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Mating, hybridisation and introgression in *Lasius* ants (Hymenoptera: Formicidae)

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Abstract

Recent reviews have shown that hybridisation among ant species is likely to be more common than previously appreciated, but that documented cases of introgression remain rare. After molecular phylogenetic work had shown that European *Lasius niger* (LINNAEUS, 1758) and *L. psammophilus* SEIFERT, 1992 (formerly *L. alienus* (FOERSTER, 1850)) are unlikely to be very closely related, we decided to analyse an old data set confirming the conclusion by PEARSON (1983) that these two ants can indeed form viable hybrids. We show that signatures of introgression can be detected in a Danish site and that interspecific gene-flow is asymmetrical (only from *L. niger* into *L. psammophilus*) as inferred previously by Pearson for the southern England site that he studied and from which we also collected data. We compare the observed patterns of hybridisation and introgression in the Danish and British site and infer that overlap in nuptial flights in Denmark may have contributed to the higher frequency of introgressed genes relative to the southern England site where nuptial flights are clearly separated in time. We also report the first mating system data for *L. psammophilus*, showing that this species has facultative multiple mating of queens similar to *L. niger*. We suggest that *L. psammophilus-niger* introgression may be much more common than previously appreciated, which would explain that European myrmecologists have often found it difficult to distinguish between these species at sites where they occur sympatrically. This would imply that multiple accessible field sites are available to study the molecular details of hybridisation and introgression between two ant species that have variable degrees of sympatry throughout their distributional ranges.

Key words: Allozymes, relatedness, paternity, mating frequency, heath land, Hartland Moor, Mols Bjerge.

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Introduction

It is only a decade ago that hybridisation between ant species was considered to be rare, but this notion was changed when SEIFERT (1999) compiled evidence that over 10% of the central European ants may occasionally hybridise and that strong evidence for this phenomenon was already available for *Leptothorax albipennis* (CURTIS, 1854) (now *Temnothorax albipennis*) and *Lasius jensi* SEIFERT, 1982. A few years later the journal *Ecology* published a special feature issue on the ecology and evolution of hybridisation in ants (NONACS 2006), focusing mostly on *Pogonomyrmex* harvester ants where it had become clear that in some species workers arise from hybrid matings whereas gynes produced in the same colonies are pure bred (LINKSVAYER & al. 2006). More recent studies further showed that ant species may have very bizarre breeding systems with different genetic pathways towards producing males, gynes and workers (reviewed by FELDHAAR & al. 2008). This corroborated the notion that many such cases may have been overlooked and that explicit genetic studies of hybridisation and introgression have the potential to provide important insights in the evolutionary potential of ant breeding and mating systems. The most recent examples are studies in *Formica* wood ants documenting segregation distortion leading to large scale differentiation between male and female genomes of the same species (KULMUNI & al. 2010) and the convergent appearance of multiple hybridisation events between the same pair of species (SEIFERT & al. 2010).

Some of the first studies combining morphology and genetic marker techniques to elucidate hybridisation were done by PEARSON (1982, 1983), showing that sympatric *Lasius niger* (LINNAEUS, 1758) and *L.* "*alienus*" (FOERSTER, 1850) occasionally form F1 hybrids at Hartland heath in Dorset, UK and that this hybridisation appeared to be restricted to *L. alienus* queens mating with *L. niger* males. At that time, however, the taxonomy of the subgenus *Lasius* s.str., to which these species belong, was so confusing that these species were generally considered to be each other's closest relatives (i.e., sister species, although this term was hardly used in the 1980s). The taxonomy of *Lasius* was completely revised by SEIFERT (1992), who described several new species and renamed others, among

them sandy-heath-land *L. alienus*, which became *L. psammophilus* SEIFERT, 1992. However, it was only after the publication of the first *Lasius* phylogeny (STEINER & al. 2004) that it became clear that *L. niger* and *L. psammophilus* were less closely related than previously assumed, which implied that the possibility of hybridisation and asymmetric introgression between these two species became more interesting.

Two of us (TMvdH and JJB) had the opportunity to initiate follow-up studies of PEARSON (1982, 1983) in the second half of the 1980s, both at the Hartland population in Dorset and in a similar population at Mols, Denmark, where worker morphology of sympatric *Lasius niger* and *L. psammophilus* (hair numbers on tibia and scapus) was so variable that hybridisation was suspected (M.G. Nielsen, pers. comm). However, although presented at the 1986 IUSSI congress in Munich (VAN DER HAVE 1987), this work was never published, except for some of the *L. niger* data on multiple queen mating, which were used in a comparative analysis of the variation in queen mating frequencies among *L. niger* populations in Northwest (NW) Europe (BOOMSMA & VAN DER HAVE 1998).

In the present paper we present both the hybridisation and the queen mating data that we obtained from the Hartland and Mols populations of *Lasius niger* and *L. psammophilus*. The hybridisation data confirm that the two species also hybridise in the Danish site and that clear indications for asymmetric introgression can be found. The mating system data show that *L. psammophilus* has facultative multiple mating of queens, similar to *L. niger*, confirming the general notion that eusocial insect species of the same (sub)genus normally have very similar mating systems (BOOMSMA & RATNIEKS 1996, BOOMSMA & al. 2009). However, we used a more sophisticated technique for estimating queen mating frequency (PEDERSEN & BOOMSMA 1999) compared to the reanalyses of literature data provided by BOOMSMA & RATNIEKS (1996), confirming the prediction of PEDERSEN & BOOMSMA (1999) that the older statistical techniques tended to underestimate the frequency of multiply mated queens.

Materials and methods

Lasius niger and *L. psammophilus* populations in both England and Denmark were observed intensively during the period of nuptial flights to study interspecific mating opportunities and to collect inseminated gynes shortly after their nuptial flight. *Lasius alienus* colonies in Hartland Moor were studied extensively by M.V. Brian and co-workers (e.g., BRIAN & al. 1966), but were later identified as *Lasius psammophilus* by SEIFERT (1992). Alates and workers of *L. niger* and *L. psammophilus* were collected from field colonies in heath land of Hartland Moor N.N.R. (near Ridge, Dorset, UK) in 1984 and Mols (near Femmøller, Denmark) in 1985 / 1986. Newly inseminated queens were collected at the same sites a few weeks later immediately after nuptial flights while they were searching for suitable places to excavate their nest-founding burrows. In August 1985 and 1986 colonies of both species at Mols were checked daily during the period that nuptial flights of both species could occur. Alates were ready to fly when they assembled close to the surface and workers were actively walking in and out of the nest openings. This behaviour could continue for several days until conditions were optimal for a mating flight, usually warm, humid and with little wind, when alates dispersed synchronously over relatively large areas (BOOMSMA & LEUSINK 1981).

Collection dates for newly inseminated *Lasius niger* queens were 8 August 1984 (Hartland) and 15 August 1985 (Mols), and 18 (evening) to 19 (early morning) August 1985 (Mols) for *L. psammophilus*. Queens were stored together in trays at 5°C and high relative humidity for up to four months before they were set up individually in 20 ml pots with a small sponge that was watered twice a week (for further details see BOOMSMA & ISAAKS 1982, BOOMSMA & VAN DER HAVE 1998). Queen mortality was low during the months of artificial hibernation and moderate during the individual rearing period (27% in *L. psammophilus*, Mols, to 29% in *L. niger*, Mols), probably not exceeding normal mortality under field conditions. Queens produced their first batch of three to ca. 20 workers after two to four months, after which they were frozen and either analysed directly or stored at -70°C for later allozyme analysis.

Allozyme genotypes were obtained by horizontal starch gel electrophoresis of males, gynes (virgin queens) and workers sampled from the field colonies and from the queens and workers of the incipient colonies. Allele frequencies across field colonies were calculated from inferred maternal queen genotypes, whereas they could be obtained directly from queens of the incipient colonies, except for *Lasius psammophilus* at Hartland, where flights were observed but no newly inseminated queens could be collected. These comparisons were subsequently used to estimate the number of males that queens had mated with (mating frequencies) using up to three informative and genetically variable loci (Malate dehydrogenase [Mdh-1], Esterase-2 [Est-2] and Malic enzyme [Me]), which segregated three (Mdh-1) to five (Est-2) alleles. Details of the electrophoresis procedure are given by VAN DER HAVE & al. (1988). A total of 105 field colonies and 276 mother-offspring combinations were analysed, involving 1956 workers from newly inseminated queens and 1355 workers, 316 gynes and 987 males from mature field colonies. Samples of workers and gynes were pooled for further analysis to maximise sample size of female offspring per colony and hence improve accuracy of mating frequency and relatedness estimates.

We used the method developed by PEDERSEN & BOOMSMA (1999) as implemented in the program MATESOFT 1.0 (MOILANEN & al. 2004) to assign female offspring (workers and gynes) to patrilines and to correct estimates of the proportion of multiply mated queens (D) for both genetic non-detection error and non-sampling error (see also BOOMSMA & RATNIEKS 1996). The corresponding estimates for average paternity skew ($\overline{c}$), pedigree relatedness (g), and population-wide effective queen mating frequency ($m_{e,g}$) were obtained from the deduced pedigrees. We further estimated the intracolonial relatedness between pooled worker and gyne females (r_{ff}) and from the female offspring to males (r_{fm}; for field colonies only) using the program RELATEDNESS 5.0.4 (GOODNIGHT & QUELLER 1998). The genetically effective queen mating frequency was then calculated from the estimates of r_{ff}. Finally, we applied the allele and genotype frequencies for reconstructing the extent of hybridisation and introgression between the two sympatric species. These reconstructions were helped by the fact that the 100 allele at the Mdh-1 locus was expressed with consistently different additional bands for *Lasius niger* and *L. psam-*

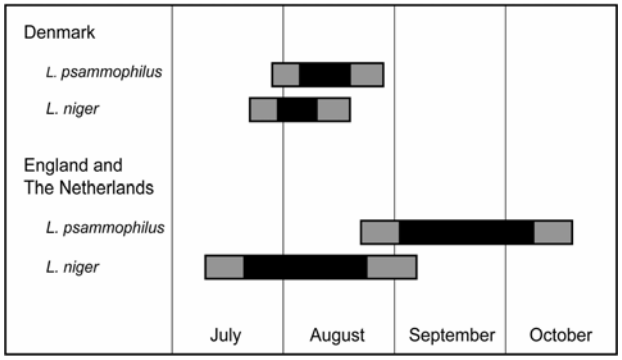

Fig. 1: Timing of nuptial flights of *Lasius niger* and *L. psammophilus* in England (Hartland) and the Netherlands and Denmark (Mols) based on BRIAN & al. (1966; observations of both species in the period 1957 - 1964), NOORDIJK & al. (2008), NIELSEN & JENSEN (1975) and personal observations (*L. niger* in the period 1982 - 1993 in England, Denmark and The Netherlands, see also BOOMSMA & VAN DER HAVE 1998, and *L. psammophilus* in Denmark in 1985 - 1986). Main flight period: black; infrequent nuptial flights: grey.

mophilus in the Hartland population. We coined these alleles 100n and 100p, respectively.

As we found evidence for hybridisation in both our study populations we also present a plot of nuptial flight observations to help explain why introgression between *Lasius niger* and *L. psammophilus* happens at different rates in the two sites that we investigated.

Results

We observed nuptial flights of *Lasius niger* close to Hartland Moor on 8 August 1984 and in the Mols study site on 15 August 1985 and 4 August 1986. *Lasius psammophilus* nuptial flights were not observed in Hartland Moor in 1984 but are known to occur in September – October well separated from the nuptial flights of *L. niger* in July – August (Fig. 1; BRIAN & al. 1966). In the Mols area *L. psammophilus* nuptial flights were observed on 18 August 1985 and on 6 and 12 August 1986. In both years the difference in timing was only two to three days with *L. niger* nuptial flights consistently before *L. psammophilus*, which agrees with the standard late-July through August nuptial flight periods for these ant species in Denmark (NIELSEN & JENSEN 1975).

Lasius niger nuptial flights typically occur around midday or early afternoon, whereas *L. psammophilus* flights normally happen a few hours later in the afternoon or early evening. Newly inseminated queens of both species remove their wings immediately after landing. Almost all *L. niger* queens start digging a burrow within a few hours after landing, but most newly inseminated *L. psammophilus* queens at Mols first spent the night with their heads down on grass stems or low shrubs, and did not start searching for sites to dig a burrow until early in the next morning.

Our mating system estimates showed that both ant species have low to moderate degrees of multiple queen-mating at both sites, but that effective queen mating frequency remains low (up to 1.06) because paternity contributions are highly skewed (Tab. 1). Relatedness estimates were never significantly different from 0.75 (two-tailed *t*-tests; $P > 0.15$ for all), as expected when effective multiple queen mating is rare and these multiple matings involved two mates (only one treble mating was ever observed). As expected, the indirect (relatedness-based) estimates of effective queen mating frequency produced similar values as the direct ones (corrected for non-detection and non-sampling error), but with considerable more scatter because of the inherent substantial confidence limits of regression relatedness estimates (Tab. 1). This is particularly true for the Hartland field colonies of both species where the genetic variation at two loci is based on a few rare additional alleles (Tab. 2), which causes a rather large combined sampling error when estimating allele frequencies and relatedness.

Tab. 1: Queen mating frequencies and relatedness estimates for broods of *Lasius niger* and *L. psammophilus*. *N*, number of broods with female (f, i.e., workers and gynes) and male (m) offspring analysed; *n*, number of workers (w), gynes (g), and males (m) analysed; *D*, proportion of multiply-mated queens observed and estimated; $\overline{c}$, average paternity skew observed and estimated; *g*, pedigree relatedness of female offspring; $m_{e,p}$, average pedigree effective queen mating frequency; r_{ff}, average regression relatedness ± standard error (SE) between female offspring; $m_{e,g}$, average genetically effective queen mating frequency derived from r_{ff}; r_{fm}, average regression relatedness ± SE from female offspring to males. [a] Including one treble mating.

Species and sample	N_f	N_m	n_w	n_g	n_m	D_{obs}	D_{est}	$\overline{c}_{obs}$	$\overline{c}_{est}$	g	$m_{e,p}$	r_{ff}	$m_{e,g}$	r_{fm}
Lasius niger														
Mols new queens	127	–	788	–	–	0.055	0.134	0.732	0.732	0.737	1.06	0.727 ± 0.031	1.05	–
Mols field colonies	23	18	405	38	297	0.087	0.199	0.918	0.933	0.743	1.03	0.720 ± 0.064	1.06	0.308 ± 0.244
Hartland new queens	110	–	915	–	–	0.054	0.205	0.823	0.878	0.738	1.05	0.711 ± 0.031	1.09	–
Hartland field colonies	21	10	200	159	246	0.000	0.000	–	–	0.750	1.00	0.510 ± 0.172	1.92	0.307 ± 0.378
Lasius psammophilus														
Mols new queens	31	–	253	–	–	0.065	0.102	0.714	0.758	0.742	1.04	0.737 ± 0.032	1.03	–
Mols field colonies	29	15	458	35	279	0.138[a]	0.228	0.841	0.843	0.731	1.06	0.706 ± 0.066	1.10	0.183 ± 0.153
Hartland field colonies	25	13	292	84	160	0.040	0.358	0.944	0.944	0.748	1.04	0.588 ± 0.110	1.48	0.456 ± 0.227

Tab. 2: Allele frequencies of *Lasius niger* and *L. psammophilus* in the Hartland (Southern England) and Mols (Denmark) populations. Frequencies are based on inferred queen genotypes in samples from field colonies and on direct genotyping for newly inseminated queens from which we also bred their first batch of workers. Allelic notation follows the custom of denoting the commonest allele as 100. For Mdh-1, the 100 allele expressed different additional bands in the two species allowing us to separate the *L. niger* (100n) and *L. psammophilus* (100p) alleles in the Hartland population but not in the Mols population. The *L. niger* alleles that could be documented to have introgressed in the *L. psammophilus* population at Mols are indicated in bold. Sample sizes (*N*) are denoted as number of colonies.

Species	**Locus and sample**	**Hartland (UK)**						**Mols (DK)**					
L. niger	Mdh-1	*N*		*100n*	*102*			*N*		*100n*	*102*		
	field colonies	21		0.98	0.02			30		0.98	0.02		
	new queens	111		1.00				133		1.0			
L. psammophilus	Mdh-1	*N*	*96*	*100p*	*104*			*N*	*96*	***100n+p***	*104*		
	field colonies	25	0.94	0.04	0.02			29	0.30	**0.67**	0.03		
	new queens	–						31	0.33	**0.60**	0.07		
L. niger	Est-2	*N*	*95*	*100*	*105*	*110*	*115*	*N*	*95*	*100*	*105*	*110*	*115*
	field colonies	21		0.88	0.08	0.04		30		0.77	0.09	0.13	
	new queens	111	0.01	0.68	0.26	0.05		133	0.01	0.73	0.16	0.10	
L. psammophilus	Est-2	*N*	*95*	*100*	*105*	*110*	*115*	*N*	*95*	***100i***	***105i***	*110*	*115*
	field colonies	25				0.89	0.11	29		**0.03**	**0.01**	0.77	0.17
	new queens	–						31		**0.07**	**0.01**	0.69	0.22
L. niger	Me	*N*	*96*	*100*				*N*	*92*	*96*	*100*		
	field colonies	21		1.0				30	0.06	0.01	0.92		
	new queens	111	0.05	0.95				133		0.08	0.92		
L. psammophilus	Me	*N*	*96*	*100*				*N*		***96i***	*100*	*102*	
	field colonies	25		1.00				29		**0.01**	0.99		
	new queens	–						31		**0.01**	0.98	0.01	

On the gels the three marker loci showed the usual patterns of co-dominant expression (Fig. 2; monomeric for Est-2, dimeric for Mdh-1 and tetrameric for Me), but allele and genotype frequencies were sufficiently different between species to provide interesting information about the extent of interbreeding across species borders (Tab. 2). The Mdh-1 locus at Hartland was almost fixed for different alleles (100n for *L. niger* and 96 for *L. psammophilus*) and the 100 allele for the latter species had characteristic stutter bands that allowed us to consistently mark this allele as 100p to distinguish it from 100n. All hybrid colonies had the four-banded phenotype of the 100n and 96p heterozygotes (Fig. 2A), which is slightly different from the 96 / 100 heterozygotes in *L. psammophilus*. These differences were less pronounced in the Mols population where the 100n and 100p alleles could not be unambiguously assigned and the frequency of the 96 allele in *L. psammophilus* was much lower (ca. 0.3). The Est-2 locus in the Hartland population had unique alleles for *L. niger* (95, 100 and 105) and *L. psammophilus* (115; and 110 with only very little overlap; Fig. 2B), which allowed us to infer low levels of introgression of *L. niger* alleles (100i and 105i) into *L. psammophilus* (Tab. 2; Fig. 2C). The Me locus was least informative, but also here we were able to identify a low frequency of introgressed alleles (96n) in the Mols population (Fig. 2D). In the Hartland population all hybrid colonies had a combination of the unique hybrid Mdh-1 genotype and the hybrid Est-2 genotypes 100n / 110p or 100n / 115p (Fig. 2A, C). In the Mols population all field colonies of *L. psammophilus* with *L. niger* alleles had a mix of hybrid and pure *L. psammophilus* genotypes. Five field colonies and seven new queens of the *L. psammophilus* samples from Mols had genotypes with *L. niger* alleles. The male genotypes of two field colonies suggested that the mother queens were either F1 hybrids or the result of introgression, while in the remaining three the mother queen genotype was unknown because offspring males were lacking. All seven newly mated *L. psammophilus* queens with *L. niger* alleles could either be F1 hybrids or stem from earlier hybridisation events in their pedigree. Two could further be shown to have been sired by a male with a *L. niger* genotype at one of the marker loci. Thus, while our limited genetic marker resolution did not allow us to single out distinct F1 hybrids between a *L. psammophilus* queen and a *L. niger* male, there is clear evidence for these matings to occasionally take place and introduce *L. niger* genes into the *L. psammophilus* gene pool. This inference is also based on finding queens with F1-like hybrid genotypes producing males with both *L. niger* and *L. psammophilus* alleles and being sired by males with a mix of *L. niger* and *L. psammophilus* alleles.

Overall, the *L. niger* gene frequencies appeared to be very similar in both populations, whereas the *L. psammophilus* populations, with the exception of the Mdh-1 96 allele, seemed to differ mostly by the presence of introgressed *L. niger* alleles. An overview of the relevant alleles and their pure-bred and hybrid genotypes is provided in Figure 2.

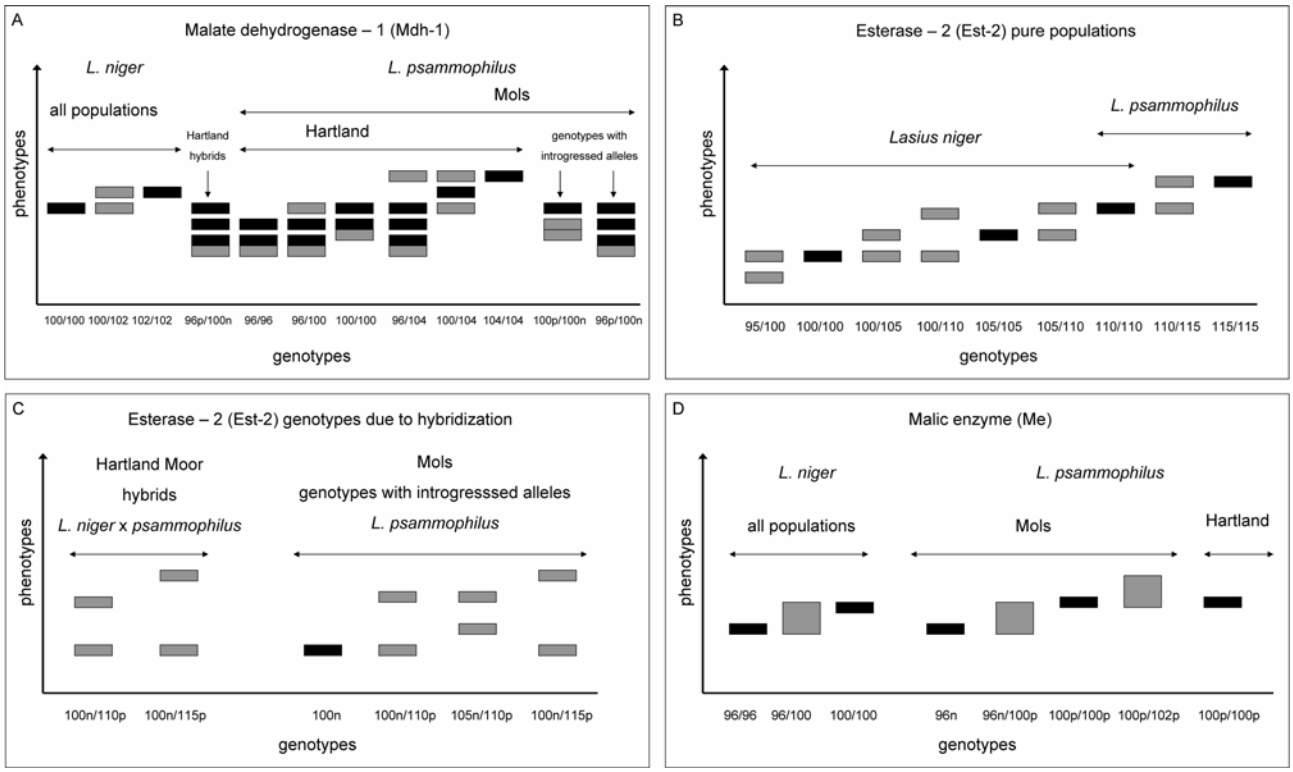

Fig. 2: Observed allozyme electrophoresis banding pattern (strong bands: black; weak bands: grey) of selected alleles and their pure-bred and hybrid genotypes in *Lasius niger* and *L. psammophilus*: Mdh-1 (A), Est-2 for pure populations of the two species (B), Est-2 hybrid genotypes (C) and Me (D). The Est-2 100n (Fig. 2C) and Me 96n genotypes (Fig. 2D) were only found in males with otherwise *L. psammophilus* genotypes. In the Hartland population all hybrid colonies had a combination of the unique hybrid Mdh genotype and the hybrid Est-2 genotypes 100n / 110p or 100n / 115p. In the Mols population all field colonies of *L. psammophilus* had a mix of hybrid and pure *L. psammophilus* genotypes.

These results (Tab. 2, Fig. 2) indicate that the Mols field site in Denmark has a significant extent of interbreeding between *Lasius niger* and *L. psammophilus* and that this involves gene flow from *L. niger* into *L. psammophilus* at three independent loci. The two species were much better separated at the Hartland site, although we know that F1 hybrids can occur there as well as PEARSON (1983) found one colony with heterozygote 4 / 1 and 4 / 2 workers, which would correspond to 100 / 115 and 100 / 110, respectively, in our notation. We believe this difference has to do with the fact that the timing of nuptial flights of the two ant species is clearly separated in the Hartland area, but has significant overlap at the Mols site (Fig. 1).

Discussion

No mating system data for *Lasius psammophilus* have been published before. Our results indicate that this species has similar frequencies of double mating as *Lasius niger*, which fits the general observation that mating systems tend to be highly conserved within ant (sub)genera (BOOMSMA & RATNIEKS 1996, BOOMSMA & al. 2009). Both species belong to the subgenus *Lasius* s.str. (see SEIFERT 1992, MARUYAMA & al. 2008) that may have facultative multiple mating of queens throughout (a small data set of *Lasius emarginatus* (OLIVIER, 1792) from France showed the same; J.J. Boomsma, unpubl.).

Queen mating frequency in *Lasius niger* has been studied quite extensively across populations in Northwest Europe (BOOMSMA & VAN DER HAVE 1998). Some of the data of that study, which were all analysed following the recommendations of BOOMSMA & RATNIEKS (1996), were reanalysed here with a later developed estimation method (PEDERSEN & BOOMSMA 1999) for obtaining fully unbiased estimates of mating frequencies in systems with single-double mating. Indeed the percentages of double mating reported here for the *L. niger* population at Mols (up to ca. 20%; Tab. 1) are somewhat higher than the ones obtained by BOOMSMA & VAN DER HAVE (1998) (up to ca. 10%). The observed frequency of queens assumed to be multiply mated (D_{obs}) was not systematically higher in field colonies compared to new colonies, and this frequency was not significantly different in any of the three samples (Fisher's exact test, $P > 0.41$ for all). This indicates that possible drifting workers in field colonies have not biased our estimates of mating frequencies.

Our data on hybridisation match those obtained by PEARSON (1983) for Est-2 (the only locus that he used distinguishing alleles 1, 2, 3 and 4, corresponding to our alleles 115, 110, 105 and 100). This study inferred that some F1 hybrids occur at the Hartland site and that these colonies had a *Lasius psammophilus* queen (then called *L. alienus*) mated with a *L. niger* male, similar to what we concluded for our samples. The fact that opposite hybrids were not discovered, neither by us nor by PEARSON (1983), is interesting as it indicates that introgression is asymmetric, a phenomenon that has also been genetically documented in *Solenopsis*, *Temnothorax* and *Formica* ants (FELDHAAR & al. 2008). More studies to elucidate whether this is generally caused by asymmetric encounter rates of the sexes during mating flights, or to reproductive isolation mechanisms that are on-

ly unilaterally effective would be highly interesting. Cytonuclear epistasis has been discussed by LINKSVAYER & al. (2006) as a possible explanation for hybrid caste determination in *Pogonomyrmex* seed harvester ants, so it might be that the cytoplasmatic genome of *L. psammophilus* is compatible with the nuclear genome of *L. niger*, whereas the opposite combination is not viable.

Our evidence for introgression is largely indirect, but credible as we found evidence for introgression for all three marker loci. For *Lasius niger*, we know that allele frequencies are approximately constant throughout NW Europe (F_{ST} = 0.003; BOOMSMA & VAN DER HAVE 1998). Average long-distance dispersal of *L. psammophilus* is likely to be somewhat less than in *L. niger*, as the former species is lacking on Frisian islands where the optimal habitat is available (BOOMSMA & al. 1987), but there is nothing to indicate that this should have lead to greatly enhanced genetic differentiation between populations. It thus seems reasonable to assume that allele frequencies among populations may differ (as also indicated in Tab. 2), but that the emergence of novel alleles is due to introgression, especially when these alleles are common in sympatric *L. niger*.

Although we only have data from a few years, the difference in overlap between *Lasius niger* and *L. psammophilus* nuptial flights across the two sites is quite striking. We do not have a ready explanation for this difference, except that there is a considerable difference in latitude between Southern England and central Denmark (50.7 ºN versus 56.2 °N), which implies that the Danish summers are short compared to those at the South coast of Britain, limiting the opportunity for nuptial flights later in the season similar to the effect of higher elevation on nuptial flight timing reported by DUNN & al. (2007). NIELSEN & JENSEN (1975) report that at Mols alate sexuals of *L. psammophilus* can often be found in the nests in September and early October, but that these have never been observed to fly and are likely cannibalised. This means that only early flights may produce mated *L. psammophilus* queens that successfully overwinter to found colonies, but these queens are then also exposed to some frequency of *L. niger* males that fly on the same day, an overlap that seems less likely at the Hartland site where the bulk of *L. psammophilus* sexuals fly in September, when all *L. niger* sexuals will normally have flown already. Unidirectional gene flow from *L. niger* into *L. psammophilus* is expected as *L. niger* nuptial flights occur a few hours before the *L. psammophilus* flights. When the *L. niger* nuptial flights are over and queens start digging burrows, *L. niger* males are still air bound and have the opportunity to mate with *L. psammophilus* queens. By contrast, *L. psammophilus* males are not yet around when *L. niger* queens are mating.

PEARSON (1983) reported that hybrid workers were also morphologically intermediate, which helped him to identify more hybrid colonies than would have been possible with only a single marker locus. No such data are available for the Mols population, but we would expect that a similar gradient might occur. However, we never observed unambiguous F1 hybrids in the Mols population, so that morphological differences might be less distinct if these F1 hybrids are only formed in some years. The overall genotype frequencies in PEARSON (1983) and the present study suggest that F1 hybrids arise in detectible frequencies at Hartland, but that selection against hybrid phenotypes is strong enough to prevent lasting introgression. In contrast, the Mols genotype distributions seem to suggest that F1 hybrids are rarer, but that there is less selection against them so that long-term introgression of low frequencies of *Lasius niger* genes into the *L. psammophilus* genome is possible. In the Mols population where we were able to sample both incipient and mature colonies the frequency of introgressed genes was slightly higher in the former category, which would be as expected from weak selection against hybrid phenotypes, but this difference was not significant.

Our study confirms recent reports that hybridisation in ants may be more common than we have previously appreciated (UMPHREY & DANZMANN 1998, SEIFERT 1999, NONACS 2006, PUSCH & al. 2006, FELDHAAR & al. 2008, SEIFERT & al. 2010, STEINER & al. 2010, BERNASCONI & al. 2011) and underlines that explicit studies combining modern genetic methods with classic morphology might prove to be highly worthwhile. Direct measurements of the timing, biomass and sex ratio of alate production in colonies with introgressed genes could shed light on the fitness consequences of hybridisation, whereas explicit comparisons of worker cuticular-hydrocarbon profiles in pure-bred and hybrid colonies could shed light on the heritability of these compounds (cf. VAN ZWEDEN & al. 2010). Finally, it would be interesting to survey hybridising *Lasius* populations for the presence of cytoplasmatic symbionts such as *Wolbachia*, to see whether any prevalence difference between *Lasius psammophilus* and *L. niger* might be consistent with introgression being asymmetric (FELDHAAR & al. 2008).

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