No sympatric speciation here: multiple data sources show that the ant *Myrmica microrubra* is not a separate species but an alternate reproductive morph of *Myrmica rubra*

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Abstract

No aspect of speciation is as controversial as the view that new species can evolve sympatrically, among populations in close physical contact. Social parasitism has been suggested to yield necessary disruptive selection for sympatric speciation. Recently, mitochondrial DNA phylogeography has shown that the ant *Myrmica microrubra* is closely related to its host, *Myrmica rubra*, leading to the suggestion that sympatric speciation has occurred. We investigated the relationships between the two ant forms using mitochondrial and nuclear DNA sequences, microsatellite genotyping and morphometrics. Molecular phylogenetic and population structure analyses showed that *M. microrubra* does not evolve separately to its host but rather shares a gene pool with it. Probability analysis showed that mitochondrial DNA data previously adduced in favour of sympatric speciation do not in fact do so. Morphometrically, *M. microrubra* is most readily interpreted as a miniature queen form of *M. rubra*, not a separate species. *Myrmica microrubra* is not an example of speciation. The large (typical *M. rubra*) and small (*M. microrubra*) queen forms are alternative reproductive strategies of the same species. *Myrmica microrubra* Seifert 1993 is consequently synonymized here with *M. rubra* Linnaeus, 1758.

Introduction

Allopatric speciation, the divergence of populations with completely separate geographical ranges (Mayr, 1963), was a dogma of speciation theory over recent decades. Speciation among sympatric populations had already been suggested by Darwin (1859) but was criticized by Mayr (1963) and thereafter considered unrealistic by most evolutionists. Proponents, however, gathered arguments over the following decades (reviewed by Bush, 1975) and in the past 15 years attempts to outline conditions under which sympatric speciation could actually take place have withstood counterarguments (Bush, 1994; Johannesson, 2001; Via, 2001). Sympatric speciation has become a hot topic in evolutionary biology (Berlocher, 2003; Coyne & Orr, 2004). Social parasitism, especially the relationship of workerless social parasites (‘inquilines’) to their hosts, is regarded as one of the conditions favouring this mode of speciation (Buschinger, 1965,1990; Elmes, 1978; Pearson, 1981; Bourke & Franks, 1991). However, notes of caution have been sounded repeatedly for this scenario (Alloway, 1980; Hölldobler & Wilson, 1990; Carpenter et al., 1993; Agosti, 1994;
Choudhary et al., 1994; Ward, 1996) and indisputable model organisms for sympatric speciation in general are still lacking (Schluter, 2001; Coyne & Orr, 2004). The radiation of the order Hymenoptera includes a profuse array of social parasites, but most of the more recent case studies (Choudhary et al., 1994; Baur et al., 1996; Lowe & Crozier, 1997; Sanetra & Buschinger, 2000; Danforth, 2002; Parker & Rissing, 2002; Schwarz et al., 2003; Janda et al., 2004; Sumner et al., 2004) could not exclude alternative interpretations involving allopatric differentiation.

Savolainen & Vepsäläinen (2003) presented two cases of sympatric speciation of socially parasitic ants from their hosts, one of these being Myrmica microrubra. Mitochondrial DNA sequences of both M. microrubra and its host Myrmica rubra segregated by regions (Finland vs. England) but the authors concluded that ‘M. microrubra presumably is an incipient species’ and that different populations are still ‘undergoing lineage sorting, the mtDNA not yet having coalesced. Alternatively, M. microrubra may evolve in parallel in different regions’. Doubts, however, remained due to a low sample size and the plausibility of alternative interpretations (hinted at by Savolainen & Vepsäläinen, 2003 and explicitly outlined by Berlocher, 2003 and Coyne & Orr, 2004). In particular, the finding that M. microrubra and M. rubra mtDNA haplotypes from the same locality are more phylogenetically related than they are to haplotypes of either entity from other sites fails to distinguish between the occurrence of two species as against polymorphism within one species, because only one specimen of each form was sampled per site (Savolainen & Vepsäläinen, 2003). Additional doubts pertain to the nature of M. microrubra as a social parasite and M. microrubra might instead be the miniature queen (microgyne) form of M. rubra (Buschinger, 1997). The morphology, reproductive biology and physiology of M. microrubra have been intensively characterized (Pearson, 1981; Elmes, 1973,1976; Pearson & Child, 1980; Brian, 1986; Cammaerts et al., 1986,1987; Elmes & Brian, 1991). Seifert (1993) formally described the ant as separate species. Today M. microrubra is known throughout most of Europe (17 states, Table S1 in the supplementary web material). All analyses were performed at the population level because M. rubra partly lives in huge supercolonies (Seppä & Pamilo, 1995; Walin et al., 2001; A. Tartally, pers. comm; own observations), i.e. in ‘unicolonia’ societies without colony limits (Wilson, 1971; Crozier & Pamilo, 1996).

**Material and methods**

**Samples**

We collected 116 M. microrubra and 107 M. rubra specimens from all over Europe (49 localities in 9 countries: Table S2, supplementary web material). All analyses were performed at the population level because M. rubra partly lives in huge supercolonies (Seppä & Pamilo, 1995; Walin et al., 2001; A. Tartally, pers. comm; own observations), i.e. in ‘unicolonia’ societies without colony limits (Wilson, 1971; Crozier & Pamilo, 1996).

**Morphometry**

Morphometric analyses were carried out on 141 gynes and 49 males from 47 localities (nine countries), including the entire type series of M. microrubra. Four measurements were made (Table S3, supplementary web material). Dry-mounted specimens were fixed onto a pin-holding goniometer. A Wild M10 stereomicroscope with a 1.6 × planapochromatic lens and a cross-scaled ocular micrometer was used at magnifications of 50–320 ×. Gyne and male data were subjected separately to discriminant analysis using the software package SPSS.

**Mitochondrial DNA**

Twenty-two specimens from six localities were selected from the morphometrics material (Table S2). For DNA extraction the gaster was taken, allowing subsequent morphometric analyses. DNA extractions and PCR with a touchdown program followed the protocols of F. M. Steiner, B. C. Schlick-Steiner, J. C. Trager, K. Moder, M. Sanetra, E. Christian & C. Stauffer (in press), except for cytochrome b apoenzyme (Cytb), where we used the conditions of Savolainen & Vepsäläinen (2003). Mitochondrial cytochrome oxidase subunit I (COI), cytochrome oxidase subunit II (COII) and Cytb primers were those of Savolainen & Vepsäläinen (2003). PCR products were purified (QIAquick PCR purification kit, Qiagen, Hilden, Germany), sequenced in both directions using the Big Dye termination reaction chemistry (Applied Biosystems, Foster City, CA, USA), and analyzed with an ABI 377 automated sequencer (Applied Biosystems).
We studied two outgroup species, namely *M. ruginodis*, the closest relative of *M. rubra* (Savolainen & Vepsäläinen, 2003) and *Manica rubida* a close relative of the genus *Myrmica* (Bolton, 2003).

**ITS1**

The ITS1 of the nuclear coded rDNA was amplified with the primer pair 5′-GAACCTGGAGAAAGTC-3′ and 5′-GTATCCACCGTTCAGGG-3′ (Baur et al., 1996). Forty-three specimens were directly sequenced (Table S2). To evaluate the presence of differing copies of the ITS1 unit within single specimens, one PCR product each of 10 specimens was cloned with the pGEM-T easy vector kit. 1–10 (on average 6) bacterial colonies were picked and sequenced after plasmid extraction.

**Microsatellites**

The microsatellite loci MS 26, MS 86 and MS 3.62, polymorphic in *M. rubra* (Azuma et al., 2005), were genotyped for 5–17 specimens per locality (mean 7.2 ± 2.3 SD for *M. microrubra*, 10.0 ± 4.2 for *M. rubra*, total 86; Tables S2 and S4). We used the amplification conditions and fluorescence detection conditions described by Azuma et al. (2005). Alleles were scored using *Genescan 2.0.1* and *Genotypet 3.7* (Applied Biosystems) software.

**Phylogenetic reconstruction**

mtDNA sequences of the two *M. rubra* and the two *M. microrubra* specimens of Savolainen & Vepsäläinen (2003) were included and sequence alignment was achieved with the default settings of Clustal X (Thompson et al., 1997).

The mtDNA haplotypes found were used to evaluate the 2-species (no haplotypes in common between the forms) and 1-species (all haplotypes held in common) hypotheses for each locality. Difficulties were the low number of sequenced specimens and the unknown extant diversity and frequency of haplotypes per locality and species. We took the occurrence of at least one haplotype common to both ants as an indication to reject the 2-species hypothesis for that locality.

As an approximation, for localities where no common haplotypes were observed the probabilities for these findings under the 1-species hypothesis were estimated under the assumption that all haplotypes are equally frequent at a locality and with equal frequency occur in *M. microrubra* and *M. rubra*. This assumption is restrictive but has to be adopted due to the lack of information on the true relative frequencies of haplotypes – adopting less restrictive assumptions would thus imply calculations for an infinite number of combinations of different frequencies. For a number *h* of extant haplotypes, the probability *a*1 that we find only one specific haplotype when sequencing a number *r* of *M. rubra* specimens can be calculated by \((1/h)^r\). This holds true for every of the *h* haplotypes and we have to multiply *a*1 with \((1/h)^r\). The probability that the haplotypes obtained by sequencing a number *m* of *M. microrubra* specimens are different from the haplotypes found in *M. rubra* is given by \((1–1/h)^m\). Combining these terms results in the probability for finding not more than one haplotype in *M. rubra* and for not finding this haplotype in *M. microrubra*. The probability *a*2 that we find exactly two haplotypes when sequencing *r* can be calculated by \((2/h)^r\), reduced by \((1/h)^r\) to address the case of obtaining the same haplotype twice, already accounted for by *a*1. As we are interested in all combinations of two haplotypes we have to multiply *a*2 with \((1/h)^r\). The probability that the haplotypes obtained by sequencing a number *m* of *M. microrubra* specimens are different from the haplotypes found in *M. rubra* is then given by \((1–2/h)^m\). The product of all these terms estimates the probability for finding not more than two different haplotypes in *M. rubra* and not finding these haplotypes in *M. microrubra*. In a similar way we calculate probabilities for 3, 4,..., *n* haplotypes. The overall probability *P* of finding no haplotype common to *M. rubra* and *M. microrubra* then is calculated by eqn (1):

\[
P = \left( \frac{h}{1} \right)^r (1–1/h)^m + \left( \frac{h}{2} \right)^r \left( \frac{2}{h} \right) (1–2/h)^m + \ldots + \left( \frac{h}{n} \right)^r \left( \frac{n}{h} \right) (1–n/h)^m
\]

\[
n = \begin{cases} r & r < h \\ h-1 & \text{otherwise} \end{cases}
\]

We implemented eqn (1) in a recursion based Java applet (http://homepage.boku.ac.at/h50513/HapPrC/index.html), the core part of which is given in Fig. S1, supplementary web material.

Under the 1-species hypothesis if any haplotypes are observed once only, i.e. are ‘singletons’, then sample coverage theory (Chao & Lee, 1992) tells us that the estimated number of haplotypes is higher than that observed. In cases where no haplotype is seen more than once, the estimate will be very much larger than the observed number. Because the numbers of haplotypes sampled are small, we did not attempt to use the estimates (which have a high level of uncertainty) but instead used plausible numbers of five and ten.

We addressed the question of sample size needed to arrive at a desired probability cut-off for refuting the 1-species hypothesis for localities where no common haplotypes were observed, under the above assumptions (all haplotypes are equally frequent at a locality and with equal frequency occur in *M. microrubra* and *M. rubra*). For the simplest case of analysing equal numbers of
specimens of *M. microrubra* and *M. rubra* we calculated the number needed each of *m* and *r*, using eqn (1), to arrive at number probability values of *P* < 0.05 that, despite no detection, haplotype overlap exists at a locality. We visualized these calculations for numbers between two and ten haplotypes per locality.

Distance (Neighbor Joining algorithm, NJ, based on Tamura-Nei distances) and character (Bayesian analysis using Markov Chain Monte Carlo, MCMC, optimization) analyses were performed using PAUP* (test Version 4.0b3a; Swoford, 1998) and MRBAYES v.3.1 (Ronquist & Huelsenbeck, 2003). Prior to Bayesian analysis the general genealogy structure of the ITS1 genotypes in the investigated *F. m. rubra* and mesosoma length, ML (0.015285 CW + 0.0556 ML) was chosen using MODELTREE 1.06 (Posada & Crandall, 1998), which uses hierarchical likelihood-ratio tests (Huelsenbeck & Rannala, 1997) to determine how well competing substitution models fit the data. For Bayesian analysis, nine partitions according to the three codon positions and the three single genes (*COI, COII, Cytb*) were defined within the data set and all model parameters were estimated separately for the single partitions. 500 000 generations with a sample frequency set to 100 were run twice. As after 250 000 generations stationarity was achieved (average standard deviation of split frequencies below 0.015, dropping to 0.013 towards the end of the run), we used the last 2500 trees of each run to compute a majority rule consensus tree assigning posterior probabilities of tree topology. Tree topology of additional Bayesian analyses with three partitions according to codon positions were identical with topology of analyses using nine partitions, with minor differences in node support values.

For intraspecific genealogies network methods are superior to bifurcating trees, because population phenomena such as persistent ancestral nodes, multifurcations and reticulations are allowed for (Maynard Smith, 1989; Posada & Crandall, 2001). To construct a haplotype network based on a 95% plausible parsimonious set for all haplotype connections, and to calculate outgroup probabilities for each haplotype, we used the TCS 1.13 software developed by Clement et al. (2000). Standard nesting rules were applied to the haplotype network (Templeton & Sing, 1993).

Due to the presence of genetically distinct ITS1 units within some single specimens, ITS1 sequences could not be subjected to phylogenetic analyses. Frequency of occurrence of distinct types of ITS1 sequences in the investigated *M. microrubra* and *M. rubra* samples was analyzed.

**Population structure analysis**

Hardy–Weinberg proportions were tested for the three microsatellite loci using exact tests at each locality, for *M. rubra* and *M. microrubra* separately (GENEPOP 3.2; Raymond & Rousset, 1995). Bonferroni correction for multiple comparisons was applied (Benjamini & Hochberg, 1997). Linkage disequilibrium between pairs of loci was tested for each locality using GENEPOP 3.2. Pairwise *F*<sub>ST</sub> estimates between sites and the overall *F*<sub>ST</sub> were computed using ARLEQUIN 2.0 (Schneider et al., 2000). We used an analysis of molecular variance (AMOVA, Excoffier et al., 1992) to assess the distribution of microsatellite and mtDNA variation. A nested three-level AMOVA was performed by partitioning the total sum of squares into components representing variation across localities, between *M. microrubra* and *M. rubra* of each locality and within *M. rubra* and *M. microrubra* of each locality separately, using ARLEQUIN 2.0. To study haplotype differentiation, this method also yields analogues to *F*-statistics (termed *Φ*-statistics), for which we used the number of pairwise differences between haplotypes as Euclidean distance measure. Significance was determined by comparing the observed values to a null distribution generated by permutation. The significance of the Pearson correlation coefficient between genetic differentiation of microsatellites (*F*<sub>ST</sub>) and geographical distance was assessed with a Mantel test (IBD; Bohonak, 2002).

Analysis of multilocus genotypes allows inference of genetic ancestry without relying on information about sampling locations, even when among-population variance components are small. We used a model-based clustering algorithm implemented in STRUCTURE (Pritchard et al., 2000) that identifies subgroups with distinctive allele frequencies. The program places specimens into K clusters, where *K* is established in advance but can be varied between runs of the algorithm. We carried out three analyses. The first analysis combined all the data and used *K* = 2, to test for the overall existence of two species. The second examined each of the five localities separately, with *K* = 2 for each locality. The third combined all data with *K* = 5, to test the tendency of the specimens to assort into five clusters. All runs used 100 000 iterations after a burn-in of 900 000.

**Results**

**Morphometry**

The discriminant function *D*<sub>1</sub> based on head width, CW and mesosoma length, ML (0.015285 CW + 0.0556 ML) resulted in a clear segregation of *M. microrubra* from *M. rubra* (Fig. 1; 100% classified with *P* = 0.012). For males, using the discriminant function *D*<sub>2</sub> based on scape length, SL, head length, CL, head width and mesosoma length (36.740 SL – 34.239 CL + 17.264 CW + 0.983 ML) the clustering was less pronounced (86.0% with *P* < 0.05).

**Phylogeny**

Excluding the noncoding region, the sequence alignment of *COI, COII* and *Cytb* consisted of 2724 bp. Sequences
Population structure

Genotyping 86 specimens for the three microsatellite loci yielded 11 alleles (Table S4, supplementary web material). Alleles were widely distributed geographically. All alleles were found in both *M. microrubra* and *M. rubra*, but the alleles present differed between the two ants for individual localities (Table S4). But on average the proportion of alleles found at a locality found in both forms was high, 80.0 ± 27.4%. Hardy–Weinberg proportions were rejected for five site-locus combinations (*P* < 0.05), and after a Bonferroni multiple-comparisons correction four of these remained significant. The latter did so because of an excess of homozygotes (from *Fis* = 0.238 to *Fis* = 0.727 for these combinations). Linkage was not detected for the three loci, and hence they were assumed to yield independent information.

The overall estimate of *FST* = 0.144, which measures all effects of population substructure combined, indicated genetic differentiation at the microsatellite loci. Pairwise *FST* values ranged from 0.036 to 0.359 within *M. microrubra*, from 0.033 to 0.304 within *M. rubra*, and from −0.034 to 0.407 for *M. microrubra/M. rubra* pairs, but 18 values (40%) were not significant at *P* < 0.05 (six within *M. microrubra*, two within *M. rubra*, ten for *M. microrubra/M. rubra*). The mean of all pairwise *FST* values was 0.172 ± 0.108, and 0.237 ± 0.085 after excluding nonsignificant values.

Exploration of isolation by distance effects suggested no difference between *M. microrubra* and *M. rubra*. The plot (Fig. 3) displayed three clusters: (i) very small to medium *FST* values between *M. microrubra* and *M. rubra* within localities (left); (ii) values in the same range representing among-site variation within *M. microrubra*, within *M. rubra* and between the two for geographical distances of 20 km (centre); and (iii) the most pronounced range of genetic variation over large geographical distances, from very small to very large (right). A matrix comparison using Mantel test showed no significant association between genetic differentiation and geographical distance (*r* = −0.014, *P* = 0.476); in addition, no differences existed between pairwise values within *M. microrubra*, within *M. rubra*, or between the two (partial correlation *r* = −0.002, *P* = 0.508).

*AMOVA* revealed that the genetic component of microsatellite variation within *M. microrubra* and within a locality was high. The haplotype network revealed multifurcations (data not shown).

Three ITS1 types were detected (I–III, 741–771 bp; Table 2) and the sequences were deposited in GenBank (DQ075936–DQ075938). Two types were very often found within one specimen (7 of the 10 cloned specimens). All types were found in *M. microrubra* and *M. rubra*. At one site (locality 6) all three types occurred. No distinct geographical pattern was apparent for the three types.
M. rubra of single localities, estimated at 82.38% (Table 3), accounted for most of the genetic diversity. Variation between M. microrubra and M. rubra within localities was 16.56%, variation among localities 1.06%.

In stark contrast, AMOVA for mtDNA revealed that 45.99% of genetic variation was due to variation within M. microrubra and M. rubra of single localities; this test produced a negative value of −10.24% between M. microrubra and M. rubra within localities, and 64.25% among localities. Negative values for variation components indicate a lack of population structuring at the respective hierarchical level.

### Table 1
**Probabilities of obtaining the observed result of no mtDNA haplotypes in common between forms for four localities, under the 1-species hypothesis (i.e. that in fact all haplotypes at a locality occur in both forms).**

<table>
<thead>
<tr>
<th>Locality</th>
<th>m/r</th>
<th>HT_{obs}</th>
<th>HT_{true}</th>
<th>5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1/1</td>
<td>2</td>
<td>0.500</td>
<td>0.800</td>
<td>0.900</td>
</tr>
<tr>
<td>2</td>
<td>1/2</td>
<td>3</td>
<td>0.440</td>
<td>0.640</td>
<td>0.810</td>
</tr>
<tr>
<td>4</td>
<td>4/1</td>
<td>2</td>
<td>0.063</td>
<td>0.448</td>
<td>0.656</td>
</tr>
<tr>
<td>8</td>
<td>1/2</td>
<td>3</td>
<td>0.444</td>
<td>0.640</td>
<td>0.810</td>
</tr>
</tbody>
</table>

The numbers of Myrmica microrubra (m) and Myrmica rubra (r) are shown; HT_{obs} is the number of haplotypes observed and HT_{true} is the true number under three assumptions. The probabilities are those from eqn 1 and the collection sites are given in Fig. 2.

### Table 2
**Distribution of ITS1 types between localities and individuals of Myrmica microrubra and Myrmica rubra, with the numbers of specimens showing the various combinations given in parentheses.**

<table>
<thead>
<tr>
<th>Locality</th>
<th>ITS1 type in M. microrubra</th>
<th>M. rubra</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>n.a.</td>
<td>II(1)</td>
</tr>
<tr>
<td>3</td>
<td>II(2)</td>
<td>II(2)</td>
</tr>
<tr>
<td>4</td>
<td>II(7), II + III(1)</td>
<td>II(4)</td>
</tr>
<tr>
<td>5</td>
<td>II(1), II(4)</td>
<td>II(2), II(1)</td>
</tr>
<tr>
<td>6</td>
<td>II(5), II + III(1)</td>
<td>II(2), II(1)</td>
</tr>
<tr>
<td>8</td>
<td>II(2)</td>
<td>II(1), II(1)</td>
</tr>
<tr>
<td>10</td>
<td>II(1)</td>
<td>II(2), II(2)</td>
</tr>
</tbody>
</table>

Collection sites are given in Fig. 2a; n.a. = no material available.

When localities were dealt with individually, the model-based clustering algorithm did not separate *M. microrubra* and *M. rubra* specimens (Fig. 4). Similarly, no discrimination was apparent when all specimens were analyzed together, neither for a clustering according to species identity (*K* = 2), nor for geographical origin (*K* = 5).

**Discussion**

The morphometric analyses on a larger sample size confirm Sellet’s (1993) finding that gynes of *M. microrubra* and *M. rubra* are well separated based on absolute size. The morphological separation of males, however, now appears to be less clearcut. The morphological situation in *M. microrubra* and *M. rubra* resembles that in ant species with dimorphic queens (McInnes & Tschinkel, 1995), e.g. the case of *Tetramorium moravicum* where the description of microgyne species had to be retracted recently (Schlick-Steiner et al., 2005). The morphometric results alone are thus insufficient to decide whether *M. microrubra* is a separate, socially parasitic species (2-species hypothesis) or the microgyne form of *M. rubra* (1-species hypothesis).

The combined molecular data are decisive, as we now show. The two ants were found to share three of 18 mtDNA haplotypes, at three localities. Although at these three localities additional haplotypes were found in exclusively one of the two ants, this does not conflict with all haplotypes actually being common to both ants, as the numbers of sequenced specimens were small. Probability calculations for the four sites where no common haplotypes were found demonstrate that sample size per locality was too small to refute the 1-species hypothesis for these sites at a *P* < 0.05 (Fig. S2) – this likewise holds true for the data presented by Savolainen & Vepsäläinen (2003) which were interpreted in favour of sympatric speciation, i.e. in favour of the 2-species hypothesis according to our terms. In fact, although for our four localities where no common haplotypes were detected the probabilities for these results under the 2-species hypothesis are *P* = 1.0, the failure to find any

**Table 3 Analyses of Molecular Variance (AMOVA) for microsatellites and mtDNA.**

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Microsatellites</th>
<th>mtDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of total variation</td>
<td><em>P</em></td>
</tr>
<tr>
<td>Between localities</td>
<td>4</td>
<td>1.06</td>
</tr>
<tr>
<td>Between <em>M. microrubra</em> and <em>M. rubra</em> within localities</td>
<td>5</td>
<td>16.56</td>
</tr>
<tr>
<td>Within <em>M. microrubra</em> and <em>M. rubra</em> of single localities</td>
<td>162</td>
<td>82.38</td>
</tr>
</tbody>
</table>

The *P*-values give the probability that a random value from 10 000 permutations is more extreme than the observed variance component and F- and *ϕ*-statistics.


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common haplotype also is entirely compatible with the 1-species hypothesis (Table 1): given that we obtained up to four haplotypes by sequencing five specimens per locality (locality 5), considerable haplotype diversities per locality may be assumed from sample coverage theory. For example, if there are 10 haplotypes per site (a number compatible with the observations), and these are equally frequent then the probability of not finding a haplotype in common for our sample sizes are very high (from \( P = 0.656 \) to \( P = 0.900 \)) and thus compatible with the 1-species hypothesis. The substantial haplotype diversity within both \( M. \) microrubra and \( M. \) rubra at some localities despite small sample sizes is remarkable itself. This finding helps explain the genetic differences between \( M. \) microrubra and \( M. \) rubra reported by Savolainen & Vepsäläinen (2003): the differences within \( M. \) rubra are comparable to those between the two ants, which is compatible with \( M. \) microrubra being a morph of \( M. \) rubra. On the other hand, haplotype diversity within localities renders parallel speciation (reviewed by Johannesson, 2001) as inferable from mtDNA data (Savolainen & Vepsäläinen, 2003) implausible, since this would suggest multiple evolution of \( M. \) microrubra within localities. The interlaced phylogenetic pattern of the two ants as inferred by all methods of phylogenetic reconstruction reveals no indication of \( M. \) microrubra evolving separately from \( M. \) rubra. Like mtDNA, the analysis of ITS1 showed variability within \( M. \) microrubra and \( M. \) rubra, but no inherent differentiation of the two ants was detected. The microsatellite analysis revealed sufficient differentiation, within the whole sample and between single localities, as measured by the number of extant alleles and by the overall fixation index, as measured by the number of extant alleles and by the overall fixation index.

AMOVA allows considerations beyond the question of one or two species. AMOVA of the microsatellite data show that the largest portion of genetic differentiation is made up by variation within the populations of \( M. \) rubra consisting of macro- and microgyynes, which is likewise reflected by the lack of association between genetic and geographical distance by the Mantel test. Nevertheless, some differentiation at the population level is suggested by AMOVA. This is not in conflict with the 1-species hypothesis because the gene pools of macro- and microgyynes in other ants with dimorphic queens or with social forms differing in queen number are separated to a similar or even greater degree (Rüppell et al., 2001, 2003; Ross et al., 1997; Goodisman et al., 2000; Gyllenstrand et al., 2005). Different mating strategies (microgyynes inside and macrogyynes outside the nest; Buschinger, 1997) resulting in assortative mating probably cause a partial separation of the gene pools, as claimed for the micro- and macrogyne forms of \( M. \) ruginodis, the closest relative of \( M. \) rubra (Elmes, 1991). However, \( M. \) rubra macrogyynes can also mate inside the nest (B. Seifert, in press), and microgyynes have repeatedly been observed to join mating swarms of macrogyynes (Czechowski et al., 1999; Czechowski & Czechowska, 2002) despite their decreased flying abilities due to smaller mesosoma size (Stille, 1996).

Microgyynes of \( M. \) rubra are promising study subjects for the origin and maintenance of queen dimorphisms, a significant problem in the developmental biology of ants. This mechanism could either be exclusively genetic as established for queen polymorphism (Winter & Buschinger, 1986), queen-worker dimorphism (Volny & Gordon, 2002), and variation in queen number (Krieger & Ross, 2002) in other ants, or could be due to a combination of genetic and environmental effects as in worker polymorphism (e.g. Fraser et al., 2000; Hughes et al., 2003) and in one case of gradual queen size variation (Bargum et al., 2004). Significant environmental influences might include nutritional and climatic conditions, which can influence larval development shortly before pupation even when these are brief (Brian, 1956). Such short-period influences could affect early and late broods differently and this would explain the...
absence of microgynes in *M. rubra* populations in some years despite their being abundant in others (A. Buschinger, unpubl. data). The two morphs can thus be seen under the Evolutionarily Stable Strategy framework as conditional strategies (reviewed by Gross, 1996) in that good conditions enable development as large queens capable of independent colony foundation whereas poor conditions only allow development as small queens, which must enter established colonies to survive.

Our findings highlight the necessity of in depth analysis of promising examples of sympatric speciation, although we have not addressed the general importance of sympatric speciation in the origin of inquilines (Buschinger, 1965; Elmes, 1978; Pearson, 1981; Buschinger, 1990; Bourke & Franks, 1991).

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### References


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**Supplementary material**

The following supplementary material is available for this article online:

**Fig. S1** Core part of the recursion based Java applet implementing eqn (1).

**Fig. S2** Probability $P$ under the 1-species hypothesis to not see haplotypes common to *Myrmica microrubra* and *M. rubra* when sequencing equal numbers of specimens of each ant, for numbers between two and ten extant haplotypes per locality, calculated using eqn (1) under the assumption that all haplotypes are equally frequent at a locality and with equal frequency occur in *M. microrubra* and *M. rubra*.

**Table S1** Geographical distribution of the ant *Myrmica microrubra*.

**Table S2** Collecting sites and numbers (n) of specimens analyzed of *Myrmica microrubra*, *M. rubra* and the outgroup species *M. ruginodis*, for the different disciplines; type and paratype specimens marked *; locality code given for sites analyzed genetically.

**Table S3** Abbreviations and definitions of the morphometric characters analyzed in *Myrmica microrubra* and *M. rubra* gynes and males.

**Table S4** Genetic diversity of three microsatellite loci (MS 26, MS 86, MS 3.62) analyzed in *Myrmica microrubra* and *M. rubra* from five localities. This material is available as part of the online article from http://www.blackwell-synergy.com