Insect. Soc. 52 (2005) 186–193
0020-1812/05/020186-08
DOI 10.1007/s00040-004-0793-6


Insectes Sociaux

Research article

# Queen size dimorphism in the ant *Tetramorium moravicum* (Hymenoptera, Formicidae): Morphometric, molecular genetic and experimental evidence

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Received 10 June 2004; revised 28 October 2004; accepted 12 November 2004.

**Summary.** By combining different methods we evaluate whether the ant *Tetramorium rhenanum* is specifically separated from *T. moravicum* or whether it is a conspecific microgyne form. High-precision morphometry shows a clear difference in queen size. Sequence comparison of 1031 bp of COI reveals that *T. rhenanum* falls into a clade with *T. moravicum,* which is significantly separated from *T. forte* and *T. chefketi*. *T. rhenanum* shares at least two haplotypes with *T. moravicum* and is considered as a junior synonym. Sexual behaviour and colony foundation experiments corroborate conspecificity. The queen dimorphism is discussed in the context of social parameters such as queen number and reproductive strategy.

*Key words*: morphometry, mtDNA, mating behaviour, colony foundation, *Tetramorium rhenanum*.

## Introduction

In ants, queen number per colony can vary from monogyny to polygyny within and between species and some species are able to produce more than one queen morph (Buschinger, 1974; Hölldobler and Wilson, 1990). Gynomorphic queens can exhibit significant size dimorphism: the so-called macrogynes and microgynes. The mating and dispersal strategies of ants range from aerial mating during a nuptial flight with a tendency toward long-distance dispersal, to intranidal copulation, which is often associated with remaining close to the mother nest (Bourke and Franks, 1995 and references therein). Colony foundation may be independent of the assistance of workers or dependent on existing colonies (Hölldobler and Wilson, 1990). Although a number of different combinations are known, macrogynes frequently mate on a nuptial flight, disperse over long distances, found their colonies independently and maintain monogynous colonies; microgynes often mate within the nest, stay in their maternal colony and found dependently by worker assistance (e.g. Bourke and Franks, 1995; Stille, 1996; Rüppell and Heinze, 1999; Rüppell et al., 2001). Microgynes frequently occur in polygynous societies that propagate by budding.

Within the myrmicine genus *Tetramorium* taxonomic and phylogenetic relationships are far from being fully resolved (Sanetra and Buschinger, 2000). The two palearctic species *Tetramorium moravicum* and *T. rhenanum* are morphologically close, based on the strong body sculpture in workers, especially on the petiolar and postpetiolar nodes. They were also found to be closely related using allozyme markers but were not grouped together with the similar species *T. forte* Forel, 1904 and *T. chefketi* Forel, 1911 (Sanetra et al., 1994; Sanetra and Buschinger, 2000). The mainly East and Central European distribution area of *T. moravicum* ranges from Transcaucasia to the Pannonian basin, the Alps and the Balkan Peninsula (Sanetra and Buschinger, 2000; Steiner et al., in press). *T. rhenanum* was described from a small and geographically separated population in the German Rhine valley (Schulz, 1996). Differentiation against *T. moravicum* was based on smaller size and denser sculpture both on the mesonotum and scutellum in the gyne, and on biogeographic reasoning. Seifert (1996) additionally ascribed some discriminative value to absolute worker head size. In his checklist of German ants Seifert (2001) synonymised *T. rhenanum* and *T. moravicum* without comment.

Social parameters of *T. moravicum* and *T. rhenanum* are best known from several locations in western Germany. *T. rhe-*

*nanum* occurs in highly polygynous colonies in the Rhine, Mosel and Nahe valleys (Felke and Sanetra, 1997; Rohe and Heller, 2000; Güsten and Heller, unpubl.). *T. moravicum* frequently maintains monogynous colonies, but in the Nahe valley and in the Kaiserstuhl area some polygynous colonies were observed (Rohe and Heller, 2000; Sanetra, unpubl.).

The present study was designed to discriminate between two hypotheses: *T. rhenanum* has reached a degree of divergence that makes its specific separation from *T. moravicum* plausible vs. *T. rhenanum* and *T. moravicum* are conspecific, "*rhenanum*" being the microgyne form. In order to establish this we combine morphometry, molecular genetics and behavioural ecology.

## Material and methods

We studied individuals from 51 *Tetramorium* colonies from eight European countries (Austria, AU; Czech Republic, EZ; France, FR; Germany, GM; Greece, GR; Slovak Republic, LO; Spain, SP; Ukraine, UP) and *T. semilaeve* André, 1883 as outgroup. Samples from the type localities of *T. rhenanum* (Lorchhausen, Rhine valley, Germany) and *T. moravicum* (Mohelno, Czech Republic) were included. Gynes of *T. rhenanum* and *T. moravicum* were determined according to Seifert (1996), workers were separated according to their cephalic size (CS = the arithmetic mean of cephalic length and width): colonies with CS < 806 µm as *T. rhenanum*, with CS > 806 µm as *T. moravicum*. *T. forte* and *T. chefketi* were identified by comparison with type specimens (Muséum d'Histoire Naturelle, Genève). Since the type material of *T. semilaeve* probably consists of several species (Sanetra et al., 1999), we compared with specimens of *T. semilaeve* from a population in Banyuls-sur-Mer, France, which was defined as typical by Bondroit (1918).

### *Morphological analysis*

For morphological analyses we considered only those 40 colonies of *T. rhenanum* and *T. moravicum* of which a sufficient number of individuals were available (Table 1). Five *T. rhenanum* samples and 15 *T. moravicum* samples contained gynes.

Dry-mounted specimens were fixed on a pin-holding goniometer that permitted rotations around three axes. A Wild M10 high-performance stereomicroscope with a 1.6 × planapochromatic lens and a cross scaled ocular micrometer was used at magnifications of 50–320 ×. The

**Table 1.** *Tetramorium* species included in the morphometric (morph; n(i) = numbers n analyzed colonies and i analyzed workers and gynes) and molecular genetic analyses (mol; n = number analyzed colonies); haplotypes (HT) of *T. moravicum* and *T. rhenanum* numbered; locality given (for country codes see Material and methods)

| Species | Locality | morph: n(i) workers | morph: n(i) gynes | mol: n; HT |
|---|---|---|---|---|
| *T. chefketi* Forel, 1911 | GR: Pieria, Litohoro | | | 1 |
| | GR: Stena Petras | | | 1 |
| *T. forte* Forel, 1904 | SP: Cuenca, Embalse de la Toba | | | 1 |
| | SP: Huelva, Donana National Park | | | 1 |
| | SP: Teruel, Albarracin | | | 1 |
| *T. semilaeve* André, 1883 | SP: Sant Cugat | | | 1 |
| *T. moravicum* Kratochvil, 1941 | AU: Gobelsburg | 1(8) | 1(3) | 1; HT7 |
| | AU: Hackelsberg | 2(6) | 2(6) | 1; HT7 |
| | AU: Hundsheimer Berg | 4(12) | 4(7) | 4; HT7 |
| | AU: Paudorf | 1(4) | | |
| | AU: Spitz an der Donau | 1(4) | | |
| | AU: Staatz | 1(4) | 1(2) | |
| | EZ: Mohelno | | | 1; HT1 |
| | EZ: Praha | 1(4) | | |
| | EZ: Satov | 3(12) | | |
| | FR: Chazey sur Ain | | | 1; HT2 |
| | GM: Nahe valley, Dorsheim | 1(4) | 1(6) | |
| | GM: Nahe valley, Norheim | 3(15) | 1(3) | 2; HT2, HT4 |
| | GM: Nahe valley, Schloßböckelheim | 3(9) | 3(13) | |
| | GM: Kaiserstuhl, Vogtsburg | 1(3) | 1(2) | |
| | GR: Fokida, Kaloskopi | 1(3) | | 1; HT6 |
| | GR: Ioannina, Kipi | 1(3) | | 1; HT6 |
| | LO: Medovarce u Krupina | 1(4) | 1(3) | |
| | UP: Crimea, Catyr Dah | 1(3) | | |
| | UP: Crimea, Simferopol | 1(3) | | 1; HT5 |
| *T. rhenanum* Schulz, 1996 | FR: Alpes maritimes, vic. Maurioun | 2(11) | | 2; HT2 |
| | GM: Nahe valley, Dorsheim | 3(12) | 1(4) | 2; HT3, HT4 |
| | GM: Nahe valley, Schloßböckelheim | 1(3) | | |
| | GM: Rhine valley, Lorchhausen | 2(8) | 2(4) | 1; HT2 |
| | GM: Rhine valley, Lorch | 2(8) | 2(2) | |
| | GM: Kaiserstuhl, Badberg | | | 1; HT2 |
| | GM: Kaiserstuhl, Bitzenberg | 2(8) | | |
| | GM: Kaiserstuhl, Schelingen | | | 1; HT2 |
| | GM: Kaiserstuhl, Vogtsburg | 1(4) | | |

highest magnification that kept a structure within the range of the ocular micrometer was used. In order to reduce reflections of the cuticular surfaces and to improve visualization of the microsculpture, a plastic diffuser was positioned as close as possible to the specimen.

Morphometric characters:
CL: Maximum cephalic length along the median line. The head must be carefully tilted to the position with the true maximum. Incisions of occiput and/or clypeus reduce CL.
CS: (CL + CW)/2, as a less variable indicator of body size.
CW: Maximum head width across eyes.
dCAMN: Mean distance of carinulae on dorsal plane of mesonotum.
ML: Mesosoma length of alates from caudalmost part of mesosoma (found either on median propodeum or caudal metapleuron) to steep frontal profile of pronotum as measured in lateral view.
MW: Maximum mesosoma width of alates in front of the tegulae.
PEW: Maximum width of petiole.
PPW: Maximum width of postpetiole.
SIZE: Geometric mean of ML and CW.
SL: Maximum straight line scape length without articular condyle.
To increase the discriminative power, the values of some characters (indexed "$_{cor}$") were corrected for size-dependent variance. The following functions were derived in the mode described by Seifert (2002).

Gynes:
$CW_{cor} = (CW/SIZE)/(+0.000016178 * CS + 0.7454)$
$ML_{cor} = (ML/SIZE)/(-0.000027321 * CS + 1.3406)$
$dCAMN_{cor} = (dCAMN/SIZE)/(-0.000017810 * CS + 0.04799)$

Workers:
$CL/CW_{cor} = (CL/CW)/(-0.000066000 * CS + 1.0656)$
$SL/CS_{cor} = (SL/CS)/(-0.000033222 * CS + 0.7873)$
$MW/CS_{cor} = (MW/CS)/(+0.000141169 * CS + 0.5298)$
$PEW/CS_{cor} = (PEW/CS)/(+0.000102579 * CS + 0.2335)$
$PPW/CS_{cor} = (PPW/CS)/(+0.000072033 * CS + 0.3241)$

Having size-dependent variance eliminated, canonical discriminants were established. For gynes: $Dg = -244.389 - 0.02 * SIZE + 142.119 * CW_{cor} + 133.669 * ML_{cor} + 1.167 * dCAMN_{cor}$; for workers: $Dw = +0.025CS + + 24.038CL/CW_{cor} + 12.563SL/CS_{cor} + 16.130MW/CS_{cor} - 0.232PEW/CS_{cor} - 2.537PPW/CS_{cor} - 69.882$.

Subsequently, canonical analyses were performed for the gyne data set excluding SIZE, and for the two data sets under the assumption of an alternative hypothesis generated by a cluster centre analysis (existence of two groups assumed). The correlation of sample means of worker CS and gyne SIZE was calculated.

### *Molecular genetic analysis*

26 individuals from 26 *Tetramorium* colonies were analyzed genetically: two *T. chefketi*, three *T. forte*, 13 *T. moravicum*, seven *T. rhenanum* and one *T. semilaeve* (Table 1).

DNA of single individuals was extracted using the Sigma Genelute Extraction kit. PCR was performed in reaction volumes of 50 µl: 4 µl template DNA; 1 × reaction buffer; 0.2 mM dNTPs; 0.2 µM forward and reverse primers; 2 U Taq DNA polymerase (Sigma) and $H_2O$dd. The PCR was run in a MJ thermocycler using a touchdown programme: Initial step 1 min at 94 °C, 31 cycles of 1 min at 94 °C, 30 s at varying annealing temperatures (47–55 °C) and 2 min at 72 °C, final step 2 min at 72 °C. Primers used for amplification of the 1279 bp long cytochrome oxidase I (COI) gene segment were COI1f 5′-ccccctctattagattattatt-3′ (developed for this study; position 2103 in COI sequence of *Apis mellifera*; Crozier and Crozier, 1993) and L2-N-3014r (Simon et al., 1994).

PCR products were purified using the QIA quick PCR purification kit (Qiagen), subsequently sequenced in both directions using the Big Dye termination reaction chemistry (Applied Biosystems) and analyzed with an ABI 377 automatic sequencer (Applied Biosystems). Sequences were deposited in GenBank under accession numbers AY641665-AY641668; AY641701-AY641719; AY641656-AY641658. Sequence alignment was achieved with Clustal X (Thompson et al., 1997). To infer phylogenetic relationships, 1031 bp of COI were used and both distance (neighbour-joining algorithm, NJ) and maximum parsimony (MP) analysis were performed with the software package PAUP (test version 4.0b3a; Swofford, 1998). Tamura-Nei distance (Tamura and Nei, 1993) was used for the NJ trees. Unweighted MP trees were generated with heuristic search using the tree bisection reconnection (TBR) algorithm and a random taxon addition sequence. Bootstrapping (Felsenstein, 1985) with 1000 replicates was applied for all trees.

### *Mating and colony foundation experiments*

Between April and May 2003, we sampled 100–200 ants (workers, males and virgin gynes or brood of sexuals) from each of four colonies from four populations: two *T. rhenanum*, one monogynous and one polygnous *T. moravicum* (Table 2). The colony fragments were kept in separate plaster formicaries in 20 × 20 cm plastic boxes. One monogynous *T. moravicum* colony had been transferred in 1997 from Schloßböckelheim, Nahe valley, to a garden in Ingelheim, Germany, from which additional test individuals were taken when required.

Mating experiments were carried out on 30 May 2003 and 3 June 2003 between 8.30 and 10.00 a.m., when the behaviour of *T. moravicum* sexuals in monogynous and polygynous formicary colonies indicated willingness for nuptial activity. Shortly prior to the experiments, males and alate virgin gynes of the four colonies, which were found to be in good condition, were separately transferred to dark plastic cylinders. Test individuals of the field colony were sampled at the same time and treated like the others.

Twenty mating trials were performed. Each time, two alate virgin gynes from one colony were placed in a fluon-coated plastic box (9.5 × 9.5 × 6 cm) and 1–3 males from the same or another colony were successively added. The experiments were terminated after 5 min or as soon as copulation attempts were observed, but before successful copulation. Combinations and numbers of males are given in Table 2.

A colony foundation experiment should show whether two *T. moravicum* gynes (from a monogynous population, Chazey sur Ain) produce viable offspring after copulation with *T. rhenanum* males (from Dorsheim, Nahe valley and Pommern, Mosel valley, respectively). Only female offspring would be a proof of successful insemination and the absence of post-mating barriers. After apparent insemination (indicated by dealation) the two *T. moravicum* gynes were colocated in a glass tube and inspected at irregular intervals over 10 weeks. Egg-laying and further stages of development were recorded.

## Results

### *Morphological analysis*

Values of gyne SIZE differed significantly between *Tetramorium rhenanum* and *T. moravicum* (Table 3, Fig. 2). CW/SIZE, ML/SIZE, $CW_{cor}$, $ML_{cor}$ and $dCAMN_{cor}$ overlapped but were significantly different. Differences in density and strength of dorsal mesosomal sculpture (Schulz, 1996) vanished entirely when this character was weighted against absolute size (dCAMN/SIZE).

In workers, CS values differed significantly between *T. rhenanum* and *T. moravicum* (Table 4). CL/CW and SL/CS were not significantly different, even when allometrically corrected. Raw values of MW/CS and PEW/CS overlapped but differed significantly. These differences vanished when corrected for size-dependent variation. PPW/CS overlapped but differed significantly, even when corrected allometrically.

The canonical discriminant Dg confirmed all initial determinations (according to the key in Seifert, 1996), but only

**Table 2.** Mating experiments with males and alate virgin gynes from monogynous and polygynous *T. moravicum* colonies and from *T. rhenanum* populations; c = number of males attempting copulation, n = total number of males

| | males | | | | |
|---|---|---|---|---|---|
| | *moravicum* | | | *rhenanum* | |
| | monogynous (FR: Chazey sur Ain) c/n | monogynous (GM: Schloßböckelh.) c/n | polygynous (GM: Norheim) c/n | polygynous (GM: Dorsheim) c/n | polygynous (GM: Pommern) c/n |
| gynes | | | | | |
| *moravicum* | | | | | |
| monogynous (FR: Chazey sur Ain) | 0/3 | 0/3 | 2/3 | 2/3 | 2/2 |
| monogynous (GM: Schloßböckelh.) | 0/3 | 0/3 | 2/3 | 1/3 | 1/1 |
| polygynous (GM: Norheim) | 0/2 | 0/3 | 0/2 | 2/3 | 0/1 |
| *rhenanum* | | | | | |
| polygynous (GM: Dorsheim) | 0/2 | 0/3 | 1/2 | 3/3 | 1/1 |
| Σ | 0/10 | 0/12 | 5/10 | 8/12 | 4/5 |

| | *rhenanum* (n=10) | *moravicum* (n=45) | t | P |
|---|---|---|---|---|
| SIZE | **1548 ± 24** [1524, 1610] | **1749 ± 55** [1636, 1916] | 11.25 | 0.0001 |
| CW/SIZE | **0.770 ± 0.012** [0.755, 0.789] | **0.754 ± 0.010** [0.736, 0.776] | 4.41 | 0.0001 |
| ML/SIZE | **1.299 ± 0.021** [1.268, 1.325] | **1.327 ± 0.017** [1.288, 1.360] | 4.51 | 0.0001 |
| dCAMN/SIZE [%] | **2.09 ± 0.17** [1.77, 2.30] | **1.98 ± 0.24** [1.57, 2.94] | 1.37 | n.s. |
| $CW_{cor}$ | **0.999 ± 0.016** [0.980, 1.024] | **0.974 ± 0.013** [0.951, 1.005] | 5.28 | 0.0001 |
| $ML_{cor}$ | **1.001 ± 0.016** [0.977, 1.020] | **1.026 ± 0.014** [0.994, 1.052] | 4.98 | 0.0001 |
| $dCAMN_{cor}$ | **1.023 ± 0.073** [0.925, 1.121] | **1.184 ± 0.153** [0.902, 1.668] | 3.23 | 0.01 |
| Dg | **1.627 ± 0.517** [0.36, 2.20] | **–2.311 ± 1.053** [–5.37, –0.36] | | |

**Table 3.** Morphometric comparison (µm) of the gynes of *Tetramorium rhenanum* and *T. moravicum*. Upper line, bold: arithmetic mean ± standard deviation, lower line, in [ ]: minimum and maximum values, n = number of measured specimens. Abbreviations of morphometric characters in Material and methods. Dg = discriminant function

87.3% had error probabilities $P < 0.05$ (Table 3). When SIZE was excluded, consistent classifications decreased to 83.6% and classifications with error probabilities $P < 0.05$ to 34.5%. The alternative hypothesis derived by the cluster centre analysis changed 5.4% of the initial determinations. Testing this alternative by a canonical discriminant function resulted in 98.2% consistent classifications and 89.1% classifications with $P < 0.05$, which is comparable to the first hypothesis. One sample from Dorsheim, originally considered to consist of six *T. moravicum* gynes, was displayed as a mixture of three *T. rhenanum* and three *T. moravicum* gynes. The mixed status of this nest, however, was not confirmed by alternative classifications with $P < 0.05$.

The canonical discriminant Dw confirmed 97.5% of the initial determinations (colonies with CS < 806 µm as *T. rhenanum*, with CS > 806 µm as *T. moravicum*), but only 77.5% of the classifications had error probabilities $P < 0.05$ (Table 4). When absolute size was excluded, 42.5% of the initial determinants were confirmed, and only 5% of the classifications had error probabilities $P < 0.05$. The alternative hypothesis of the cluster centre analysis changed 12.5% of the initial determinations. This alternative classified two worker samples with large gynes (including one from E Austria) as *T. rhenanum*. Testing this alternative hypothesis by a canonical discriminant function resulted in 82.5% of classifications with $P < 0.05$.

**Table 4.** Morphometric comparison (µm) of the workers of *Tetramorium rhenanum* and *T. moravicum* based upon nest sample means. Upper line, bold: arithmetic mean ± standard deviation, lower line, in [ ]: minimum and maximum values, n = number of evaluated nest samples, i = number of evaluated individuals, t = results of t-test, P = level of significance. Dw = Discriminant function

| | without corrections for size-dependent variance | | | | corrected for size-dependent variance | | | |
|---|---|---|---|---|---|---|---|---|
| | *rhenanum* (n = 13; i = 54) | *moravicum* (n = 27; i = 101) | t | P | *rhenanum* (n = 13) | *moravicum* (n = 27) | t | P |
| CS | **756 ± 37** [687, 805] | **878 ± 41** [807, 951] | 9.08 | 0.0001 | | | | |
| CL/CW | **1.011 ± 0.017** [0.982, 1.030] | **1.011 ± 0.015** [0.978, 1.035] | 0.00 | n.s. | **0.995 ± 0.016** [0.968, 1.014] | **1.004 ± 0.014** [0.970, 1.027] | 1.82 | n.s. |
| SL/CS | **0.755 ± 0.013** [0.732, 0.780] | **0.759 ± 0.013** [0.735, 0.795] | 0.91 | n.s. | **0.991 ± 0.017** [0.961, 1.024] | **1.001 ± 0.016** [0.973, 1.045] | 1.81 | n.s. |
| MW/CS | **0.633 ± 0.012** [0.612, 0.646] | **0.654 ± 0.013** [0.632, 0.680] | 4.90 | 0.0001 | **0.995 ± 0.020** [0.963, 1.024] | **1.000 ± 0.018** [0.960, 1.028] | 0.79 | n.s. |
| PEW/CS | **0.309 ± 0.008** [0.296, 0.320] | **0.322 ± 0.009** [0.300, 0.339] | 4.43 | 0.001 | **0.994 ± 0.020** [0.955, 1.017] | **0.996 ± 0.027** [0.918, 1.055] | 0.24 | n.s. |
| PPW/CS | **0.374 ± 0.010** [0.353, 0.387] | **0.390 ± 0.010** [0.369, 0.412] | 4.74 | 0.0001 | **0.989 ± 0.022** [0.944, 1.022] | **1.006 ± 0.023** [0.948, 1.067] | 2.22 | 0.05 |
| D | | | | | **–1.306 ± 1.041** [–3.04, 0.15] | **2.132 ± 0.992** [–0.15, 3.92] | | |

We found no dimorphism in worker size, but nest sample means of worker CS and gyne SIZE were highly correlated ($r = 0.764$, $n = 18$, $P < 0.001$). No other morphological differences in sexuals and workers were detected.

### *Molecular genetic analysis*

Topologies of the NJ and MP trees were congruent. *T. rhenanum* showed three haplotypes (HT2, HT3, HT4), one in France and all three in Germany, differing by only one base substitution (sequence divergence 0.1%). In a phylogenetic analysis, *T. rhenanum* was placed within the *T. moravicum* clade (Fig. 1). Two *T. rhenanum* haplotypes (HT2, HT4) from Germany and France were identical with those of *T. moravicum*, and these overlapping haplotypes occurred in some German and French populations (Table 1). *T. moravicum* exhibited six haplotypes, with a maximum sequence divergence of 1.2%. The seven *T. rhenanum* + *moravicum* haplotypes as a whole showed mutations at 15 positions, with all mutations in the third codon position (Table 5). No mutation led to a change in amino acid composition. *T. chefketi* and *T. forte* formed another clade. Sequence divergences were: *T. chefketi* – *T. forte* = 4.3 to 4.7%, *T. rhenanum*/*moravicum* – *T. chefketi* = 5.1 to 5.7%, *T. rhenanum*/*moravicum* – *T. forte* = 5.3 to 5.8%. *T. forte* exhibited a maximum intraspecific sequence divergence of 0.9%. The outgroup species *T. semilaeve* showed 9.4–10.3% sequence divergence compared with all other species.

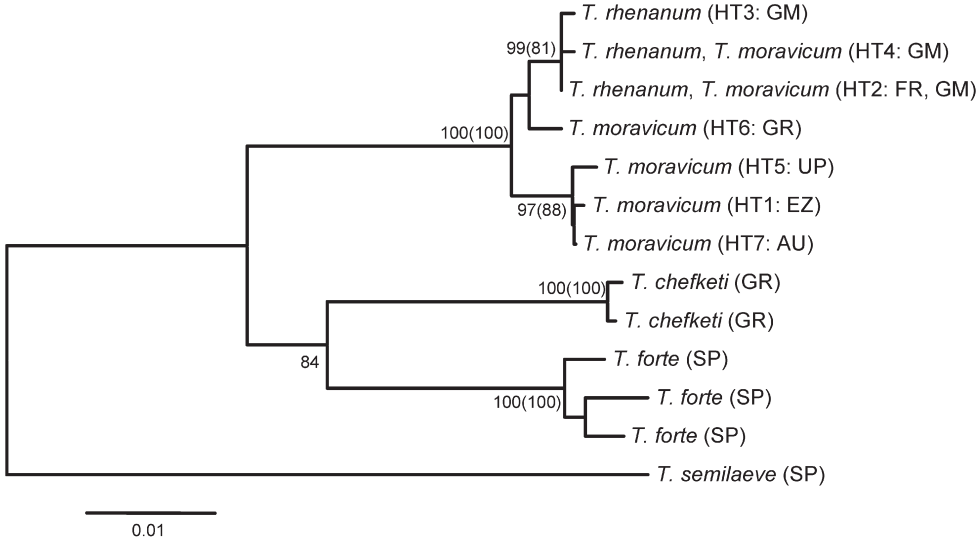


**Figure 1.** Phylogenetic tree showing the relationships of *T. moravicum*, *T. rhenanum, T. forte* and *T. chefketi* in relation to *T. semilaeve* based on Neighbour Joining calculated with the Tamura-Nei algorithm of 1031 bp of the COI gene; haplotypes (HT) of *T. moravicum* and *T. rhenanum* numbered as in Tables 1 and 5; bootstrap values > 75 at nodes, bootstrap values of the unweighted MP branches in parentheses

**Table 5.** Variable sites of COI sequences of the different haplotypes of *Tetramorium moravicum* and *T. rhenanum*, with corresponding COI sequence positions in *Apis mellifera* (Crozier and Crozier, 1993); codon positions (1–3) indicated below; haplotypes (HT) numbered as in Table 1

| | Nucleotide at polymorphic position | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2204 | 2210 | 2213 | 2324 | 2456 | 2459 | 2462 | 2507 | 2615 | 2651 | 2693 | 2792 | 2813 | 2840 | 2897 | 2951 |
| HT1: *T. moravicum* (EZ) | G | G | A | T | A | T | G | T | C | A | G | T | A | G | A | G |
| HT2: *T. moravicum, T. rhenanum* (FR, GM) | | A | G | | G | A | A | C | T | | A | | | | G | A |
| HT3: *T. rhenanum* (GM) | | A | G | C | G | A | A | C | T | | A | | | | G | A |
| HT4: *T. moravicum, T. rhenanum* (GM) | | A | G | | G | A | A | C | T | | A | C | | | G | A |
| HT5: *T. moravicum* (UP) | A | | | | | | | | T | | | | G | | | |
| HT6: *T. moravicum* (GR) | | | | | G | A | | C | T | G | A | | | A | G | A |
| HT7: *T. moravicum* (AU) | | | | | | | | | T | | | | | | | |
| Codon position | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |

*Mating and colony foundation experiments*

In eight mating experiments with *T. moravicum* males from monogynous colonies (22 males from two colonies, Table 2) no copulation attempts were observed. In contrast, in the four experiments with 10 *T. moravicum* males from the polygynous colony, 50% of the males attempted to copulate. In eight experiments with *T. rhenanum* males (17 males from two colonies), 71% of the males attempted to copulate. Different copulation activity was caused by the readiness of males (Fisher's exact test, $P < 0.0001$) and depended on the social structure of the parent colonies (Fisher's exact test for monogynous vs. polygynous *T. moravicum*: $P = 0.0013$, for monogynous *T. moravicum* vs. *T. rhenanum*: $P < 0.0001$), but not on the taxonomic classification (Fisher's exact test for polygynous *T. moravicum* vs. *T. rhenanum*; tested by means of a Bonferroni-Holm-procedure). Copulation attempts were not influenced by characteristics of gynes (same Fisher's exact tests as for males).

In the colony foundation experiment, the two *T. moravicum* gynes from a monogynous colony inseminated by *T. rhenanum* males initially showed a cooperative behaviour. A few days later the first eggs were laid, and after one month the first workers appeared. One gyne died two months after oviposition, the other still continues to produce workers (19 February 2004).

## Discussion

*Two separate species vs. one size-dimorphic species*

Neither of the two competing hypotheses on the identity of *T. rhenanum* was sufficiently supported by gyne morphology. However, the absence of any differential diagnostic character in both sexes except for absolute size differences suggests conspecificity of *T. moravicum* and *T. rhenanum*. In most other carefully investigated cases of ant sibling species, such characters do exist (e.g. Seifert, 2003). Only in *Lasius neglectus* Van Loon et al., 1990 and *L. turcicus* Santschi, 1921, workers and gynes can be separated solely by absolute size differences (Seifert, 2000), while male morphology (Seifert, 2000) and mtDNA data (Steiner et al., 2004) substantiate heterospecificity.

Accordinlgy weak is the morphological separation of workers (Table 4). Comparable discriminant systems usually produce ≥ 95% consistent classifications ($P < 0.05$), when applied to ant sibling species (e.g. Seifert, 2003). Like in gynes, there is almost no separation if absolute size is excluded, but absolute size is the only known character that separates *T. rhenanum* from *T. moravicum* workers. The alternative hypothesis of the cluster center analysis produced a likewise weak separation and a stronger discordance with the gyne dimorphism argument (two samples with large gynes classified as *T. rhenanum*).

The molecular phylogenetic analysis showed that *T. rhenanum* clusters within the *T. moravicum* clade (Fig. 1), and shares haplotypes with *T. moravicum* (Table 1). Sequence comparisons of COI did not reveal any nucleotide positions that would allow to discriminate between the two species.

The results of our mating experiments indicate gene flow rather than a reproductive barrier between *T. rhenanum* and *T. moravicum*. Male *T. rhenanum* attempt to copulate with *T. moravicum* gynes and such matings produce viable offspring. This lends support to our hypothesis of conspecificity, though hybridisation of ant species has been observed in laboratory experiments (e.g. Buschinger, 2001) and in nature (Seifert, 1999).

The consistency of morphometric, molecular and behavioural data justifies to sink *T. rhenanum* into junior synonymy of *T. moravicum*. As a consequence, the bimodal distribution of gyne size (Fig. 2) is interpreted as queen dimorphism. The morphometric graph is comparable to that of other queen size dimorphic ants (e.g. Rüppell et al., 2001) while several species with variable queen size exhibit no bimodal distribution (Kikuchi et al., 1999). We thus conclude that the small gynes of the "*rhenanum*" type constitute the microgyne form of *T. moravicum*.

*Queen number, mating and colony foundation*

The sampled microgyne colonies of *T. moravicum* always contained several dealate gynes. Felke and Sanetra (1997)

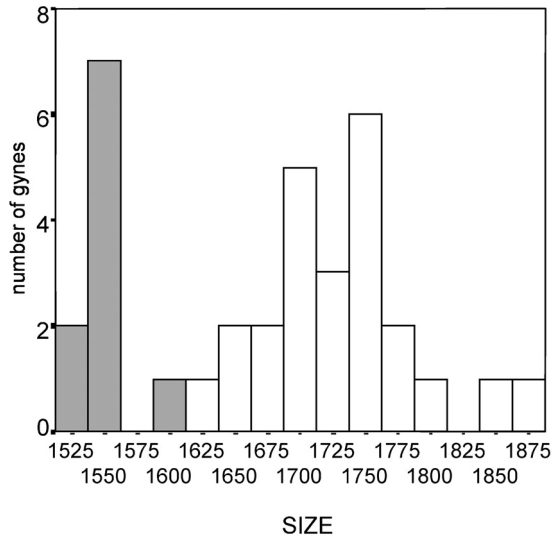


**Figure 2.** Gyne size distribution (µm) in *Tetramorium moravicum* from SW Germany (n = 34): 10 gynes originally hypothesized as *T.* "*rhenanum*" (shaded grey; Dorsheim, Nahe valley; Lorch and Lorchhausen, Rhine valley), 24 gynes hypothesized as *T. "moravicum*" (white; Dorsheim, Norheim and Schlossböckelheim, Nahe valley; Vogtsburg, Kaiserstuhl)

dissected dealate microgynes and found them inseminated in all cases. Felke (1994) experimentally proved that all inseminated gynes develop into functional queens, which means that the microgyne colonies are (at least facultatively) polygynous. Since macrogyne colonies, on the other hand, are only exceptionally polygynous (e.g. Norheim), there seems to exist some connection between queen morph and queen number. As to the other European *Tetramorium* species, polygyny has been reported as an exception in *T. impurum* (Buschinger, 1974) and as the rule in an Alpine population of *T.* cf. *caespitum* (Steiner et al., 2003). However, many Mediterranean species can have more than one functional queen, such as *T. meridionale* Emery, 1870, *T. diomedeum* Emery, 1908, *T. forte* and species of the *T. semilaeve* complex (Sanetra et al., 1999; Sanetra, unpubl.).

Felke and Sanetra (1997) proposed that unsynchronised development of alate microgynes over a prolonged period indicates intranidal copulation in microgyne colonies. Alates might copulate in the nest as soon as they have reached sexual maturity, instead of waiting for a synchronised nuptial flight with latecomers. The results of the mating trials support this idea. As an exception among monogynous macrogyne colonies, unsynchronised development of alates occurs in the Schloßböckelheim population, including the colony transferred to Ingelheim (Heller, unpubl.). Nuptial flight was observed in this population in several years, and males from this population never attempted to copulate in our experiments. However, the unsynchronised development of alates might promote intranidal copulation in *T. moravicum*, including macrogynes.

## Acknowledgements

Our thanks go to the University of Natural Resources and Applied Life Sciences, Vienna, and to the Austrian Federal Ministry for Education, Science and Culture for partial funding; to T. Aßmuth, A. Buschinger, A. Schulz, X. Cerda, R. Güsten, V.E. Kipyatkov, E.B. Lopatina, N. Pöll, F. Pühringer, A. Radchenko, K. Vock and P. Werner for donating samples and help with the field work; to N. Aktac, M. Balint, G. Bracko, A. Buschinger, W. Czechowski, J. Petal and I. Petrov for valuable information; to K. Moder for help with statistical analyses; to M. Stachowitsch for linguistic improvements; and to A.F.G. Bourke and an anonymous referee for valuable comments on the manuscript. BCS and FMS contributed equally to this work.

## References

Bondroit, J., 1918. Les Fourmis de France et de Belgique. *Ann. Soc. Entomol. France 87*: 1–174.

Bourke, A.F.G. and N.R. Franks, 1995. *Social Evolution in Ants*. Princeton University Press, Princeton, New Jersey, 529 pp.

Buschinger, A., 1974. Monogynie und Polygynie in Insektensozietäten. In: *Sozialpolymorphismus bei Insekten* (G.H. Schmidt, Ed), Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart. pp. 862–896.

Buschinger, A., 2001. Multiple Hybridisierung von Arten der Ameisen-Gattung *Epimyrma* (Hymenoptera: Formicidae), und Beobachtungen zur Ausbeutung der Wirtsarten durch die Parasiten. *Myrmecol. Nachr. 4*: 25–35.

Crozier, R.H. and Y.C. Crozier, 1993. The mitochondrial genome of the honeybee *Apis mellifera*: complete sequence and genome organization. *Genetics 133*: 97–117.

Felke, M., 1994. *Populationsgenetische Studien auf enzymelektrophoretischer Basis an mitteleuropäischen Vertretern der Gattung Tetramorium (Hymenoptera: Formicidae)*. Diploma thesis, TH Darmstadt, Darmstadt, 88 pp.

Felke, M. and M. Sanetra, 1997. Beitrag zur Taxonomie und Ökologie von *Tetramorium rhenanum* Schulz, 1996 (Hym.: Formicidae). *Hess. Faun. Briefe 16*: 1–7.

Felsenstein, J., 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution 39*: 783–791.

Hölldobler, B. and E.O. Wilson, 1990. *The Ants*. The Belknap Press of Harvard University Press, Cambridge, Massachusetts, 732 pp.

Kikuchi, T., S. Higashi and T. Murakami, 1999. A morphological comparison of alates between monogynous and polygynous colonies of *Myrmica kotokui* in northernmost Japan. *Insect. Soc. 46*: 250–255.

Rohe, W. and G. Heller, 2000. Vergleichende Untersuchung zur Ameisenfauna (Hymenoptera: Formicidae) von Weinbaustandorten in Rheinland-Pfalz. *Mainz. naturwiss. Arch. 38*: 237–287.

Rüppell, O. and J. Heinze, 1999. Alternative reproductive tactics in females: the case of size polymorphism in winged ant queens. *Insect. Soc. 46*: 6–17.

Rüppell, O., J. Heinze and B. Hölldobler, 1998. Size-dimorphism in the queens of the North American ant *Leptothorax rugatulus* (Emery). *Insect. Soc. 45*: 67–77.

Rüppell, O., J. Heinze and B. Hölldobler, 2001. Alternative reproductive tactics in the queen-size-dimorphic ant *Leptothorax rugatulus* (Emery) and their consequences for genetic population structure. *Behav. Ecol. Sociobiol. 50*: 189–197.

Sanetra, M., R. Güsten and A. Schulz, 1999. On the taxonomy and distribution of Italian *Tetramorium* species and their social parasites (Hymenoptera Formicidae). *Mem. Soc. Entomol. Ital. 77*: 317–357.

Sanetra, M., J. Heinze and A. Buschinger, 1994. Enzyme polymorphism in the ant genus *Tetramorium* Mayr and its social parasites (Hymenoptera: Formicidae). *Biochem. Syst. Ecol. 22*: 753–759.

Sanetra, M. and A. Buschinger, 2000. Phylogenetic relationships among social parasites and their hosts in the ant tribe Tetramoriini (Hymenoptera: Formicidae). *Eur. J. Entomol. 97*: 95–117.

Schulz, A., 1996. *Tetramorium rhenanum* nov. spec. vom Mittleren Rheintal in Deutschland (Hymenoptera: Formicidae). *Linzer biol. Beitr. 28*: 391–412.

Seifert, B., 1996. *Ameisen: beobachten, bestimmen.* Naturbuch Verlag, Augsburg, 352 pp.

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

Seifert, B., 2000. Rapid range expansion in *Lasius neglectus* (Hymenoptera, Formicidae) – an Asian invader swamps Europe. *Mitt. Mus. Nat.kd. Berl., Dtsch. entomol. Z. 47*: 173–179.

Seifert, B. 2001. Formicidae. In: *Entomofauna Germanica, Band 4 – Verzeichnis der Hautflügler Deutschlands* (H. Dathe, A. Taeger, S. Blank, Eds). *Entomol. Nachr. Ber., Dresden, Beiheft 7*: 125–129, 172.

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*: 1–9.

Seifert, B., 2003. The ant genus *Cardiocondyla* (Insecta: Hymenoptera: Formicidae) – a taxonomic revision of the *C. elegans*, *C. bulgarica, C. batesii, C. nuda, C. shuckardi, C. stambuloffi, C. wroughtonii, C. emeryi, and C. minutior* species groups. *Ann. Naturhist. Mus. Wien 104 B*: 203–338.

Simon, C., F. Frati, A. Beckenbach, B. Crespi, H. Liu and P. Flook, 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Ann. Entomol. Soc. Am. 87*: 651–701.

Steiner, F.M., B.C. Schlick-Steiner and A. Buschinger, 2003. First record of unicolonial polygyny in *Tetramorium* cf. *caespitum* (Hymenoptera, Formicidae). *Insect. Soc. 50*: 98–99.

Steiner, F.M., B.C. Schlick-Steiner, M. Sanetra, T. Ljubomirov, V. Antonova, E. Christian and C. Stauffer (in press): Towards DNA-aided biogeography: an example of *Tetramorium* ants (Hymenoptera, Formicidae). *Ann. Zool. Fennici. 42*

Steiner, F.M., B.C. Schlick-Steiner, S. Schödl, X. Espadaler, B. Seifert, E. Christian and C. Stauffer, 2004. Phylogeny and bionomics of *Lasius austriacus* (Hymenoptera, Formicidae). *Insect. Soc. 51*: 24–29.

Stille, M., 1996. Queen/worker thorax volume ratios and nest-founding strategies in ants. *Oecologia 105:* 87–93.

Swofford, D.L., 1998. PAUP*: Phylogenetic analysis using parsimony (*and other methods). Version 4.0b3a. Sunderland, Massachusetts, Sinauer.

Tamura, K. and M. Nei, 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol. 10*: 512–526.

Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin and D.G. Higgins, 1997. The Clustal-X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl. Acids Res. 25*: 4876–4882.