Available online at www.sciencedirect.com
SCIENCE DIRECT®
Organisms, Diversity & Evolution 4 (2004) 295–305
ORGANISMS DIVERSITY & EVOLUTION
www.elsevier.de/ode

Ideal phenotypes and mismatching haplotypes – errors of mtDNA treeing in ants (Hymenoptera: Formicidae) detected by standardized morphometry

Bernhard Seifert[a,*], Anna V. Goropashnaya[b]

[a]*Abteilung Pterygota, Staatliches Museum für Naturkunde Görlitz, Postfach 30154, 02806 Görlitz, Germany*
[b]*Institute of Arctic Biology, Center for Alaska Native Health Research, 311 Irving I Bldg, University of Alaska Fairbanks, Fairbanks, AK 99775, USA*

Received 18 December 2003; accepted 28 April 2004

Abstract

A total of 401 nest samples of *Formica lugubris* Zetterstedt, *F. pratensis* Retzius, *F. aquilonia* Yarrow, *F. rufa* Linnaeus, and *F. polyctena* Förster, covering the entire Palaearctic range of these species and including 2100 individual workers, was phenotypically investigated by a system of standardized morphometry, pairwise removal of allometric variance, and canonical discriminant functions. A mitochondrial DNA fragment including the cytochrome *b* gene was sequenced in 148 samples from basically the same range. In the more difficult *F. pratensis* vs. *F. lugubris* case, the phenotypic system correctly determined 99.6% of all nest samples, and 95.1% with $p<0.05$. In all other pairwise species discriminations any nest sample was correctly determined with $p<0.01$, and three samples with hybrids *F. rufa* × *lugubris* were identified. At four localities in the Pyrenees and the Urals, 9 samples with *F. pratensis* phenotypes (7 of them ideal) but *F. lugubris* mtDNA haplotypes could be identified, resulting in 14.5% of phenotype/haplotype mismatches. A local dominance of this mismatch combination was observed at one Pyrenean and one Ural locality. There was no indication of an *F. pratensis* haplotype associated with an *F. lugubris* phenotype. One ideal *F. polyctena* phenotype was associated with an *F. aquilonia* haplotype in a sample from the Urals, and one ideal *F. aquilonia* phenotype was combined with an *F. lugubris* haplotype in a sample from central Siberia, resulting in overall phenotype/haplotype mismatch frequencies of 12.5% and 11.1%, respectively. We conclude that all these samples cannot represent actual F_1 hybrids but are the result of hybridizations in the past followed by unidirectional purging of the nuclear genome. Whether this process of purging worked very fast or over longer periods of population history, and whether or not it was complete or incomplete, cannot be assessed from the available information. These facts of hybridizing in two thirds of the W Palaearctic wood ant species, of extreme regional hybrid frequencies (up to 26%), of unidirectional purging of nDNA associated with mismatching mtDNA haplotypes, and of occasional achievement of local dominance of these mismatch combinations, may serve as urgent warning not to perform isolated mtDNA phylogenetic studies without a geographically and locally wide sampling basis and without control by nDNA information or reliable phenotypic determination. The latter two systems definitely have superior significance when conflicts with mtDNA indications arise.

Keywords: *Formica rufa* group; Phylogenetic trees; mtDNA; Haplotype mismatches; Hybridization; nDNA purging

*Corresponding author.
E-mail address: bernhard.seifert@smng.smwk.sachsen.de (B. Seifert).

1439-6092/$ - see front matter © 2004 Elsevier GmbH. All rights reserved.
doi:10.1016/j.ode.2004.04.005

Introduction

We aim to show here that mitochondrial DNA (mtDNA) studies may frequently lead to wrong species indications in ants. For clarity, we first have to state our definition of a species. The biospecies concept used by us defines a species as "a biological unit that passed a threshold of minimal irreversible evolutionary divergence". In our opinion, this concept proposed by the protozoologist Tracy Sonneborn (Sonneborn 1957) is also applicable to Metazoa – regardless of whether they reproduce sexually or parthenogenetically. This concept does not focus on strict reproductive isolation and allows one to consider two hybridizing entities as different species when there are mechanisms saving their evolutionary divergence expressed by gene combinations of different adaptive function. This is, for instance, most probably the case in the wood ant species *Formica rufa* Linnaeus, 1761 and *F. polyctena* Förster, 1850, which show extreme hybridzation frequencies in some special regions but not in many others (Seifert 1991).

In this paper, we identify species completely without use of genetic data. The practical procedure usually starts with identification of reliably separable morphological entities. Such an entity or morphospecies serves as an hypothesis on a biospecies which is then tested by distributional, ecological, ethological and demographic characters. In this way, some morphospecies of *Formica* ants have been downgraded to morphs or ecological races (e.g. Seifert 1992, 2003), others confirmed as good biospecies (Seifert 1996a, 1997, 2000). The taxa considered here, *F. lugubris* Zetterstedt, 1838, *F. pratensis* Retzius, 1783, *F. aquilonia* Yarrow, 1955, *F. rufa* and *F. polyctena*, are currently recognized as separate species by all contemporary wood ant taxonomists. Such consensus is rare in ant taxonomy. The two species with the highest morphological similarity among the workers, *F. lugubris* and *F. pratensis*, strongly differ in habitat selection, vertical distribution, reproductive cycles, response to physical factors, street building behaviour or gyne morphology (Otto 1962; Collingwood 1979; Gösswald 1981; Seifert 1996b).

Inheritance of mtDNA is thought to be basically matrilinear and more or less independent from nuclear DNA (nDNA), although there is growing evidence for transfer of mitochondrial genes into the nucleus (Zhang and Hewitt 1996; Adams et al. 2000; Bensasson et al. 2000, 2003; Berg and Kurland 2000; Williams and Knowlton 2001; Kvist et al. 2003). According to the present knowledge, basic biological traits such as morphology, mechanisms for reproductive isolation, reproductive success, and behavioural or ecological adaptations are largely determined by nDNA and not (or only very little) by mtDNA. In other words, the biological identity of a eukaryotic organism (or that which makes a species) is basically reflected by nDNA and not by mtDNA. However, mtDNA advantages such as clonal inheritance, lack of recombination, and simple organization in animals have led to the situation that phylogenies are more frequently investigated based upon mtDNA markers.

Incongruence between phylogenies derived from mtDNA and species trees derived from either nDNA or morphological evidence has been shown by empirical evidence (Sota and Vogler 2001; Shaw 2002; Machado and Hey 2003) and also predicted by mathematical considerations (Hudson and Coyne 2002; Hudson and Turelli 2003). The biggest threat to the applicability of mtDNA trees comes from interspecific hybridization. In species groups with high frequency of interspecific hybridization, mtDNA phylogenies can dramatically deviate from those indicated by nDNA or morphology. In a study of species in the beetle subgenus *Carabus* (*Ohomopterus*), nDNA generally conformed better with the morphologically defined species, whereas mtDNA results were in considerable conflict with those from either morphology or nDNA (Sota and Vogler 2001). This was explained with a complex process of geographic isolation and hybridization in the Japanese Archipelago, that has led to occasional gene flow and recombination between separated entities. Similarly, in an exemplary study of 23 Hawaiian cricket species of the genus *Laupala*, Shaw (2002) has demonstrated that mtDNA and nDNA phylogenies were largely incongruent. On the other hand, she found a strict consensus of nDNA phylogeny with the morphological concept of Otte (1994), whereas mtDNA and morphology were strongly incompatible. Shaw explained her results by interspecific hybridization being a persistent feature in the history of *Laupala* crickets, and by selection acting on mtDNA haplotypes.

Seifert (1999) has emphasized that hybridization occurs in at least 10% of the Central European ant species, and that hybridizing frequencies in *Lasius*, *Formica* and *Leptothorax* can reach unexpected levels. In addition, he argued that frequent hybridizing does not necessarily dissolve the evolutionary divergence of the *Leptothorax*, *Formica* and *Lasius* species involved, which finally was the main reason to appreciate the species concept of Sonneborn. Recent research on the Central European fauna (this paper; and Seifert, unpublished) has shown that a minimum of 12% of all ant species and a minimum of 60% of *F. rufa* group species can hybridize – sometimes in unexpected frequencies.

We are not prepared to perform here the ideal case of parallel investigation of nDNA and mtDNA, but we can offer a reasonable substitute for nDNA studies: verifiable and standardized morphological investigation (Seifert 2002). Morphology as a reflection of nDNA provides a lot of characters and should plausibly play a major role in phylogenetic treeing. In taxonomic groups

with a high frequency of hybridization, standardized morphological investigation definitely has higher significance than mtDNA indication.

We aim to show here that, in cases of hybridizing species groups and lacking nDNA control, reasonable mtDNA treeing is only possible when (a) it is assisted by a reliable, non-subjective system of morphological species identification, and (b) each species is studied based upon a sufficient number of samples from a large geographical area. Only then can it be detected when samples of a species are associated with an mtDNA haplotype from another species.

Material and methods

Phenotypic identification

A total of 176 nest samples of *F. lugubris* Zetterstedt from W Europe to E Siberia (3°W to 160°E), 91 nest samples of *F. pratensis* Retzius from W Europe to Central Siberia (3°W to 93°E), 64 nest samples of *F. aquilonia* Yarrow from Europe to Central Siberia (9°E to 109°E), 37 nest samples of *F. rufa* Linnaeus from across Europe (3°W to 38°E), and 32 nest samples of *F. polyctena* from Europe to W Siberia (10°E to 61°E) were phenotypically investigated. Depending upon the degree of homogeneity and uncertainty of determination, 3–10 workers per sample were examined. Voucher specimens from these samples are stored in the collection of Staatliches Museum für Naturkunde Görlitz, Germany.

All measurements were made on mounted and dried specimens using a goniometer-type pin-holding device, permitting unlimited rotation around the *X*-, *Y*-, and *Z*-axis. A Wild M10 stereomicroscope equipped with a 1.6× planapochromatic objective was used at magnifications of 50–320×. Mean measuring error is ±2 μm for small and well-defined structures, such as hair length, but may reach 4 μm for measures >1.7 mm affected by difficult positioning and high influence of air humidity. To avoid rounding errors, all measurements were recorded in μm, even for characters for which a precision of ±1 μm is impossible. All metric measurements of body parts (e.g. CW, CL, mPnHL) refer to real cuticular surface and not to the diffuse pubescence surface. Setae, also called pilosity or simply 'hairs', are differentiated from pubescence hairs in having a much larger basal diameter: 4–8 μm in setae vs. 1–2 μm in pubescence. Setae counts and measurements only include setae protruding more than 10 μm from the cuticular surface, and are always unilateral numbers. Data of both body halves were averaged when asymmetries not caused by mechanical damage were obvious. In case of unilateral mechanical ablations, the undamaged half was evaluated.

Morphological characters

CL = maximum cephalic length along median line. The head must be carefully tilted to the position yielding the true maximum. Excavations of occiput and/or clypeus reduce CL.

CS = cephalic size; the arithmetic mean of CL and CW, used as a less variable indicator of body size.

CW = maximum measurable cephalic width, across or behind eyes, whichever is greater.

EyeHL = length of longest hair on eyes.

GuHL = length of longest hair on gula (= venter of head).

nGU = unilateral number of standing setae on gula protruding more than 10 μm from gular profile in lateral view.

nMes = unilateral number of standing setae on mesopleuron protruding more than 10 μm from cuticular surface in dorsal view (the specimen is not turned during counting).

nMet = unilateral number of standing setae on a metapleuro-propodeal area below a straight reference line that is parallel to the straight section of the lower metapleural margin and that touches the lower margin of the propodeal spiracle. Include also the infraspiracular area of the caudal propodeal slope, and take care to visualize the antero-lateral suture between meso- and metapleuron. Setae positioned directly on the suture are counted as 0.5. Definitely excluded from the count are hairs immediately fringing the metathoracal gland, hairs standing on the ventrolateral edge of the metapleuron, and hairs which are very near the petiolar junction.

nOcc = unilateral number of standing hairs protruding more than 10 μm from head silhouette as seen in full face view. Counting begins at level of anterior eye margin and ends at median occiput. The full depth of focus is to be used for counting.

nPn = unilateral number of standing setae on pronotum protruding more than 10 μm from cuticular surface.

nPr = unilateral number of setae on propodeum, above the level of the lower spiracular margin, protruding more than 10 μm from cuticular surface.

nSC = unilateral number of setae on dorsal plane of scape protruding more than 10 μm from cuticular surface.

MetHL = length of longest standing seta on area described for counting of nMet. Use the average of both body sides.

mPnHL = mean pronotal hair length in the worker. Applied measuring schedule: select one of the longest hairs on dorsal pronotum and calculate the arithmetic mean from the lengths of this hair and its six nearest neighbours. Take care to visualize the hair's true base at the cuticular surface; measuring from the diffuse pubescence surface reduces hair length.

OccHL = largest protruding distance of standing setae on occipital margin of head, including the postocular head sides, as seen in the position where maximum CL is measured. This mode of measuring, although not yielding the full setae lengths, was selected to save time.

PEW = maximum width of petiole scale.

SL = maximum scape length excluding articular condyle and its neck.

Smax = maximum scape diameter at midpoint

Removal of allometric variance

Removal of allometry-caused variance was performed by linear functions specific for the species pairs considered – pairwise treatment was preferred because allometric functions differ between the species, and functions valid for more than two species usually provide less accurate corrections (Seifert 2002).

In the case of *F. lugubris* vs. *F. pratensis*, mono- or diphasic procedures were used:

$CL/CW_{cor} = CL/CW/(-0.0992*CS + 1.2788)$ for $CS < 1.915$
$CL/CW_{cor} = CL/CW/(-0.1078*CS + 1.2952)$ for $CS >= 1.915$
$SL/CS_{cor} = SL/CS/(-0.0503*CS + 0.9996)$ for $CS < 1.851$
$SL/CS_{cor} = SL/CS/(-0.1082*CS + 1.1068)$ for $CS >= 1.851$
$PEW/CS_{cor} = PEW/CS/(+0.0007*CS + 0.4678)$ for $CS < 1.749$
$PEW/CS_{cor} = PEW/CS/(+0.0323*CS + 0.4101)$ for $CS >= 1.749$
$SL/SMAX_{cor} = SL/Smax/(-0.2505*CS + 10.46)$
$nSC_{cor} = nSC/(+1.126*CS + 0.92)$
$nOcc_{cor} = nOcc/(+12.78*CS + 1.71)$
$OccHL_{cor} = OccHL/(+49.07*CS + 36.8)$
$mPnHL_{cor} = mPnHL/(+42.98*CS + 34.4)$
$nMet_{cor} = nMet/(+9.12*CS - 4.28)$
$MetHL_{cor} = MetHL/(+69.70*CS + 53.4)$

In the case of *F. aquilonia* vs. *F. polyctena*, only monophasic procedures were used:

$CL/CW_{cor} = CL/CW/(-0.0871*CS + 1.2532)$
$SL/SMAX_{cor} = SL/Smax/(-0.1132*CS + 9.84)$
$OccHL_{cor} = OccHL/(-7.476*CS + 43.0)$
$nGU_{cor} = nGu/(+1.361*CS + 1.07)$
$GuHL_{cor} = GuHL/(-15.985*CS + 100.4)$
$nPn_{cor} = nPm/(+1.830*CS + 1.47)$
$mPnHL_{cor} = mPnHL/(-6.990*CS + 46.02)$
$nMes_{cor} = nMes/(+5.56*CS + 0.85)$
$nMet_{cor} = nMet/(-0.08*CS + 1.02)$
$MetHL_{cor} = MetHL/(-32.51*CS + 107.1)$
$nPr_{cor} = nPr/(+2.70*CS - 0.80)$
$nSc_{cor} = nSc/(-1.060*CS + 5.71)$
$EyeHL_{cor} = EyeHL/(+5.820*CS + 10.01)$

In the case of *F. lugubris* vs. *F. rufa*, monophasic procedures were used:

$CL/CW_{cor} = CL/CW/(-0.1063*CS + 1.2906)$
$SL/CS_{cor} = SL/CS/(-0.0739*CS + 1.0449)$
$nOcc_{cor} = nOcc/(+8.16*CS - 0.80)$
$OccHL_{cor} = OccHL/(+33.70*CS + 13.45)$
$mPnHL_{cor} = mPnHL/(+36.97*CS + 26.55)$
$nMet_{cor} = nMet/(+5.53*CS - 3.33)$
$MetHL_{cor} = MetHL/(+54.09*CS + 72.3)$
$nSc_{cor} = nSc/(+1.404*CS - 0.28)$

In the case of *F. aquilonia* vs. *F. lugubris*, monophasic procedures were used:

$CL/CW_{cor} = CL/CW/(-0.0932*CS + 1.2624)$
$SL/CS_{cor} = SL/CS/(-0.0700*CS + 1.0230)$
$nOcc_{cor} = nOcc/(+9.76*CS - 0.79)$
$OccHL_{cor} = OccHL/(+34.14*CS + 32.2)$
$mPnHL_{cor} = mPnHL/(+22.48*CS + 31.95)$
$nMet_{cor} = nMet/(+5.19*CS - 2.95)$
$MetHL_{cor} = MetHL/(+31.22*CS + 77.0)$
$nSc_{cor} = nSc/(+1.404*CS - 0.28)$

Discriminant analysis

The allometry-corrected data were used for canonical discriminant analysis with the SPSS 10.0 statistical package. Recent tests in several genera belonging to different subfamilies of ants have shown that species-specific removal of allometric variance as proposed by Seifert (2002) does not always improve the performance of discriminant functions significantly (Seifert, unpublished). It is obvious that discriminant functions (such as those provided by SPSS) can compensate for moderate allometric effects in many ant groups. However, in some groups with strong allometries, such as wood ants, removal of allometric variance leads to a substantial increase of discriminative power. Head size, for instance, varied from 1177 to 2184 µm in 900 individuals of *F. lugubris*, from 1177 to 2239 µm in 312 individuals of *F. pratensis*, and removal of allometric variance in the characters SL/CS and CL/CW reduces the coefficient of variation in the corrected values to 77% of that observed in the primary ratios. The effects in some of the pilosity characters were similar.

Apart from discriminative advantages, removal of allometric variance virtually represents some correction against environmental modification of character expression. This is derived from the following considerations. Phenotypes are directed by nDNA, with character expression modified by environmental influence during ontogeny. Ontogeny of a wood ant worker, from the egg to eclosion from the pupa, proceeds inside the thermoregulated, chemically stable environment of the nest core. Hence, environmental modification of character expression in wood ant workers is mainly caused by

differences in quantity and quality of larval nutrition, which primarily causes substantial body-size variation. Since character expression is a function of body-size in most of the characters, removal of allometric variance means some correction against nutritional modification, and some constriction of character expression around its "genetical core" (Seifert 2002).

Genetic identification

We used mtDNA data from two independent studies describing the variation in 65 samples of *F. lugubris*, 60 samples of *F. pratensis*, 8 samples of *F. polyctena*, and 9 samples of *F. aquilonia* (Goropashnaya et al. 2004a, b). Two additional sequences of *F. pratensis* from the Urals were included. Sampling regions generally coincided with those stated above. In total, 34 samples of *F. lugubris* and 28 of *F. pratensis* were studied both phenotypically and genetically. In the less difficult *F. polyctena* vs. *F. aquilonia* and *F. aquilonia* vs. *F. lugubris* cases, where subjective determination frequently is sufficient, only those genetically investigated samples subjectively suspected to have contradicting phenotypes were checked by the complete phenotypic discriminant procedure.

Total genomic DNA was extracted from only the head and mesosoma of single individuals with the DNeasy Tissue Kit (QIAGEN Inc.). An mtDNA fragment including the cytochrome *b* gene was amplified, and both strands were sequenced as described earlier (Goropashnaya et al. 2004a). Successful PCR products were cleaned with the QIAquick Gel Extraction Kit (QIAGEN Inc.) and sequenced on an Applied Biosystems 3100 automated DNA sequencer. In total, 2051 base pairs were scored in 17 individuals, and 1505 base pairs in 127 individuals (GenBank Accession Nos. AY517507–AY517513AY517515AY488759–AY488763 AY488780–AY488783AY573856–AY573896AY584196–AY584233AY604524–AY604525). In order to exclude the possibility of sequencing mitochondrial inserts into the nuclear genome, a 4-kb mtDNA fragment was amplified and sequenced from two *Formica* samples and then aligned with that of the honeybee (see Goropashnaya et al. 2004a). Sequence variation and substitution pattern of the mtDNA fragment were analysed using the program MEGA v. 2 (Kumar et al. 1993). Jukes-Cantor distances (Nei and Kumar 2000) were calculated, and a neighbour-joining tree (NJ) was constructed with the MEGA program.

In order to compare sample positions in the NJ tree of the species pair *F. pratensis*/*F. lugubris* (Fig. 1) with those along the canonical discriminant vector, the distance from the bifurcation point in the NJ tree was selected as the genetic measure and is given in units of 10^{-3} nucleotide substitutions per site, with positive values for *F. pratensis* haplotypes and negative ones for *F. lugubris* haplotypes.

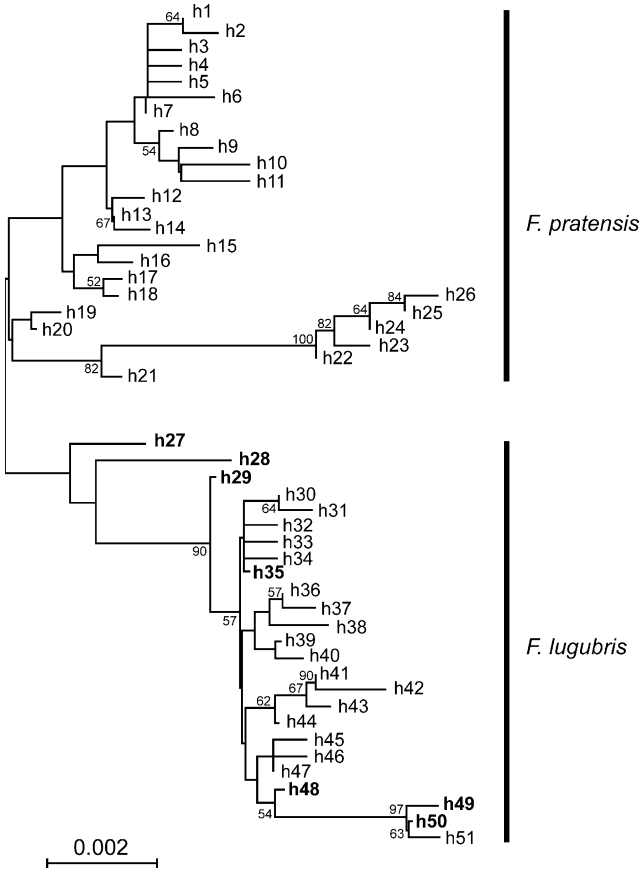

Fig. 1. Neighbour-joining tree of *F. pratensis* and *F. lugubris* mtDNA sequences. Bootstrap percentages with values greater than 50 are shown on nodes. *F. lugubris* haplotypes associated with *F. pratensis* phenotypes are indicated in bold.

Results

The *F. pratensis*/*F. lugubris* case

The most difficult phenotypic separation, the one concerning *F. pratensis*/*F. lugubris*, is treated here in more detail. Discriminatory power was tested in 11 morphometric characters. All six seta characters (nOcc, OccHL, mPNHL, nMet, MetHL) did not substantially contribute to separation in the discriminant functions despite significant differences between the mean values for $mPnHL_{cor}$, $nMet_{cor}$, and $MetHL_{cor}$ (Table 1). This lack of value in characters that are usually valuable in wood ant identification is caused by extreme intraspecific pilosity polymorphism just in the species pair considered here: three pilosity morphs are expressed in *F. lugubris* (Seifert 2003), and two morphs in *F. pratensis* (Seifert 1992). However, five characters, all of them metric

Table 1. Nest sample means of size-corrected values (=allometric variance removed) of 11 morphometric characters and of head size in 267 samples of the wood ants *F. pratensis* and *F. lugubris* from the whole Palaearctic region

	F. pratensis (*n*=91)	*t*	*P*	*F. lugubris* (*n*=176)
CS	1.816±0.149 [1.285, 2.088]	1.47	n.s.	1.794±0.123 [1.336, 2.053]
CL/CW_{cor}	1.005±0.016 [0.973, 1.045]	6.05	0.001	0.994±0.013 [0.926, 1.028]
SL/CS_{cor}	1.018±0.019 [0.979, 1.079]	14.18	0.001	0.982±0.020 [0.966, 1.042]
$SL/SMAX_{cor}$	1.070±0.039 [0.996, 1.178]	32.76	0.001	0.929±0.030 [0.846, 1.005]
PEW/CS_{cor}	0.961±0.042 [0.847, 1.043]	13.26	0.001	1.041±0.049 [0.935, 1.170]
$nOcc_{cor}$	0.924±0.280 [0.198, 1.504]	1.48	n.s.	1.056±0.341 [0.268, 2.587]
$OccHL_{cor}$	0.989±0.223 [0.501, 1.557]	1.41	n.s.	1.022±0.188 [0.600, 1.670]
$mPnHL_{cor}$	1.064±0.122 [0.829, 1.480]	7.91	0.001	0.938±0.124 [0.698, 1.496]
$nMet_{cor}$	1.102±0.200 [0.689, 1.500]	7.28	0.001	0.904±0.216 [0.527, 1.784]
$MetHL_{cor}$	0.968±0.088 [0.809, 1.236]	5.90	0.001	1.036±0.090 [0.776, 1.295]
nSc_{cor}	0.686±0.361 [0.380, 1.805]	3.44	0.01	1.212±1.435 [0.384, 7.468]

t, $p = t$ values and error probability in a *t* test testing the difference of means.

measurements of body parts, proved discriminative when computed in a canonical discriminant function

$$D(5) = 29.90\ \mathrm{SL}/SMAX_{\mathrm{cor}} + 3.36\ \mathrm{CL}/CW_{\mathrm{cor}} - 8.69\ \mathrm{SL}/CS_{\mathrm{cor}} - 6.28\ \mathrm{PEW}/CS_{\mathrm{cor}} - 18.08.$$

$D(5)$ allowed a correct phenotypic distinction in 99.6% of the 267 nest samples of *F. pratensis* and *F. lugubris* throughout the entire Palaearctic range:

F. pratensis phenotype 2.407 ± 1.138 [0.04, 5.01], $n = 91$,

F. lugubris phenotype -2.045 ± 0.921 [−4.406,−0.04], $n = 176$.

95.1 % of all determinations showed error probabilities of $p<0.05$. The only misidentified sample (U59, see Appendix), clearly an *F. pratensis* according to surface structure and haplotype, had morphometric probabilities of 0.649 for *F. lugubris* and 0.351 for *F. pratensis*, with $D(5) = 0.04$.

The separation of mtDNA haplotypes, regardless of which phenotypes they were associated to, was very clear. The NJ values were:

F. pratensis haplotype 5.096 ± 2.097[0.95, 8.09], $n = 53$,

F. lugubris haplotype -5.281 ± 1.108[−8.10, −2.43], $n = 74$.

The most uncertain *F. pratensis* haplotype was significant with $p<0.001$, and the most uncertain *F. lugubris* haplotype with $p<0.042$, if NJ is considered as normally distributed.

As a consequence, we can state both the morphometric and the mtDNA identification system to be most discriminative. Fig. 2 shows the morphometric discriminants and mtDNA haplotype positions for 62 samples in which both phenotype and DNA were evaluated. It is obvious that the nine samples within the first (upper left) quadrant represent striking mismatches between phenotype and haplotype. Each of these nine samples is morphometrically determined as *F. pratensis* and remains so when microsculpture of dorsal head is subjectively assessed. *F. pratensis* shows a deeper and more reticulate microsculpture giving a mat overall surface appearance at magnifications of 60×, whereas in *F. lugubris* microsculpture is slightly flatter and less reticulate, giving a weakly shining overall surface appearance comparable to the situation found in *F. rufa*. The mismatching samples have the following geographic origins (bracketed terms in sample codes designate haplotypes as shown in Fig. 1): P1(h50), P2(h49), P3(h48) from 5 km W of Font Romeu (French Pyrenees), P19(h50) from 11 km SW of Font Romeu; U1(h29), U10(h35), U12(h27) and U21(h35) from Snezhinsk (55.56°N, 60.59°E; Ural region), and U30(h28) from 60 km E Yekaterinburg (56.50°N, 61.36°E; Ural region).

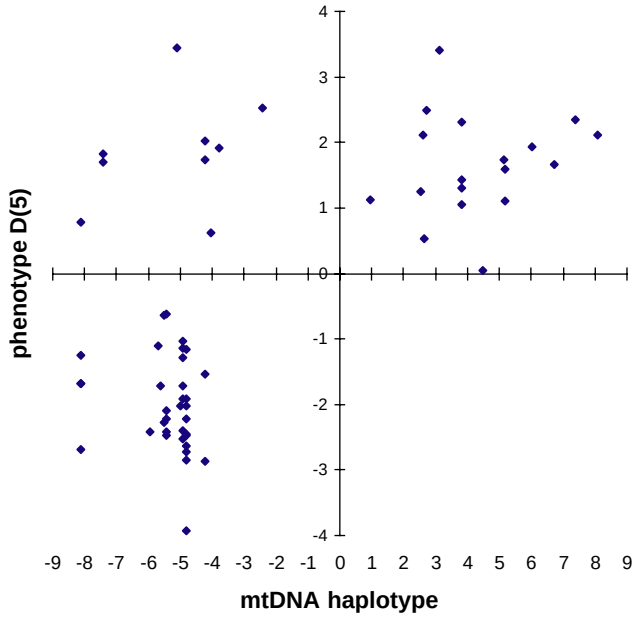

Fig. 2. Plotting of mtDNA haplotype distance (=distance from the bifurcation point in a neighbour joining tree in 10^{-3} nucleotide substitutions per site) against phenotypic distance (=canonical discriminant $D(5)$ considering five morphometric characters).

The *F. polyctena*/*F. aquilonia case*

Separation of *F. polyctena* and *F. aquilonia* was possible based upon worker nest sample means with

$$\begin{aligned} D(16) = {} & 23.99 - 2.056\ \mathrm{CS} + 0.027\ \mathrm{CL}/CW_{\mathrm{cor}} \\ & - 11.88\ \mathrm{SL}/CS_{\mathrm{cor}} - 0.195\ \mathrm{nOcc}_{\mathrm{cor}} \\ & + 0.084\ \mathrm{OccHL}_{\mathrm{cor}} + 0.097 * \mathrm{EyeHL}_{\mathrm{cor}} \\ & + 0.194 * \mathrm{nGu}_{\mathrm{cor}} + 0.005 * \mathrm{GuHL}_{\mathrm{cor}} \\ & - 0.027\ \mathrm{nPncor} - 0.022\ \mathrm{mPnHL}_{\mathrm{cor}} \\ & + 0.011 * \mathrm{nMes}_{\mathrm{cor}} + 0.121\ \mathrm{nMet}_{\mathrm{cor}} \\ & + 0.007\ \mathrm{MetHL}_{\mathrm{cor}} - 0.122 * \mathrm{nPr}_{\mathrm{cor}} \\ & - 1.399 * \mathrm{SL\_}SMAX_{\mathrm{cor}} + 0.273 * \mathrm{nSc}_{\mathrm{cor}}. \end{aligned}$$

All 96 samples were reliably allocated to either species with $p<0.008$:

F. aquilonia	$3.546 \pm 1.065[1.04, 6.30]$,	$n = 64$,
F. polyctena	$-2.802 \pm 0.861[-4.70, -1.04]$,	$n = 32$.

In one sample (U23, Yekaterinburg, 56.51°N, 60.36°E, 1998, leg. A.Goropashnaya), a striking phenotype/haplotype mismatch was observed. This sample, representing an ideal *F. polyctena* phenotype, showed a $D(16)$ of -2.78 and was determined with an error probability of $p<0.001$. Its haplotype, however, was identical to an *F. paralugubris* Seifert haplotype that together with *F. aquilonia* haplotypes formed one tight cluster (Goropashnaya et al. 2004a). Since *F. paralugubris* is not known to occur east of 12°E, this particular *F. polyctena* sample was found 3200 km outside the range of this species. We interpret this haplotype as belonging to *F. aquilonia*.

The *F. aquilonia*/*F. lugubris* case

A separation of *F. aquilonia* and *F. lugubris* was possible in 97.9% of 1118 evaluated worker individuals with $p<0.05$, and in 100% of 210 nest samples with $p<0.004$. The discriminant function based upon nest sample means is

$$\begin{aligned} D(9) = {} & 11.27 + 0.998\ \mathrm{CS} - 2.887\ \mathrm{CL}/CW_{\mathrm{cor}} \\ & - 16.516\ \mathrm{SL}/CS_{\mathrm{cor}} + 0.258\ \mathrm{nSC}_{\mathrm{cor}} \\ & + 1.077\ \mathrm{nOcc}_{\mathrm{cor}} - 1.070\ \mathrm{OccHL}_{\mathrm{cor}} \\ & + 3.526\ \mathrm{mPnHL}_{\mathrm{cor}} + 1.606\ \mathrm{nMet}_{\mathrm{cor}} \\ & + 1.216\ \mathrm{MetHL}_{\mathrm{cor}}, \end{aligned}$$

F. aquilonia	$-3.749 \pm 1.047\ [-5.89, -1.22]$,	$n = 64$,
F. lugubris	$3.233 \pm 0.979\ [1.22, 5.78]$,	$n = 146$.

In sample E5 (Severobaikalsk, 55.38°N, 109.21°E, 1998, leg. V. Semerikov), a striking phenotype/haplotype mismatch was observed. This sample, representing a clear *F. aquilonia* phenotype, showed a $D(9)$ of -2.22 and was determined with an error probability of $p<0.001$. Its haplotype, however, clustered with an *F. lugubris* haplotype from the same locality (Goropashnaya et al. 2004a).

The *F. lugubris*/*F. rufa* case

So far, we have no data in this species pair for associations of ideal phenotypes with mismatching haplotypes, but we give the first evidence for the hybrid combination *F. lugubris* × *rufa*, which is a possible starting point for phenomena as reported above. The separation of *F. lugubris* and *F. rufa* was clearly possible based upon worker individuals with:

$$\begin{aligned} D(8) = {} & 30.106 - 0.667\ \mathrm{CS} - 6.120\ \mathrm{CL}/CW_{\mathrm{cor}} \\ & - 24.407\ \mathrm{SL}/CS_{\mathrm{cor}} + 0.311\ \mathrm{nOcc}_{\mathrm{cor}} \\ & + 2.037\ \mathrm{OccHL}_{\mathrm{cor}} - 1.418\ \mathrm{mPnHL}_{\mathrm{cor}} \\ & + 0.775\ \mathrm{nMet}_{\mathrm{cor}} - 0.044\ \mathrm{MetHL}_{\mathrm{cor}}. \end{aligned}$$

Within 183 samples of pure species identity, 99.3% of 1027 investigated worker individuals could be allocated to either *F. rufa* or *F. lugubris* with $p<0.05$:

F. rufa	$-2.848 \pm 0.0926[-4.70, -0.89]$,
$n = 207$, $P<0.05$,	
doubtful phenotypes	$0.060 \pm 0.309[-0.41, 0.45]$,
$n = 7$, $P>0.05$,	
F. lugubris	$2.870 \pm 0.981[0.54, 6.60]$,
$n = 812$, $P<0.05$.	

Table 2. Individual composition of *F. lugubris* et *F. rufa* nest samples with at least one specimen not safely determinable as either species

	rufa	XX	lugu	*D*(8)
Germany: Ebersbach-Bhf-1.5W-19960612-2	4	1		−2.865
Finland: Lille Halstö-1996.07.07-129	5	1		−1.889
Finland: Eno-14 km E-1987-18	4	1		−1.651
Finland: Lille Halstö-1996.07.07-131	4	1		−1.607
Finland: Tvärminne-1991.05.23-12	**3**	**4**	**2**	**−0.191**
Sweden: Hallamölla-1978.07.27-05		**3**	**7**	**0.667**
Finland: Tvärminne-1991.05.23-43	**1**	**5**	**3**	**1.068**
Switzerland: Jura: 7.14°E, 46.22°N, 2000-21		1	5	1.333
Switzerland: Jura: (Cherix)-1993.05.05-G61		1	5	1.692
Norway: Röros-1996.08.24-1		1	5	1.765

Determinations were performed with a canonical discriminant *D*(8) computing eight characters. Individuals with $p<0.05$ were classified as *F. rufa* (rufa) or *F. lugubris* (lugu), those with $p>0.05$ as doubtful (XX = putative hybrids). *D*(8) is given as arithmetic sample mean.

This allows a detailed interpretation of nest sample compositions. Only 3.8% out of 183 samples of *F. rufa* and *F. lugubris* contained a maximum of one doubtful worker, and the overall frequency of doubtful individuals is 0.69%. There is no indication that any of these samples contain hybrids. Radically different, however, is the composition of three samples from Fennoscandia in which an average of 42.9% of individuals could not be assigned to either *F. rufa* or *F. lugubris* (Table 2: lines in boldface). Since phenotypic copies by other wood ant species can be ruled out, it is reasonable to conclude that these nests contain a high fraction of hybrids, or even hybrid individuals exclusively. We have no genetic data for these hybrid nests.

Discussion

It seems conceivable that an interspecific hybrid between two species A and B may phenotypically copy a third species C – i.e. a hybrid of *Myrmica specioides* Bondroit and *M. rugulosa* Nylander is expected to be phenotypically most similar to *M. hellenica* Finzi (Seifert 1988). However, is it possible to state that an F_1 hybrid between species A and B cannot show an ideal phenotype of either parent? We say yes, since no examples are known in botany or zoology that F_1 hybrids between morphologically distinguished species have ever shown a comprehensive phenotypic character combination inseparable from that of one parent species. It is always only a fraction of characters approaching or being equal to either parent. In ants, an intermediate or almost intermediate character position is frequently found in morphological (Pearson 1983; Seifert 1984, 1991, 1999) or biochemical phenotypes (Pearson 1983; Ross et al. 1987). Hence, an ant showing a clear character combination of *F. pratensis* but the matriline of *F. lugubris* should not have the nuclear genome of an F_1 hybrid of these species. This ant should have, mainly or completely, the nuclear genome of *F. pratensis*.

In the *F. pratensis* vs. *F. lugubris* case, seven out of nine mismatching samples showed ideal *F. pratensis* phenotypes, and only two a trend towards *F. lugubris*. We conclude that all these samples are the result of historic hybridization events between *F. pratensis* males and *F. lugubris* gynes, and of later elimination of *F. lugubris* genes from the nuclear genome. Whether this process of genomic purging worked very fast or over longer periods of population history, and whether or not it was complete or incomplete, cannot be assessed from the available information. The widely used term 'genomic purging' (an Internet search in April 2004 resulted in 959 web pages mentioning this term) is apparently not applied uniformly, but most frequently refers, as in our case, to mechanisms by which the problem of hybrid incompatibility of divergent genomes can be circumvented.

In this context it must be noted that any phenotypic indication presented in the *F. pratensis*/*F. lugubris* case refers to nest samples and not to individual ants. This compromise was necessary because *F. pratensis* and *F. lugubris* are phenotypically too similar to get a clear-cut separation on the individual level. Nests of both species may be polygynous in some localities (Crozier and Pamilo 1996, pp. 114–115) and some frequency of polyandry – which has been shown for ten out of eleven other *Formica* species (reviewed in Strassmann 2001) – may be expected also in *F. pratensis* and *F. lugubris*. Hence, particularly regarding the two doubtful samples, a more complicated composition of nest populations is conceivable, though not very likely in light of data from other wood ants. Nests containing both individuals of

the parent species and true hybrid specimens are reported in 1.2% of 432 German nest samples of *F. rufa*/*F. polyctena* which are high-frequency hybridizers (Seifert 1991).

Our material does not indicate any *F. pratensis* haplotype association with an *F. lugubris* phenotype. The most probable explanation seems to be that the *F. pratensis* haplotype is selected when exposed to the nDNA background of the hybrid genome, or that purging of the nuclear genome is unidirectional towards *F. pratensis*. Other explanations could be that matings of *F. pratensis* gynes with *F. lugubris* males are infertile, or that these partners do not mate at all. Development of local dominance of mismatching haplotypes, as found at one locality each in the Pyrenees and Urals, may be explained through colonization of a new isolated site by a single cross-mated *F. lugubris* gyne, and subsequent crossing of their F_1 to F_n offspring accompanied by unidirectional purging of the nuclear genome. Random events, such as dilution of nuclear *F. lugubris* genes by strong invasion of external *F. pratensis* males, may accelerate the purging process. The overall abundance of phenotype/haplotype mismatches among the 28 *F. pratensis* samples investigated both genetically and phenotypically was 32.1%. This enormous proportion, however, is most probably an overestimation because the parallel phenotypic investigation was biased to problematic samples – i.e. to samples near the bifurcation point of the NJ tree or to those subjectively suspected to have phenotype/haplotype mismatches. The remaining 34 samples not phenotyped by a discriminant analysis most probably did not contain mismatches according to subjective assessment. Hence a realistic proportion of phenotype/haplotype mismatches should be 14.5%.

One phenotype/haplotype mismatch was observed in eight *F. polyctena* samples (U23 from Yekaterinburg, Urals, with an *F. aquilonia* haplotype), and another in nine *F. aquilonia* samples (E5 from Severobaikalsk, Central Siberia, with an *F. lugubris* haplotype), which results in overall mismatch frequencies of 12.5% and 11.1%, respectively. The clear phenotypes of both samples, which are clustered in the centre of either parent species, speak against an F_1 hybrid identity and indicate partial or complete nDNA purging.

Hybridization between *F. rufa* and *F. lugubris* is unknown from Central European countries, where their distribution shows strong vertical separation. In the lowlands of southern Fennoscandia, in contrast, syntopic occurrence of the two species is not rare, increasing the likelihood of hybridization. The extreme habitat mosaic as found at Tvärminne in southern Finland – where as many as six wood ant species of boreomontane, temperate as well as submediterranean origin can be found within a radius of five kilometers – is possibly the best place to study wood ant hybridization and nest mixing. Two hybrid samples of *F. rufa* and *F. lugubris* are from this locality, and Czechowski (1996), without going into details, mentioned at least one example of a mixed nest of *F. lugubris* and *F. rufa*.

The results presented in this paper and those of Seifert (1991) sufficiently document the following interspecific hybridization events in Palaearctic wood ants: *F. rufa* × *F. polyctena*, *F. lugubris* × *F. rufa*, *F. pratensis* × *F. lugubris*, *F. polyctena* × *F. aquilonia,* and *F. aquilonia* × *lugubris*. This means that 56% of the nine W Palaearctic wood ant species hybridize more or less frequently.

These 56% of detected hybridizing species, extreme regional hybrid frequencies of *F. rufa* × *polyctena* of up to 26%, and an overall phenotype/haplotype mismatch frequency of 11–14% as found in three Palaearctic wood ant species with occasional achievement of local dominance of these mismatch combinations, may serve as urgent warning not to perform isolated mtDNA phylogenetic studies without a geographically and locally wide sampling basis and without control by nDNA information or reliable phenotypic determination. The latter two systems definitely have superior significance when conflicts with mtDNA indication arise. The situation in the ant subgenera *Lasius* (*Chthonolasius*) Ruzsky and *Leptothorax* (*Myrafant*) Smith seems comparable to that in wood ants (Seifert 1999, and unpublished data).

Appendix A. Geographic origin of samples specially mentioned in the text

E5: Russia, Severobaikalsk (55.37.652N, 109.20.627E), leg. V. Semerikov, 1998

P1: France, Pyrenees, Font Romeu, 5 km W, leg. T. Monnin, 2000

P2: France, Pyrenees, Font Romeu, 5 km W, leg. T. Monnin, 2000

P3: France, Pyrenees, Font Romeu, 5 km W, leg. T. Monnin, 2000

P19: France, Pyrenees, Font Romeu, 11 km SW, Caldegas, leg. T. Monnin, 2000

U1: Russia, Snezhinsk (55.56N, 60.59E), leg. A. Goropashnaya, 1998

U10: Russia, Snezhinsk (55.56N, 60.59E), leg. A. Goropashnaya, 1998

U12: Russia, Snezhinsk (55.56N, 60.59E), leg. A. Goropashnaya, 1998

U21: Russia, Snezhinsk (55.56N, 60.59E), leg. A. Goropashnaya, 1998

U23: Russia, Yekaterinburg (56.51N, 60.36E), leg. A. Goropashnaya, 1998

U30: Russia, Yekaterinburg, 60 km E (56.50N, 61.36E), leg. A. Goropashnaya, 1998

U59: Russia, Tjumen, 2 km W (57.10N, 65.30E), leg. Belyayev, 2001.

References

Adams, K.L., Daley, D.O., Qiu, Y.-L., Whelan, J., Palmer, J.D., 2000. Repeated, recent and diverse transfers of a mitochondrial gene to the nucleus in flowering plants. Nature 408, 354–357.

Bensasson, D., Feldman, M.W., Petrov, D.A., 2003. Rates of DNA duplication and mitochondrial DNA insertion in the human genome. J. Mol. Evol. 57, 343–354.

Bensasson, D., Zhang, D.-X., Hewitt, G.M., 2000. Frequent assimilation of mitochondrial DNA by grasshopper nuclear genomes. Mol. Biol. Evol. 17, 406–415.

Berg, O.G., Kurland, C.G., 2000. Why mitochondrial genes are found most often in nuclei. Mol. Biol. Evol. 17, 951–961.

Collingwood, C.A., 1979. The Formicidae of Fennoscandia and Denmark. Fauna Entomol. Scand. 8, 1–174.

Crozier, R.H., Pamilo, P., 1996. Evolution of Social Insect Colonies: Sex Allocation and Kin Selection. Oxford University Press, Oxford, New York, Tokyo.

Czechowski, W., 1996. Colonies of hybrids and mixed colonies; interspecific nest takeover in wood ants (Hymenoptera, Formicidae). Memorabilia Zool. 50, 1–116.

Gösswald, K., 1981. Artunterschiede der Waldameisen in Aussehen, Lebensweise, Organisation, Verhalten, Nest- und Straßenbau, Ökologie und Verbreitung. Waldhygiene (Würzburg) 14, 161–192.

Goropashnaya, A.V., Fedorov, V.B., Pamilo, P., 2004a. Recent speciation in the *Formica rufa* group ants (Hymenoptera, Formicidae): inference from mitochondrial DNA phylogeny. Mol. Phylogenet. Evol. 32, 198–206.

Goropashnaya, A.V., Fedorov, V.B., Seifert, B., Pamilo, P., 2004b. Limited phylogeographical structure across Eurasia in two red wood ant species *Formica pratensis* and *F. lugubris* (Hymenoptera, Formicidae). Mol. Ecol. 13, 1849–1858.

Hudson, R.R., Coyne, J.A., 2002. Mathematical consequences of the genealogical species concept. Evolution 56, 1557–1565.

Hudson, R.R., Turelli, M., 2003. Stochasticity overrules the "three-times rule": genetic drift, genetic draft, and coalescence times for nuclear loci versus mitochondrial DNA. Evolution 57, 182–190.

Kumar, S., Tamura, K., Nei, M., 1993. MEGA: Molecular Evolutionary Genetics Analysis, vol. 2. Pennsylvania State University.

Kvist, L., Martens, J., Nazarenko, A.A., Orell, M., 2003. Parental leakage of mitochondrial DNA in the great tit (*Parus major*). Mol. Biol. Evol. 20, 243–247.

Machado, C.A., Hey, J., 2003. The causes of phylogenetic conflict in a classic *Drosophila* species group. Proc. R. Soc. London B 270, 1193–1202.

Nei, M., Kumar, S., 2000. Molecular Evolution and Phylogenetics. Oxford University Press, Oxford, New York.

Otte, D., 1994. The Crickets of Hawaii: Origin, Systematics, and Evolution. The Orthopterists' Society, Academy of Natural Sciences of Philadelphia, Philadelphia.

Otto, D., 1962. Die Roten Waldameisen. Ziemsen-Verlag, Wittenberg-Lutherstadt.

Pearson, B., 1983. Hybridisation between *Lasius niger* and *Lasius alienus*. Insectes Soc. 30, 402–411.

Ross, G., Vander Meer, R.K., Fletcher, D.J.C., Vargo, E.L., 1987. Biochemical phenotyping and genetic studies of two introduced fire ants and their hybrid (Hymenoptera: Formicidae). Evolution 41, 280–293.

Seifert, B., 1984. Nachweis einer im Freiland aufgetretenen Bastardierung von *Leptothorax nigriceps* Mayr und *Leptothorax unifasciatus* (Latr.) mittels einer multiplen Diskriminanzanalyse. Abh. Ber. Naturkundemus. Görlitz 58 (7), 1–8.

Seifert, B., 1988. A taxonomic revision of the *Myrmica* species of Europe, Asia Minor and Caucasia. Abh. Ber. Naturkundemus. Görlitz 62 (3), 1–75.

Seifert, B., 1991. The phenotypes of the *Formica rufa* complex in East Germany. Abh. Ber. Naturkundemus. Görlitz 65 (1), 1–27.

Seifert, B., 1992. *Formica nigricans* Emery, 1909—an ecomorph of *Formica pratensis* Retzius, 1783 (Hymenoptera, Formicidae). Entomol. Fenn. 2, 217–226.

Seifert, B., 1996a. *Formica paralugubris* nov. spec.—a sympatric sibling species of *Formica lugubris* from the western Alps (Insecta: Hymenoptera: Formicoidea: Formicidae). Reichenbachia 31, 193–201.

Seifert, B., 1996b. Ameisen beobachten, bestimmen. Naturbuch-Verlag, Augsburg.

Seifert, B., 1997. *Formica lusatica* n.sp.—a sympatric sibling species of *Formica cunicularia* and *Formica rufibarbis* (Hymenoptera: Formicidae). Abh. Ber. Naturkundemus. Görlitz 69 (5), 3–16.

Seifert, B., 1999. Interspecific hybridisations in natural populations of ants by example of a regional fauna (Hymenoptera: Formicidae). Insectes Soc. 46, 45–52.

Seifert, B., 2000. A taxonomic revision of the ant subgenus *Coptoformica* Mueller, 1923. Zoosystema 22, 517–568.

Seifert, B., 2002. How to distinguish most similar insect species—improving the stereomicroscopic and mathematical evaluation of external characters by example of ants. J. Appl. Entomol. 126, 445–454.

Seifert, B., 2003. The "Hippie Ant"—a case of extreme intranidal polymorphism in Fennoscandian *Formica lugubris* Zetterstedt, 1838 (Hymenoptera: Formicidae). Sociobiology 42, 285–297.

Shaw, K.L., 2002. Conflict between nuclear and mitochondrial DNA phylogenies of a recent species radiation: what mtDNA reveals and conceals about modes of speciation in Hawaiian crickets. Proc. Natl. Acad. Sci. 99, 16122–16127.

Sonneborn, T.M., 1957. Breeding systems, reproductive methods, and species problems in protozoa. In: Mayr, E. (Ed.), The Species Problem, vol. 50. Am. Assoc. Adv. Sci. Publ., pp 155–324.

Sota, T., Vogler, A.P., 2001. Incongruence of mitochondrial and nuclear genes in the carabid beetles *Ohomopterus*. Syst. Biol. 50, 39–59.

Strassmann, J., 2001. The rarity of multiple mating by females in the social Hymenoptera. Insectes Soc. 48, 1–13.

Williams, S.T., Knowlton, N., 2001. Mitochondrial pseudogenes are pervasive and often insidious in the snapping shrimp genus *Alpheus*. Mol. Biol. Evol. 18, 1484–1493.

Zhang, D.-X., Hewitt, G.M., 1996. Nuclear integrations: challenges for mitochondrial markers. Trends Ecol. Evol. 11, 247–251.