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The role of swarming sites for maintaining gene flow in the brown long-eared bat (*Plecotus auritus*)

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Bat-swarming sites where thousands of individuals meet in late summer were recently proposed as 'hot spots' for gene flow among populations. If, due to female philopatry, nursery colonies are genetically differentiated, and if males and females of different colonies meet at swarming sites, then we would expect lower differentiation of maternally inherited genetic markers among swarming sites and higher genetic diversity within. To test these predictions, we compared genetic variance from three swarming sites to 14 nursery colonies. We analysed biparentally (five nuclear and one sex-linked microsatellite loci) and maternally (mitochondrial D-loop, 550 bp) inherited molecular markers. Three mtDNA D-loop haplolineages that were strictly separated at nursery colonies were mixed at swarming sites. As predicted by the 'extra colony-mating hypothesis', genetic variance among swarming sites (V_{ST}) for the D-loop drastically decreased compared to the nursery population genetic variance (V_{PT}) (31 and 60%, respectively), and genetic diversity increased at swarming sites. Relatedness was significant at nursery colonies but not at swarming sites, and colony relatedness of juveniles to females was positive but not so to males. This suggests a breakdown of colony borders at swarming sites. Although there is behavioural and physiological evidence for sexual interaction at swarming sites, this does not explain why mating continues throughout the winter. We therefore propose that autumn roaming bats meet at swarming sites across colonies to start mating and, in addition, to renew information about suitable hibernacula.
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Introduction

Flying organisms such as bats might be thought to have intense gene flow among populations. However, their enigmatic life history makes direct observation of population interactions difficult. In general, bats are highly gregarious, sometimes forming large colonies of individuals that interact with each other more than with individuals of other colonies. In the temperate zone, colony formation is highly seasonal. Genetic analyses of recombining nuclear genes of temperate zone species consistently show high levels of gene flow among populations (inferred from genetic variance; Burland and Worthington Wilmer, 2001), sometimes even over large distances as in the case of the migratory noctule bat *Nyctalus noctula* (Petit and Mayer, 1999).

Strong gene flow among nursery colonies was also detected for the brown long-eared bat (*Plecotus auritus* L. 1758), a nonmigratory species that is widespread over the Western Palearctic. In Scotland, inter-colony F_{ST} values estimated from nuclear microsatellite loci were low, although significantly different from zero (Burland *et al*, 1999). This suggests a nearly panmictic population structure. However, genetically detected panmixia in Scottish *P. auritus* populations is in contrast with direct observation. Adults of both sexes show high fidelity to the roost at which they were captured first, with less than 1% of recaptured bats having moved among colonies (Entwistle *et al*, 1998, 2000; pers.obs.). Since the range of *P. auritus* during foraging is high (2.8 km in Scotland and 3.3 km in Germany; Fuhrmann and Seitz, 1992; Entwistle *et al*, 1996), roost philopatry and social cohesion must prevent individuals from joining new colonies. Therefore, male-mediated copulation seems to sustain the genetic cohesion among nursery colonies (Burland *et al*, 1999, 2001).

But where and when does gene flow among brown long-eared bat populations occur? Burland *et al* (1999) could not address this question since they studied only individuals from summer colonies. They assumed that, as in most other European bat species, males and females mate during the autumn and winter. Although brown long-eared bats usually roost singly in winter, this mating schedule is supported by the fact that spermatogenesis is completed around the end of August followed by a gradual shrinkage of caudae epididymides, in which sperm can be stored throughout hibernation (Entwistle *et al*, 1998). In addition, the number of inseminated females steadily increases during the winter (Strelkov, 1962). Both males and females are able to store sperm throughout winter in the caudae epididymides or in the uterus, respectively (Swift, 1998 and references therein).

In temperate regions, many bats, including *P. auritus*, spend their winters in subterranean hibernacula (Sendor *et al*, 2000). However, during a short period in late summer and early autumn, tens of thousands of bats from different species may congregate in front of such subterranean sites. Some individuals seem to stay

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only for a single night, and individual and species turnover can be high (Kiefer *et al*, 1994). Such 'swarming sites' (Davis, 1964; Davis and Hitchcock, 1965; Fenton, 1969) are therefore a special kind of transient bat habitat.

Classically, swarming sites were thought to serve as information pools (eg Fenton, 1969; Helversen, 1989), where juveniles gain knowledge about suitable hibernacula. However, anecdotal evidence for sexual interactions exists, for example, for the North American *Myotis lucifugus* (Thomas *et al*, 1979). These authors therefore concluded that males and females of a given colony meet at swarming sites for pair formation and sperm transfer.

However, mating does not necessarily imply gene flow. Providing that only males and females of a single colony meet and mate at swarming sites, the genetic integrity of colonies is sustained. However, if brown long-eared bats from different colonies meet and subsequently mate at swarming sites (extra-colony mating), this would result in gene flow among colonies and consequently in a relaxation of colonies' genetic borders. Recently, Kerth *et al* (2003) could show for *Myotis bechsteinii* that mitochondrial genetic diversity increases at swarming sites relative to the colony. This implies that males and females meet at swarming sites and that colony boundaries break down.

To show that males and females of *P. auritus* from different colonies meet at swarming sites, we compared the genetic structure of maternally and biparentally inherited molecular markers within and among three swarming sites and 14 nursery colonies, respectively. We applied analyses of molecular variance and genetic diversity (AMOVA; Excoffier *et al*, 1992) and individual relatedness (Queller and Goodnight, 1989).

We tested, in a stepwise manner, four hypotheses about the role of swarming sites in the breeding system of *P. auritus*:

(1) If gene flow is strictly male-biased, molecular variance of maternally inherited markers should mainly be distributed among nursery colonies, while that of biparentally markers should be mainly distributed within colonies.
(2) If gene flow is male-biased and females are philopatric, admixture of colonies at swarming sites should mainly affect the molecular variance of the maternally inherited marker. If population borders break down at swarming sites as compared to summer roosts, we expect that mtDNA molecular variance should increase within swarming sites and decrease among swarming sites. Furthermore, genetic diversity for both nuclear and organelle markers should increase at swarming sites relative to the colony.
(3) If individuals from different colonies mix at swarming sites, we expect that the average individual relatedness should decrease at swarming sites relative to within colonies.
(4) Adult males are often found in colonies and could sire offspring within the same colony. To test this hypothesis, we calculated the average relatedness of subadults/juveniles to adult females and males, respectively. If some males sire same-colony juveniles, we expect positive male relateness to the young.

Materials and methods

Definition of terms

We studied nursery colonies that lived either in bat boxes within forest habitats or under the roofs of buildings. Despite intense search, no further nursery assemblages of brown long-eared bats were detected in the respective villages where the roof-dwelling colonies lived. In contrast, forest habitats support several colonies that live in bat boxes within small forest segments, often in immediate vicinity of each other. Mark-recapture experiments, conducted in the Lennebergwald population between 1997 and 2001 (Kiefer *et al*, unpublished), showed that nursery colonies of brown long-eared bats almost exclusively utilised spatially nearby bat boxes within small forest segments. In only one case three females moved from one such forest segment to a bat box in the neighbouring forest segment. Thus, classifying such colonies as demes, the smallest level of population structure, is biologically meaningful, since colony members interact to a much greater degree than with other conspecifics (Burland and Worthington Wilmer, 2001). We, therefore, classified nursery aggregations as colonies within the Ober-Olm and Hoerdt populations.

In order to distinguish between roof-dwelling village populations and spatially structured forest-dwelling populations, we apply the following terms throughout the paper:

- *Populations* comprise all breeding females living in a forest or a village.
- *Colonies* are nursery groups of females that live in spatial clusters of bat-boxes within a given forest segment or within the roof of a house. Hence, when only a single colony inhabits a village (as is the case in our study), such colonies are equivalent to populations.
- *Swarming sites* are potential hibernacula where activity of bats is markedly increased in late summer/early autumn. We also use this term to circumscribe groups of bats that utilise these places for swarming activity. Consequently, we regard swarming sites to be the operational equivalent of 'populations', with no further subdivision.

We therefore consider roof-dwelling colonies as functionally equivalent to forest colonies that utilise several bat boxes within a given forest segment. Both types of colonies utilise different shelters, either several bat boxes or different parts of a roof (Entwistle *et al*, 2000).

Spatial sampling

The study area is part of the federal state of Rhineland-Palatinate in southwest Germany (Figure 1). It comprises parts of Rheinhessen and the Saar-Nahe-Bergland. In 1999, we sampled 194 brown long-eared bats from five nursery populations: Bärweiler (village), Niederkirchen (village), Hoerdt (forest), Ober-Olm (forest) and Lennebergwald (forest) (Table 1). The forest populations roosted in bat boxes and consisted of four colonies each. In all, 30 specimens from three swarming sites were studied: an abandoned slate mine at Schwerbach (S), a natural cave at Boos (B) and an abandoned basalt mine at Mayen (M) (Figure 1). We mist-netted bats after the nursery roosts were abandoned, from August 1999 to

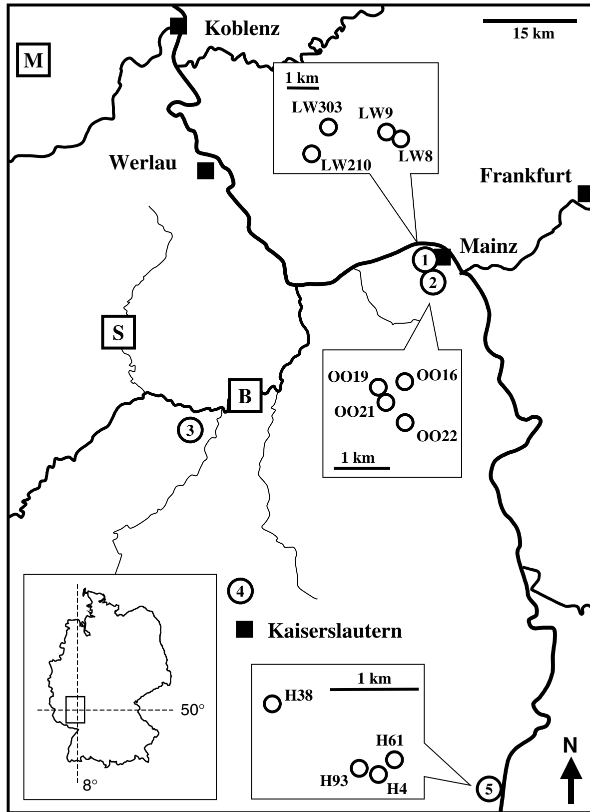

Figure 1 Swarming sites (B = Boos, M = Mayen, S = Schwerbach) and nursery colonies (1 = Lennebergwald, 2 = Ober-Olm, 3 = Bärweiler, 4 = Niederkirchen, 5 = Hoerdt) of the brown long-eared bat.

Table 1 Brown long-eared bats studied in nursery populations, nursery colonies and swarming sites

	Type	n	*Females*	*Males*	*Juveniles*
Ober-Olm					
OO16/6	nc	6	5	—	1
OO19/5	nc	18	10	—	8
OO21/4	nc	9	4	2	3
OO22/1	nc	11	8	2	1
Lennebergwald					
LW8	nc	23	22	1	—
LW9	nc	13	12	—	1
LW210	nc	14	13		1
LW303	nc	18	12	4	2
Hoerdt					
H6/4	nc	3	3	—	—
H6/38	nc	12	4	2	6
H6/61	nc	16	10	—	6
H6/93	nc	21	12	2	7
Bärweiler	nc	31	10	4	17
Niederkirchen	nc	3	—	—	3
Boos	ss	5	1	2	2
Schwerbach	ss	18	5	10	3
Mayen	ss	7	1	3	3
Σ		228	132	32	64
Σ (%)			57.9	14.0	28.0

n = total number of individuals; juv = juveniles, ad = adults; nc = nursery colony, ss = swarming site.

October 1999 and from August 2000 to October 2000. We recorded sex (male *versus* female) and age (adult *versus* juvenile). In contrast to Burland *et al* (2001), colonies were studied in natural and semi-natural habitats, and colony sizes were much lower. We examined large proportions of the colonies with respect to the estimated average annual colony size (data not shown).

Individuals were sexed, based on their secondary sexual characters. Age was classified into two categories: juveniles (pre-reproductive individuals with unfused phalangeal epiphyses) and adults (reproductive individuals with fused epiphyses and brown pelage colour) (Racey, 1974; Entwistle *et al*, 1998).

For outgroup rooting of the mitochondrial haplotype tree, we used specimens of *P. austriacus* that were mist-netted in front of the abandoned slate mine at Schwerbach.

Tissue sampling

We collected bats from nursery colonies under license during summer by mist-netting in front of the roosts or by visiting bat boxes during the course of regular annual censuses. We took a small piece of wing tissue (3 mm diameter, taken from the plagiopatagium) using a sterile biopsy punch following the procedure of Worthington Wilmer and Barratt (1996). Tissue samples were subsequently stored in 70% ethanol. Bats were released immediately after tissue sampling. The number of bats sampled from each colony or population is given in Table 1.

DNA extraction

DNA was extracted using the Boehringer extraction kit. It was subsequently purified and stored at −20°C until further processing.

Microsatellite analysis and genotyping

We analysed six microsatellite loci using previously published PCR primer pairs: Paur02 [$(TC)_n$-repeat], Paur03 [$(TG)_n$], Paur04 [$(CT)_n(C(CA)_n$], Paur05 [$(GT)_n$] and Paur06 [$(AC)_n(AG)_n$] of Burland *et al* (1998) and P5 [$(AC)_n$] of Kerth (1998). Modified PCR conditions were as follows:

- Paur02: 1 × 94°C for 120 s; 39 × 92°C for 60 s, 57°C for 30 s, 72°C for 60 s;
- Paur03: 1 × 94°C for 180 s; 29 × 94°C for 60 s, 59°C for 120 s, 72°C for 120 s; 1 × 72°C for 600 s;
- Paur06: 1 × 94°C for 120 s; 39 × 92°C for 60 s, 58°C for 30 s, 72°C for 60 s;
- P5: 1 × 94°C for 120 s; 39 × 92°C for 60 s, 40°C for 30 s, 72°C for 60 s.
- Thermocycling profiles of Paur04 and Paur05 followed Burland *et al* (1998).

We used fluorescent-labelled primers from APPLIED BIOSYSTEMS. PCR products were analysed with an ABI PRISM™377 automatic sequencer using the GENESCAN®ANALYSIS software (version 2.1, APPLIED BIOSYSTEMS INC.). We used the program GENOTYPER® for genotyping (version 2.0, APPLIED BIOSYSTEMS INC.).

DNA sequencing

The primer pair L15975 and H16425 of Wilkinson and Chapman (1991) amplified a ca. 600 bp fragment of the

control region in *P. auritus*. Sequences were analysed using an ABI PRISM™377 automatic sequencer and the SEQUENCE NAVIGATOR version 1.0.1 software (APPLIED BIOSYSTEMS INC.). We consistently scored 550 bp for each *P. auritus* haplotype.

The sequence of *P. austriacus* was 113 bp shorter, mainly due to the absence of one 81-bp repeat that is present in varying numbers in *Plecotus* species (own data).

Analysis of molecular data

Sequences were aligned using the Clustal X software of Thompson *et al* (1997). Minor refinements were made manually at single base positions. A maximum parsimony (MP) tree was calculated using PAUP* (Swofford, 2001) with gaps treated as a fifth character, random addition of taxa, and a heuristic search using the tree bisection and reconnection branch-swapping algorithm.

Microsatellite allele frequencies and exact tests for Hardy–Weinberg distribution of genotypes were calculated using GENEPOP (version 3.2; Raymond and Rousset, 1995).

The average gene diversity of microsatellite loci was estimated as $\hat{H}$ (Nei, 1987; equivalent to the expected average heterozygosity, H_e), with the variance obtained via 1023 permutations (software: ARLEQUIN; Schneider *et al*, 2001).

Hardy–Weinberg disequilibria were assessed for nursery colonies by estimating F_{IS}, which quantifies the departure from panmixia at the level of individual samples. To test for the influence of males and juveniles on the distribution of genetic variation among colonies and populations, we performed all calculations with and without males and juveniles. Microsatellite Paur03, located on the *x*-chromosome (Burland *et al*, 1999), was omitted from analyses of heterozygosity that included males.

Linkage disequilibria among all possible pairs of polymorphic loci were tested for each population separately using the ARLEQUIN software (1023 permutations; Schneider *et al*, 2001). Probabilities of exact tests were estimated from 1023 permutations. Probabilities were corrected colony-wide for multiple tests following the sequential Bonferroni procedure (Rice, 1989).

Our spatial sampling design of nursery colonies and populations enabled us to analyse the molecular variance distribution at hierarchical levels for both marker systems: individual (*I*), colony (*C*), population (*P*) and total sample (*T*) level. We applied an analysis of molecular variance (AMOVA; Excoffier *et al*, 1992) using the ARLEQUIN software of Schneider *et al* (2001). This analysis partitions the total variance into covariant components at predefined levels. We estimated three variance components and the respective inbreeding coefficients (*F*-values) for both marker systems according to Weir and Cockerham (1984): within colonies (V_{IC}), among colonies within populations (V_{CP}) and among populations (V_{PT}). The three-level AMOVA of nursery colonies is contrasted by a two-level AMOVA of swarming sites (V_{IS} and V_{ST}). We tested the hypothesis that formerly separated haplolineages and haplotypes of the maternally inherited D-loop mix at swarming sites. Since molecular variance partitioning did not vary significantly among subsets of individuals (differentiated by sex and age; data not shown), we only present AMOVA results for pooled specimens.

Due to the large number of alleles at the microsatellite loci, exact probabilities were calculated using the Markov-chain method (Guo and Thompson, 1992), with 1023 recombinations and replicates each.

Gaggiotti *et al* (1999) emphasized that molecular variances estimated for ordered microsatellite alleles produce biased results due to their high mutation rate, especially when sample sizes are small and the number of loci scored is low ($n_S = 5$, $n_l = 10$). In addition, Pons and Petit (1996) showed that ordered alleles or haplotypes may harbour information about their own history, depending on their phylogenetic background. However, this may vary among microsatellites and mitochondrial haplotypes. We therefore conducted the AMOVA only for unordered alleles.

The inference of genetic population structure using inbreeding coefficients (*F*-statistics – AMOVA) is problematic when molecular markers, such as microsatellites, are highly polymorphic (Hedrick, 1999). In this case, the among-population variance is biased downward by the number of alleles, especially if populations do not share alleles and if the expected within-population heterozygosity exceeds 0.50. Simulation experiments by Balloux *et al* (2000) showed that in such cases *F*-values are highly underestimated. To complement the among-population analysis, we calculated the average individual relatedness within colonies. The coefficient of relatedness is the probability that individuals share genes identical by descent (Pamilo, 1984), and confers information of genetic cohesion within groups rather than genetic dissimilarity among groups.

Estimates of relatedness were calculated with the program Relatedness 5.0.8 (Queller and Goodnight, 1989) between females, males, females and males and among all individuals from 14 nursery colonies ($N = 198$) and three swarming sites ($N = 30$), respectively. In addition, we calculated the (asymmetric) relatedness of juveniles and sub-adults to adult females and males, respectively, for inference of male paternity of same-colony young. Results were averaged over colonies. Relatedness estimates should be positive given colony cohesion, but because *P. auritus* conceive single pups the average colony relatedness is expected to be low when averaged across individuals.

Results

Mitochondrial haplotypes

We sequenced 550 bp for each brown long-eared bat. In the combined alignment of 15 *P. auritus* and one outgroup haplotype from swarming sites and nursery colonies (GenBank accession numbers AY531614–AY531628), 447 of 600 bp are constant and 65 variable characters are parsimony informative. Empirical base frequencies are $\pi_T = 0.271$, $\pi_C = 0.206$, $\pi_A = 0.434$ and $\pi_G = 0.090$, showing the typical anti-G bias of mitochondrial DNA (Zhang and Hewitt, 1996). Within *P. auritus* we found three haplolineages (A–C). Evidence for heteroplasmy in the Lennebergwald population comes from five specimens from forest segment LW210 that showed a mixed T/C peak with identical intensity at 419 bp. The C characterises haplotype B2; a T

characterises haplotype B3. Both haplotypes co-occurred in LW210. We omitted these presumably heteroplasmatic specimens from further analyses since none of the applied population genetic approaches is designed to handle this phenomenon.

Haplotypes were not distributed homogeneously among nursery populations (Figure 2). Haplolineage A only occurred in the roof-dwelling colonies of Bärweiler and Niederkirchen. Haplolineage C is confined to Hoerdt with C1 most frequent in all colonies. Haplolineage B is shared between the two populations around Mainz (Ober-Olm and Lennebergwald). Among colonies of single populations, alternative haplotypes were fixed or nearly fixed (eg, OO21 and OO22 , or LW8 and LW9).

In contrast, haplolineages A–C were all present at swarming sites (Figure 2). None of the haplotypes found at Hoerdt (C1–C3), the nursery population most distant from the swarming sites, occurred at any swarming sites. Only one Lennebergwald/Ober-Olm haplotype (B2) occurred at Boos. In contrast, both Bärweiler/Niederkirchen haplotypes (A1 and A2) were found at Schwerbach. At Mayen, the swarming site most distant from the nursery colonies, none of the nursery colony haplotypes was found.

Mitochondrial nucleotide diversity within colonies, mean = 0.00037, range: 0 – 0.00097, was significantly lower than at swarming sites, mean = 0.0488, range: 0.0157–0.0661 (t-test, 15 df, $t = -7.27$, $P < 0.001$).

Microsatellite diversity

All loci were highly polymorphic. The number of alleles ranged from 11 for P5 to 30 for Paur06 (over colonies and swarming sites). (An individual-specific table of allele distribution can be provided upon request) After sequential Bonferroni correction, only eight out of 210 pairs of loci were significantly affected by linkage disequilibrium at the 5% level: Paur02/Paur05 in BW, Paur02/Paur06 in OO19 and OO22, Paur03/Paur05 in H93, Paur04/P5 in LW8 and Paur05/P5 in OO16, LW303 and LW210. Since five pairs of loci were affected, we regard all loci as unlinked.

After Bonferroni correction for multiple tests, heterozygote deficit within colonies was significant for Paur02 in HD93, for Paur04 in LW210 and for Paur06 in OW19/5, LW303 and HD93 only. Microsatellite gene diversity within colonies, mean = 0.792, range: 0.994–0.833, was lower than at swarming sites, mean = 0.838, range: 0.799–0.863, but not significantly so (t-test, 15 df $t = -1.83$, $P = 0.08$).

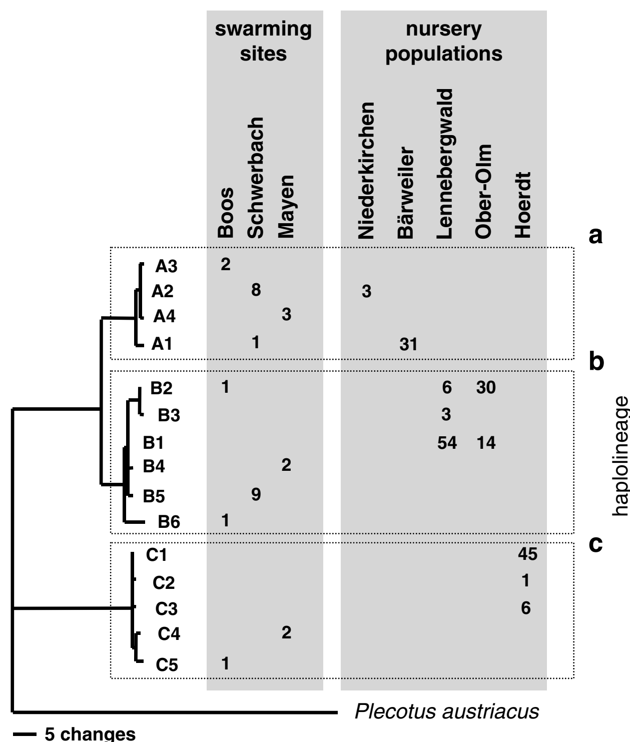

Figure 2 MP haplotype tree and haplotype distribution of the brown long-eared bat at swarming sites (Boos, Schwerbach and Mayen) and nursery populations (Niederkirchen, Bärweiler, Lennebergwald, Ober-Olm and Hoerdt).

Distribution of molecular variance (AMOVA)

With the exception of V_{PT} for microsatellite alleles at nursery colonies, all variance components were significantly different from zero (Table 2). The total molecular variance of nuclear and mitochondrial markers was almost identical under the unordered alleles/haplotypes model. While almost 100% of the molecular microsatellite variance was distributed within colonies, ca. 60% of the total mitochondrial variance was distributed among populations.

Swarming site molecular variance components for the microsatellite loci are similar to those estimated for nursery colonies (Table 2). Almost all molecular variance is distributed within colonies and swarming sites, respectively. In contrast, among-swarming site molecular variance for the D-loop is markedly decreased with respect to among-nursery population molecular variance (Table 2; 31 *versus* 60%).

All common microsatellite alleles, with one exception, were present in all haplolineages, populations and colonies. Thus, low genetic variance originated rather from homogenous allele distributions and was not caused by large numbers of private alleles linked to separate haplolineages. The only exception was allele 9 of the maternally inherited locus Paur03, which was present at 19% in population BW, again implying maternal philopatry.

Relatedness

The average relatedness among colony members was significantly greater than zero in all categories except for male–male estimates (Table 3). Within colonies, the relatedness of juveniles to adult females was significant, $R = 0.05 \pm 0.01$ (Px = 34, Py = 75), whereas relatedness of juveniles to adult males was not significant, $R = 0.03 \pm 0.05$ (Px = 39, Py = 13).

In contrast, relatedness at swarming sites was not significantly greater than zero for any category (Table 3).

Discussion

Our data clearly support all the four hypotheses set out in the introduction: (i) Gene flow in *P. auritus* is strongly male-biased. At the nuclear level, almost all genetic variance (ca. 100%) was distributed within colonies of local populations. As expected under the male-biased gene flow model, this is in marked contrast with 85%

Table 2 Variance components (AMOVA) and hierarchical *F*-statistics derived from an AMOVA for D-loop haplotypes and for six microsatellite loci of nursery colonies (a) and swarming sites (b) of brown long-eared bats

(*a*)

	Among populations					*Among colonies, within populations*					*Within colonies*					*Total variance*	
	df	V_{PT}	$\%V_{PT}$	F_{PT}	P	*df*	V_{CP}	$\%V_{CP}$	F_{CP}	P	*df*	V_{IC}	$\%V_{IC}$	F_{IC}	P	*df*	V_t
Microsatellites	4	−0.00006	−0.01	−0.0001	0.88759	9	0.00021	0.04	0.00043	<0.01	382	0.49983	99.97	0.00032	<0.001	395	0.49999
D-loop	4	0.27881	59.39	0.59391	<0.001	9	0.11878	25.30	0.62305	<0.001	184	0.07186	15.31	0.84692	<0.001	184	0.46945

(*b*)

	Among swarming sites					*Within swarming sites*					*Total variance*	
	df	V_{ST}*	$\%V_{ST}$*	F_{ST}	P	*df*	V_{IS}*	$\%V_{IS}$*	F_{IS}	P	*df*	V^*_{Total}
Microsatellites	2	0.01727	2.99	0.02544	<0.05	27	0.41091	97.01	nc	<0.001	29	0.42818
D-loop	2	0.14747	30.60	0.30600	<0.001	27	0.33445	69.40	nc	<0.001	29	0.48192

Significance of variance components and inbreeding coefficients was tested after 1023 permutations; nc = not computable; the total variance of swarming sites is marked with an asterisk.

Table 3 Average relatedness estimates over colonies and swarming sites of *P. auritus*

Location	*R ± SD*			
	Female–female	*Male–male*	*Female–male*	*All*
Colony	**0.06** ± 0.01 (157)	0.05 ± 0.03 (38)	**0.04** ± 0.02 (159)	**0.05** ± 0.01 (198)
Swarming site	0.02 ± 0.06 (10)	−0.05 ± 0.02 (19)	0.06 ± 0.04 (30)	0.02 ± 0.03 (30)

Significance was estimated by jack-knifing over groups, and is marked in bold (sample size in brackets).

($V_{PT} + V_{CP}$) of molecular variance distributed among colonies in maternally inherited genes. These results indicate that populations are genetically similar for bi-parentally inherited genes and different for maternally inherited genes. (ii) At swarming sites, colony borders break down and haplolineages mix. Gene diversity was greater at swarming sites, implying that colony structure breaks down. This effect may not have been detected by the AMOVA analysis alone, because of the very high level of heterozygosity within colonies. In contrast to the nuclear markers, among-swarming site molecular variance for the D-loop is markedly less than among-nursery population (Table 3; 31 *versus* 60%) and nucleotide diversity is greater. Secondly, relatedness was significant within colonies but not at swarming sites and was positive for juvenile-female comparisons but not juvenile-male, suggesting (iii) colony breakdown and (iv) extra-colony copulation. The finding that adult males within a colony share haplotypes with the other colony members shows that males are not foreign but that they may be site faithful upon reaching sexual maturity. However, the relatedness estimates imply that they seldom sire offspring within the natal colony. The latter finding, based on a combination of mtDNA and relatedness estimates, supports the analysis done by Burland *et al* (2001), who estimated that a modal percentage of fathers and mothers per colony were 15 and 100, respectively.

Delimitation of demes and female philopatry

The correct delimitation of demes as the smallest genetically cohesive groups of individuals is crucial for a correct interpretation of gene flow data. Mark-recapture data (Kiefer *et al*, unpublished) demonstrate for the Lennebergwald population that, within a single forest segment, females frequently interchange among different bat boxes. At the same time, exchange with other such colonies is negligible (for similar results, see Heise and Schmidt, 1988; Boyd and Stebbings, 1989). This delimitation of colonies as demes is strongly supported by our non-molecular data, providing us confidence that our definition of colonies in Ober-Olm and Hördt is also biologically meaningful.

In Lennebergwald, female philopatry is strong, and is comparable to that found in Scottish populations (Entwistle *et al*, 1998). The maximum distance between two colonies of Lennebergwald was only 2250 m, a distance well within the range of foraging female brown long-eared bats (Entwistle *et al*, 1996). Fuhrmann and Seitz (1992) radio-tracked females of the Lennebergwald population. Within a single night, females foraged across distances of 3.3 km, well within the range of neighbouring colonies. In Lennebergwald, prominent dispersal barriers are lacking. Forest segments are only separated by pathways and small forest roads. Thus, the pronounced microgeographic structuring of these forest populations must be sustained through behavioural constraints on female dispersal, such as living together in closed social groups.

Is male-biased gene flow sustained at swarming sites?

Male-mediated gene flow in bats may simply be the consequence of the largely disconnected life cycles of

sexes and the necessity to find mates. Therefore, meeting at traditional mating sites seems to be one strategy of choice for male bats (Thomas *et al*, 1979, Gerell and Lundberg, 1985). Males and females actively move to swarming sites in late summer and early autumn (see also Horacek, 1975, Kiefer *et al*, 1994). They bear microsatellite alleles that cover most of the variability known from adjacent maternal colonies. However, while nursery populations are fixed for single D-loop haplolineages, swarming sites hold all haplolineages (Figure 2). Consequently, the molecular variance of the D-loop is much lower among swarming sites than among nursery populations ($V_{ST} < V_{PT}$). This provides strong evidence that individuals from different nursery populations meet at swarming sites. However, does this also provide evidence that swarming sites act as 'hot spots' for gene flow, as was recently assumed for *Myotis bechsteinii* (Kerth *et al*, 2003)?

The presence of males and females at swarming sites, paralleled by the breakdown of population borders, supports the idea that swarming sites are the places where gene flow among colonies is sustained. Some indirect evidence indicates that breeding behaviour is a promoter of this swarming activity. Brown long-eared bats are active at swarming sites mainly from mid-August until late October (Kiefer *et al*, 1994, Parsons *et al*, 2003). This corresponds perfectly to the males' testosterone level, which is highest in late August (Helversen, personal communication). In the Nearctic bat *Myotis lucifugus,* it is known that as soon as the testosterone level reaches its maximum sperm is transferred from the testicles to the caudae epididymides (Gustafson and Shemesh, 1976), and hence is available for mating. Accordingly, the caudae epididymides of male brown long-eared bats are the largest at the end of August or beginning of September (Entwistle *et al*, 1998). During winter, their size steadily decreases, indicating mating. Direct observation of *P. auritus* matings from September and October have been made by Stebbings (1966), and Lichacev (1980) assumes that *P. auritus* predominantly mates in August.

However, if brown long-eared bats meet mainly for mating at swarming sites, we expect a dramatic increase of inseminated females during swarming activity. Some evidence exists that this is not the case, although to the best of our knowledge data on inseminated female bats at swarming sites are lacking for any species. In November, right after the time of swarming, the proportion of inseminated females only is 14%. It steadily increases until it reaches 100% in April (Strelkov, 1962; admittedly, this is the best information we have, and its general validity needs to be tested). This is paralleled by the steady decrease of caudae epididymides size (see above), and strongly indicates that mating of brown long-eared bats predominantly occurs after swarming and perpetuates during the entire period of hibernation (like in the Daubenton's bat, *Myotis daubentonii*; Roer and Egsbaek, 1966; Roer, 1969).

If, however, swarming involves mating, but not instantaneously and not on the spot, this would necessitate the guarding of mates by males. In the light of the high life expectancy of long-eared bats (Horacek, 1975), we suspect that autumn food intake and associated winter survival are more important than mate guarding. Swarming behaviour could therefore be seen more in the light of information transfer, as has been hypothesised previously.

Is information transferred at swarming sites?

To the best of our knowledge, swarming behaviour of bats is restricted to temperate zone species which depend on suitable hibernacula. Swarming is observed mainly in front of potential hibernacula, although the number of swarming bats is often much higher than that actually found within a hibernaculum (see eg Sendor *et al* (2000) for the detectability of hibernating bats in subterranean shelters). This evidence strongly suggests that swarming behaviour initially evolved in the context of hibernation. It is therefore likely that swarming is the way young bats learn the location of important hibernacula (Altringham, 2003).

Conclusion

Our data clearly show that during autumnal swarming colony borders break down and specimens from different localities (and hence haplolineages) mix at swarming sites. This result links swarming behaviour with sexual interactions between individuals from different colonies (extra colony mating), resulting in outbreeding (Parsons *et al*, 2003). Outbreeding was recently shown to be important for individual survival in a temperate zone bat species, *Rhinolophus ferrumequinum* (Rossiter *et al*, 2001). However, the hypothesis that swarming sites act as 'hot spots' for gene flow has some weaknesses. The major objection to sexual interaction as the sole reason for swarming behaviour is the low proportion of females being inseminated during swarming itself. We therefore propose that it is also explained by the need to transfer information about suitable hibernacula.

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