonization event from the Atlantic via the Mediterranean Basin, rather than from the Aralo–Caspian region as had previously been thought (Buturlin 1934; Filchagov 1993). Also phenotypically very similar to, but geographically separated from, *armenicus* is the west Siberian *barabensis*, whose affinities have always been controversial. We found it to be most closely related to *heuglini*, which is supported by evidence from a recent field and museum study (Panov & Monzikov 2000). Gulls inhabiting the Azores and Madeira (*atlantis*) were originally thought to be part of the *fuscus*-group (Dwight 1922), i.e. most closely related to *graellsii*, because of their relatively dark mantle and extensive head streaking in adult nonbreeding plumage (features they share with *graellsii*). However, *atlantis* and *graellsii* belong to different major clades in the mitochondrial haplotype phylogeny (Fig. 3) and are clearly not each other's closest relatives.

Population history

Pleistocene glacial cycles and associated ecological changes undoubtedly affected the population dynamics of gulls. Two extremes with respect to population history were evident among the gull taxa in our study. Genetic characteristics of *cachinnans* and *atlantis* indicate that these lineages had large populations over long periods. Both taxa reside today in areas of relative climatic stability: in the eastern North Atlantic Ocean (*atlantis*), glacial cycles at most may have led to north–south range shifts, but not to severe population bottlenecks or range restrictions. In the Aralo–Caspian–Pontic region (*cachinnans*), large inland seas have persisted throughout the Holocene, probably always providing habitat for large gull populations (Dawson 1992; Rutter 1995). Two lines of evidence suggest that *cachinnans* is a direct descendant of the ancestral population, from which *barabensis*, *heuglini*, *taimyrensis*, *graellsii* and *mongolicus* are derived: first, *cachinnans* has the deepest divergence in the haplotype tree, the highest nucleotide diversity and the smallest expansion coefficient of all taxa investigated, consistent with a large long-term population size (Slatkin & Hudson 1991; von Haeseler *et al*. 1996). Second, in the haplotype phylogeny (Fig. 3), *cachinnans* is paraphyletic relative to the other five taxa. This pattern is reminiscent of the global phylogeography of human mtDNA, where African populations contain the most divergent haplotypes and are paraphyletic relative to populations in the rest of the world (Ingman *et al*. 2000).

At the opposite extreme are several taxa that harbour little mitochondrial genetic diversity and show evidence of recent population expansion. In *barabensis* and *mongolicus* this seems to be due to very recent colonization of inland areas from different source populations: *barabensis* was found not to be differentiated from *heuglini*, suggesting that it colonized its present range from the north via the Ob–Irtys river system. Phenotypic divergence between *heuglini* and *barabensis* (mostly in mantle colour) must have been fairly rapid, not allowing for measurable mitochondrial genetic differentiation to accumulate. In *mongolicus*, the extreme paucity of genetic variation and a high expansion coefficient also suggest very recent immigration and population increase. Haplotypes dominating (at 94%) throughout its range are related to or identical with those of eastern Siberian and Pacific gull taxa (*vegae*, *schistisagus*; data not shown), while one haplotype (6% frequency) is common in *heuglini–taimyrensis*. This suggests that Central Asia was colonized primarily from an eastern Siberian and/or NW Pacific source, probably by relatively few individuals.

The Armenian Gull (*armenicus*) is a good example of a taxon that must have passed through a population bottleneck. Its current population genetic make-up differs little from that of recent colonizers such as *graellsii* or *barabensis*, but in contrast to the latter, *armenicus* derives from a phylogenetically relatively old lineage (Fig. 3). Had its population been large over long periods, a much more diverse and deeply branching haplotype assortment would be expected. The discrepancy is particularly striking compared to its sister lineage, the *atlantis*/*michahellis* clade (Fig. 3).

Within the *atlantis–michahellis* group, the haplotype phylogeny and the decline in nucleotide diversity from the Atlantic (*atlantis*) toward the Mediterranean (*michahellis*) suggest that Atlantic populations were ancestral to those living today in the Mediterranean Basin (with peripheral extensions into Black Sea and inland SW Europe). Consistent with this hypotheses, *michahellis* has a shallower haplotype branching pattern than *atlantis* and a unimodal, rather

than bimodal, mismatch distribution. Gene flow between these poorly differentiated taxa was estimated to be moderate, which is to be expected given the continuous oceanic connection via the Straits of Gibraltar. Interestingly, Mediterranean *michahellis* have recently recolonized the French Atlantic coast via southern France (Yésou 1991), further increasing the likelihood of genetic exchange with *atlantis*. This will oppose further differentiation and lineage sorting between *atlantis* and *michahellis*. On the other hand, the differentiation of southern (Madeira, Morocco) from northern (Azores, Iberia) *atlantis* and from Mediterranean *michahellis* populations is noteworthy. Among these three, southern *atlantis* contain the greatest diversity and largest divergence of haplotypes (Fig. 4a), suggesting that southern populations were more stable throughout periods of glacial oscillations. It is not obvious what may today restrict gene flow between the oceanic island groups (Madeira, Azores), or why the southern *atlantis* population evidently contributed few colonizers to the Mediterranean.

Age of gull lineages

Dating the split between the two major clades of gulls identified in this study (Fig. 3) is difficult, because rates of HVR-I evolution have not been calibrated accurately in gulls or other Charadriiform birds. More reliable calibrations are available for the cytochrome *b* gene (average 2% divergence per 1 Myr; Avise 2000). Average cyt *b* divergence between *michahellis* and *graellsii* is 0.007 (data not shown). The mean divergence of HVR-I sequences between the two major clades is 0.060% (K 2-p distance with gamma correction). Thus, HVR-I seems to evolve roughly 8.6 times faster than cyt *b*, yielding a divergence rate of 17% per Myr. This calibration dates the basal split between the major mitochondrial lineages at roughly 350 000 years ago. The separation into two ancestral populations from which the two major clades of gull taxa derived must be younger, because lineage divergence usually predates population-level divergence (Avise 2000). To date the latter, we need to correct the divergence estimate for sequence diversity in the ancestral population as suggested by Edwards (1997). Using as a correction the mean of current intrapopulation divergence in *atlantis–michahellis* (0.0127) and in the *cachinnans* group (0.0202), we arrive at a divergence between clades of $\delta = 0.0436$, which yields an age of 256 000–295 000 years for the population-level separation. This separation most likely corresponded to the reciprocal isolation of gulls in an Atlantic and a Pontic–Caspian refugium, because most of the current within-population genetic diversity is localized in these two regions (*atlantis* and *cachinnans* populations). The cause of the isolation of gulls in two separate refugia may have been a glacial maximum around 250 000–270 000 years BP (Schrag 2000).

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This paper is part of a comprehensive effort to understand the evolutionary history of the large gulls worldwide based on various genetic marker systems (mtDNA, AFLP, intron sequences). It comprises the core of Dorit Liebers' PhD work, which was conducted in Andreas Helbig's laboratory and was co-supervised by Peter de Knijff. AJH is head of the ornithological research station of Greifswald University located on the Baltic Sea island of Hiddensee. PdK is an assistant professor at Leiden University's Medical Centre and, apart from gulls, has a primary interest in genetics of the human Y chromosome. DL is currently continuing her research on mtDNA sequence evolution in vertebrates with Arndt von Haeseler at the MPI for Evolutionary Anthropology in Leipzig.

Supplementary material

The following material is available from http://www.blackwell-science.com/products/journals/suppmat/mec/mec1370/mec1370sm.htm

Appendix I

List of all haplotypes found among the 580 individual gulls sequenced in this study. The full length sequence (430 nc) of haplotype 01 is available from EMBL nucleotide sequence data bank (accession no. AJ277127). Bold numbers show haplotypes used in the phylogenetic analysis (Fig. 3). The left part of the table shows the variable positions relative to haplotype 01 (position no. 1 corresponds to position no. 38 in the *Calidris alpina* sequence of Wenink *et al*. 1994). The right part of the table shows the frequency of each haplotype per population (abbreviations see Table 1). Roman numerals in the rightmost column correspond to clades in the haplotype phylogeny (Fig. 3).