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A multidisciplinary approach reveals cryptic diversity in Western Palearctic *Tetramorium* ants (Hymenoptera: Formicidae)

Birgit C. Schlick-Steiner [a,b,*,1], Florian M. Steiner [a,b,1], Karl Moder [c], Bernhard Seifert [d], Matthias Sanetra [e], Eric Dyreson [f], Christian Stauffer [b], Erhard Christian [a]

[a] *Institute of Zoology, Department of Integrative Biology, Boku, University of Natural Resources and Applied Life Sciences Vienna, Gregor-Mendel-Str. 33, A-1180 Vienna, Austria*
[b] *Institute of Forest Entomology, Forest Pathology and Forest Protection, Department of Forest and Soil Sciences, Boku, University of Natural Resources and Applied Life Sciences Vienna, Hasenauerstr. 38, A-1190 Vienna, Austria*
[c] *Institute of Applied Statistics and Computing, Department of Spatial-, Landscape-, and Infrastructure-Sciences, Boku, University of Natural Resources and Applied Life Sciences Vienna, Gregor-Mendel-Str. 33, A-1180 Vienna, Austria*
[d] *Staatliches Museum für Naturkunde Görlitz, PSF 300154, D-02826 Görlitz, Germany*
[e] *Zoology and Evolutionary Biology, University of Konstanz, Universitätsstr. 10, D-78457 Konstanz, Germany*
[f] *Department of Mathematics, University of Montana-Western, 710 S. Atlantic St., Dillon, MT 59725, USA*

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Abstract

Diversity of ants of the *Tetramorium caespitum/impurum* complex was investigated in a multidisciplinary study. Focusing on morphologically hardly distinguishable Western Palearctic samples, we demonstrate the genetic and phenotypic diversity, demarcate phylogenetic entities, and discuss the clades in terms of biogeography. Sequences of 1113 bp of the mitochondrial COI gene revealed 13 lineages. COII data, worker morphometry and male genitalia morphology corroborated the COI results for seven lineages; the remaining six were disregarded because of small sample size. A comparison with published data on cuticular hydrocarbons showed correspondence. The seven entities show different distribution patterns, though some ranges overlap in Central Europe. Since no major discrepancy between the results of the different disciplines became apparent, we conclude that the seven entities within the *T. caespitum/impurum* complex represent seven species. Geographical evidence allows the identification of *T. caespitum* and *T. impurum*, and we therefore designate neotypes and redescribe the two species in terms of morphology and mtDNA. As the revision of about 50 taxon names would go beyond the scope of this study, we refer to the remaining five species under code names. We discuss our findings in terms of plesiomorphy and convergent evolution by visualizing the mtDNA phylogeny in morphological space.

Keywords: Biodiversity; Cryptic species; Ants; mtDNA; Morphometry; Male genitalia; Temperate regions

1. Introduction

Tropical and marine biomes are generally considered to contain a multiplicity of still unknown species (Hebert et al., 2004; Knowlton, 2001; Mason, 2003; Meegaskumbura et al., 2002; Moon-van der Staay et al., 2001; Sáez and Lozano, 2005; Sechrest et al., 2002). Less frequently addressed is the extent of hidden biodiversity in supposedly well-studied groups of organisms in terrestrial biomes of the temperate zones. As an example we investigate morphologically highly similar ants of the genus *Tetramorium* in the Western Palearctic region.

The myrmicine ant genus *Tetramorium* comprises 445 acknowledged species and subspecies worldwide (Shattuck and Barnett, 2001). Taxonomic problems persist especially in the Palearctic region, mainly because this region was

* Corresponding author. Fax: +43 1 47654 3203.
E-mail address: h9304696@edv1.boku.ac.at (B.C. Schlick-Steiner).
[1] These authors contributed equally to this work.

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excluded from more recent revisions (Bolton, 1976, 1977, 1979, 1980, 1985). About 70 Palearctic species are currently accounted valid (Bolton, 1995). Bolton (1976, 1977, 1979, 1980) outlined the Palearctic *Tetramorium caespitum* group by means of morphological characters and allocated 55 species (Bolton, 1995). Within this group, the species with strongest resemblance to *T. caespitum* (L.) pose a special challenge to discrimination and taxonomy (Kutter, 1977; Sanetra and Buschinger, 2000; Sanetra et al., 1999; Seifert, 1996; Steiner et al., 2002). These species (of what we term the *T. caespitum/impurum* complex) are morphologically variable. Recently, Sanetra et al. (1999) and Steiner et al. (2002, 2003) found indications that *T. caespitum* and *T. impurum* might include a number of cryptic species. Self-Organizing Maps classification of cuticular hydrocarbons data suggested several entities (Steiner et al., 2002), but vague original descriptions and the loss of the type material (*T. caespitum*: Bolton, 1979; M. Fitton, pers. comm.; *T. impurum*: B. Seifert, unpubl.) have hampered fine-scale systematics in the *T. caespitum/impurum* complex to date.

Despite considerable progress in the morphometrical analysis of insects (e.g., Seifert, 2002), groups with subtle differences between and high variation within species are often badly resolved by morphological methods alone (for review: Wiens, 1999; for ant examples: Knaden et al., 2005; Lucas et al., 2002; Ross and Shoemaker, 2005; Steiner et al., 2004, 2005b, 2006). Plesiomorphic and convergently evolved characters may additionally distort the picture (Wiens and Penkrot, 2002; Wiens et al., 2003). Morphologically similar species may, however, differ markedly in their mitochondrial DNA (mtDNA) sequences as shown for ants, among others, by Heinze et al. (2005), Knaden et al. (2005), Ross and Shoemaker (2005), and Steiner et al. (2006). Attempts have been launched to catalog biological diversity by mtDNA on a large scale, resolving also closely related species (Hebert et al., 2003). The inclusion of mtDNA sequences into species descriptions constitutes important complementary information. However, Avise and Walker (2000) argue against species-demarcation merely based on threshold values of genetic difference (cf. Hendrixson and Bond, 2005; cf. Will and Rubinoff, 2004) because biological speciation is a gradual rather than a sudden event. Moreover, genetic markers need not evolve at the same pace as the species does, and the pace may vary across species and markers (Hebert et al., 2003). In conclusion, for a profound evaluation of biological variation, mtDNA should be combined with other approaches such as morphology or semiochemistry (Janda et al., 2004; Knaden et al., 2005; Lucas et al., 2002; Schlick-Steiner et al., 2005; Seifert and Goropashnaya, 2004; Steiner et al., 2004; Ward and Brady, 2003; Ward and Downie, 2005; Wetterer et al., 1998). Congruence of mtDNA and other data supports evolutionary hypotheses much more strongly than any of these approaches alone (Feldhaar et al., 2003; Wetterer et al., 1998; Wiens and Reeder, 1997; Wiens et al., 2003).

The issue of empirically delimiting species is increasingly recognized to be crucial in evolutionary biology (e.g., Dayrat, 2005; Hendrixson and Bond, 2005; Sites and Marshall, 2003; Sites and Marshall, 2004; Wiens, 1999; Wiens and Servedio, 2000). A number of quantitative methods for delimiting species have been suggested recently (reviewed by Sites and Marshall, 2003, 2004), but few studies have assessed the performance of a multidisciplinary approach (Sites and Marshall, 2004; Wiens and Penkrot, 2002). Currently, at least 25 species concepts have been advanced (Coyne and Orr, 2004). Each one has certain limitations, and adhering to one particular concept may affect the assessment of biological diversity (Avise and Walker, 1999; Beresford and Cracraft, 1999). On the other hand, gathering evidence from different sources allows approaching entities that are acceptable as species regardless of which species concept is adopted (Avise and Walker, 1999, 2000). We follow Mallet (1995) in considering the sympatric existence of separate genotypic lineages as an indication of full species status.

This study aims at uncovering the biological diversity within the *T. caespitum/impurum* complex. For the mentioned methodological and conceptual reasons, we chose a multidisciplinary approach utilizing molecular genetic methods and morphological analyses, and also incorporating cuticular hydrocarbons data (Steiner et al., 2002).

2. Materials and methods

2.1. Study system

Tetramorium caespitum was originally described from Europe, without further geographical specification, the morphologically very similar *T. impurum* (Foerster, 1850) from Germany. The *T. caespitum/impurum* complex is morphologically variable. Workers range from small to large and from light brown to black; the head is often strongly, less frequently weakly rugulose; the mesosoma bears longitudinal rugulae; propodeal spines are moderately short; the dorsal surfaces of petiole and postpetiole are finely sculptured or nearly smooth; and the first gastral tergite shows a weakly developed, reticulate microstructure at the most. Based on morphology, two recently revised species have to be incorporated into the *T. caespitum/impurum* complex: the European *T. hungaricum* (Röszler, 1935), which had been confused with species such as *T. caespitum* and *T. semilaeve* (André, 1883) for a long time, and was redescribed by Csősz and Markó (2004); and the East Asian *T. tsushimae* (Emery, 1925), previously regarded as a subspecies of *T. caespitum* and raised to species rank by Bolton (1995), which was confirmed by morphological and molecular analyses (Steiner et al., 2006).

2.2. Molecular analysis

We investigated *Tetramorium* samples from 29 countries, mostly European, and from the Caucasus, Middle Asia, and North America (Appendix A, online supplementary material; Armenia, AM; Australia, AS; Austria, AU; Belgium, BE; Bulgaria, BU; Croatia, HR; Cyprus, CY; Czech Republic, EZ; Denmark, DA; Estonia, EN; Finland, FI; France, FR;

Germany, GM; Greece, GR; Hungary, HU; Italy, IT; Kyrgyzstan, KG; Malta, MT; Netherlands, NL; Poland, PL; Portugal, PO; Romania, RO; Russia, RS; Slovakia, LO; Slovenia, SI; Spain, SP; Sweden, SW; Switzerland, SZ; Turkey, TU; Ukraine, UP; United Kingdom, UK; and United States of America, US). Out of more than 1000 nest samples, 323 were determined as *T. caespitum* or *T. impurum* according to the keys in Kutter (1977), Agosti and Collingwood (1987), and Seifert (1996), and 23 as *T. hungaricum* based on comparison with paralectotypes (Natural History Museum Vienna) and mtDNA sequences (Steiner et al., 2006). These 346 samples were mtDNA sequenced (9 sequences already obtained for another study; Appendix A). In addition, five Palearctic *Tetramorium* species were included to assess intra- and interspecific divergence: the East Asian *T. tsushimae* of the *T. caespitum/impurum* complex, and *T. chefketi* (Forel, 1911), *T. forte* (Forel, 1904), *T. moravicum* (Kratochvil, 1941), and *T. semilaeve* from other complexes of the *T. caespitum* group (partly sequenced for earlier studies; Appendix A). GenBank accession numbers are given in Appendix A. The originally Oriental tramp species *T. bicarinatum* (Nylander, 1846) from the *T. bicarinatum* group (Bolton, 1977), and the Australian *T. capitale* (McAreavey, 1949) from the *T. striolatum* (Viehmeyer, 1914) group (Bolton, 1977), were taken as outgroups.

DNA extractions and PCR with a touchdown program followed the protocols of Steiner et al. (2005b). For sequencing a stretch of the mitochondrial COI gene we used the primers LCO1490 (Folmer et al., 1994) or COI1f (Steiner et al., 2005b) combined with L2-N-3014r alias "Pat" (Simon et al., 1994) as reverse primer, amplifying 1584 and 1280 bp, respectively. Mitochondrial genes can be functionally coupled and there is only little evidence for recombination of mtDNA (reviewed in Rokas et al., 2003). Nevertheless, different mitochondrial genes were shown to exhibit different degrees of variation (Crozier et al., 1989) and even result in different phylogenies (Cao et al., 1998). The phylogenetic utility of combining COI and COII has been widely demonstrated (e.g., Simon et al., 1994) and we sequenced COII for 76 of the samples to evaluate and possibly strengthen the phylogenetic signal of COI. For those samples, the amplified region was extended by 563 bp, using "COIIr2" 5′-gtagagtctattttaattcctaatg-3′, developed for this study, as reverse primer instead of Pat. The also amplified non-coding region and tRNA-leu gene between COI and COII was excluded from further phylogenetic analysis. All PCR products were purified (QIAquick PCR purification kit, Qiagen), sequenced in both directions using the Big Dye termination reaction chemistry (Applied Biosystems), and analyzed with an ABI 377 automated sequencer (Applied Biosystems).

For phylogenetic analysis, sequence alignments were achieved with the default settings of Clustal X (Thompson et al., 1997). Tests for saturation of substitutions, as developed by Xia et al. (2003), were performed using the program DAMBE 4.2.13 (Xia and Xie, 2001). We tested all codon positions simultaneously, as well as the first, second, and third positions separately, in COI and in COII. A partition homogeneity test implemented in PAUP* (version 4.0b10; Swofford, 1998) was used to determine whether the COI and COII data sets were significantly incongruent. For all data sets (COI, COII, and COI + COII), distance (Neighbor Joining algorithm, NJ) and character (maximum parsimony, MP; Bayesian Markov Chain Monte Carlo, BMCMC) analyses were performed using PAUP* and MrBayes 3.1 (Ronquist and Huelsenbeck, 2003). For NJ analyses we used Tamura-Nei distance, but the results did not change when using other models, among them the models used for BMCMC. For MP analysis, all characters were assigned equal weights. MP trees were generated with a heuristic search using tree bisection-reconnection branch swapping (COI: reconnection limit set to 12; rearrangement limit set to 100,000,000), with 10 random taxon addition sequence replicates, and with the Multree option in effect. Bootstrapping was applied for NJ (1000 replicates) and MP trees (100 replicates). Prior to each BMCMC analysis the best-fitting nucleotide substitution model was selected by using the hierarchical likelihood ratio test (hLRT) and Akaike Information Criterion (AIC) implemented in MrModeltest 2.2. (Nylander, 2004). In hLRT and AIC, "GTR + I + G" was selected for the COI data set, "HKY + G" for the COII data set. All BMCMC analyses were performed by two parallel runs. We defined three partitions according to codon positions for COI and for COII, and six partitions according to codon positions and single genes for COI + COII. For the COI data set we ran 3,625,000 generations with 4 chains each (one cold, three hot), the temperature set to 0.1 and a sample frequency of 100. A good measure for stationarity is the standard deviations of split frequencies, which should be below 0.01 (Ronquist et al., 2005). However, for some data sets this value cannot be achieved; in such cases, stationarity can be considered reached if the average standard deviations of split frequencies are stable and below 0.05 (F. Ronquist, pers. comm.). For the COI data set, after 2,496,000 generations, standard deviations of split frequencies reached values below 0.017, and afterwards oscillated between 0.0169 and 0.0149, the log likelihood values being stable at about −7100. Thus, we used the last 11,290 trees of both runs to compute the majority rule consensus tree assigning posterior probabilities of tree topology. For the COII data set we ran one unheated chain for 1,000,000 generations. Average standard deviation of split frequencies never exceeded 0.01 after 490,000 generations, thus the last 5100 trees of both runs were used for the majority rule consensus tree. For the COI + COII data we ran one chain for 1,000,000 generations, applying GTR + I + G to COI and HKY + G to COII. After 510,000 generations the standard deviations of split frequencies dropped below 0.01 and oscillated between 0.007 and 0.008 after 600,000 generations. We used the last 4000 trees of both runs for the majority rule consensus tree.

2.3. Morphology

We morphologically analyzed a subset of those 346 samples determined as *T. caespitum*, *T. impurum*, or *T. hungari-*

cum (means of determination described above) which had been used for the molecular analysis.

For worker morphometry, 422 workers from 128 nests were analyzed (Appendix A; three to five workers per nest), a part of which (69 workers of 23 nests) had been included in Steiner et al. (2006). All 29 countries were represented in this sample. Twenty-nine morphometric values were determined, partly serving for the calculation of five angles (Appendix B, online supplementary material). Dry-mounted specimens were fixed on a pin-holding goniometer. A Nikon SMZ 1500 high-performance stereomicroscope with a 1.6× planapochromatic lens and a cross-scaled ocular micrometer was used at magnifications of 50–320×.

The software package SAS 8.2 was used to classify the morphometric data by discriminant analysis (DA), based on pooled covariance matrices, according to mtDNA hypotheses. DA was applied to single worker data and to nest means of three to five workers.

Evolutionary principal components analysis (EPCA), a derived form of PCA, was performed with the module Rhetenor of the software package Mesquite 1.01 (Maddison and Maddison, 2001). While PCA rotates the data cloud to maximize among-group variation, EPCA rotates the cloud to maximize evolutionary change based on a phylogenetic tree mapped into a morphological space. For this analysis, we used a least-squares parsimony mapping of the COI NJ tree into the worker morphological space as a basis for EPCA.

Genitalia of 97 males from 42 nests were examined (Appendix A). The shapes of squama and stipes (sensu Collingwood, 1979) were characterized in dorsal, ventral, lateral, and posterior view.

2.4. Distribution maps

Geographical origins of samples determined as *T. hungaricum*, or as *T. caespitum* or *T. impurum*, were plotted on a map of the Western Palearctic region; for the latter two we separately visualized the distribution of demarcated mtDNA units.

3. Results

3.1. Molecular phylogenetics

A total of 1113 bp of the mitochondrial COI gene were sequenced in all *Tetramorium* samples, and additionally 454 bp of the COII gene in 76 samples; the sequence data were deposited in GenBank (Appendix A).

In COI a total of 356 sites were variable. Within the 323 samples determined as *T. caespitum* or *T. impurum*, mutations at 229 sites (35 at the first, six at the second, and 188 at the third codon position) resulted in 113 haplotypes, with a maximum sequence divergence of 9.6% (Fig. 1, Appendix C, online supplementary material). Substitutions resulted in 28 changes of amino acids. Concerning the two other species of the *T. caespitum/impurum* complex: *T. hungaricum* had six mutations (0.4%), *T. tsushimae* 12 mutations (1.1%) leading to one amino acid change. No saturation of substitutions was revealed, independently of whether positions were tested simultaneously or separately.

NJ and BMCMC trees were based on data condensed to haplotypes (Figs. 1 and 2). MP phylogenetic analyses of all taxa were based on a total of 269 parsimony-informative characters (tree not shown, bootstrap values within NJ tree, Fig. 1). The bootstrap 50% majority-rule consensus tree was 1045 steps long, with a consistency index of 0.45 and a retention index of 0.88; the topology was nearly identical to the NJ tree. In all trees, *T. hungaricum* and *T. tsushimae* of the *T. caespitum/impurum* complex formed monophyletic entities. *T. moravicum*, *T. forte*, *T. chefketi*, and *T. semilaeve* of other species complexes likewise revealed monophyletic origins, with the maximum intraspecific divergence varying from 0.4% (*T. chefketi*) to 1.2% (*T. semilaeve*; Fig. 1), while interspecific divergence ranged from 4.0 to 10.6%.

Samples determined as *T. caespitum* or *T. impurum* clustered into a series of units. A numerical delimitation based on threshold divergence values alone is not advisable because some animal taxa of unquestioned species status exhibit an interspecific divergence lower than the intraspecific variation of other species (e.g., Goropashnaya et al., 2004). We decided in favor of a combined approach to taxa delimitation and considered node support and sequence divergence. Node support values of NJ, MP, and BMCMC are not independent as all are based upon the same data, but congruence indicates robustness to different tree-building algorithms. We defined 13 taxa arbitrarily termed A, B, C, D, E, F, G, and U1–U6. Sample numbers of the 13 entities ranged from 1 (U1, U3, and U5; Appendices A and C) to 95 (F), haplotype numbers from 1 (U1, U4, and U6) to 20 (E). In the following we disregard U1–U6 due to insufficient sample size. Node support of NJ/MP was 100/100 for taxa C, E, and G, 99/98 for D, 98/93 for F, 90/95 for B, and 74/68 for A. Maximum sequence divergence within the entities ranged from 0.3% (taxon G; Fig. 1, Appendix C) to 2.9% (D). Minimum variation among taxa varied from 1.6% between A and F to 8.5% between D and E.

Fig. 1. COI: phylogenetic tree based on NJ calculated with the Tamura-Nei algorithm of 1113 bp of the COI gene, broken down to haplotypes (HT). The scale bar denotes 0.02 substitutions/site. NJ bootstrap values >50 are given at nodes, except at terminal nodes, MP bootstrap values are given after forward slashes. Haplotypes numbered as in Appendix C, online supplementary material. For country codes see Section 2. Male genitalia: characterization of stipes and squama of *T. hungaricum* and taxa A–G in (from left to right) dorsal, ventral, lateral, and posterior view; description of key characters; male of taxon C unknown. CHC: classification of cuticular hydrocarbons of 42 samples by Self-Organizing Maps, from Steiner et al. (2002; green = neurons 24, 29, 30; pink = 16, 21; grey = 43; blue = 26, 31; red = 3; yellow = 5, turquoise = 41, 44–47); circle areas proportional to numbers of samples; no samples of taxon A included. Distribution: mapping of *T. hungaricum* and taxa A–G in Europe and the Caucasus region (records in KG not shown).

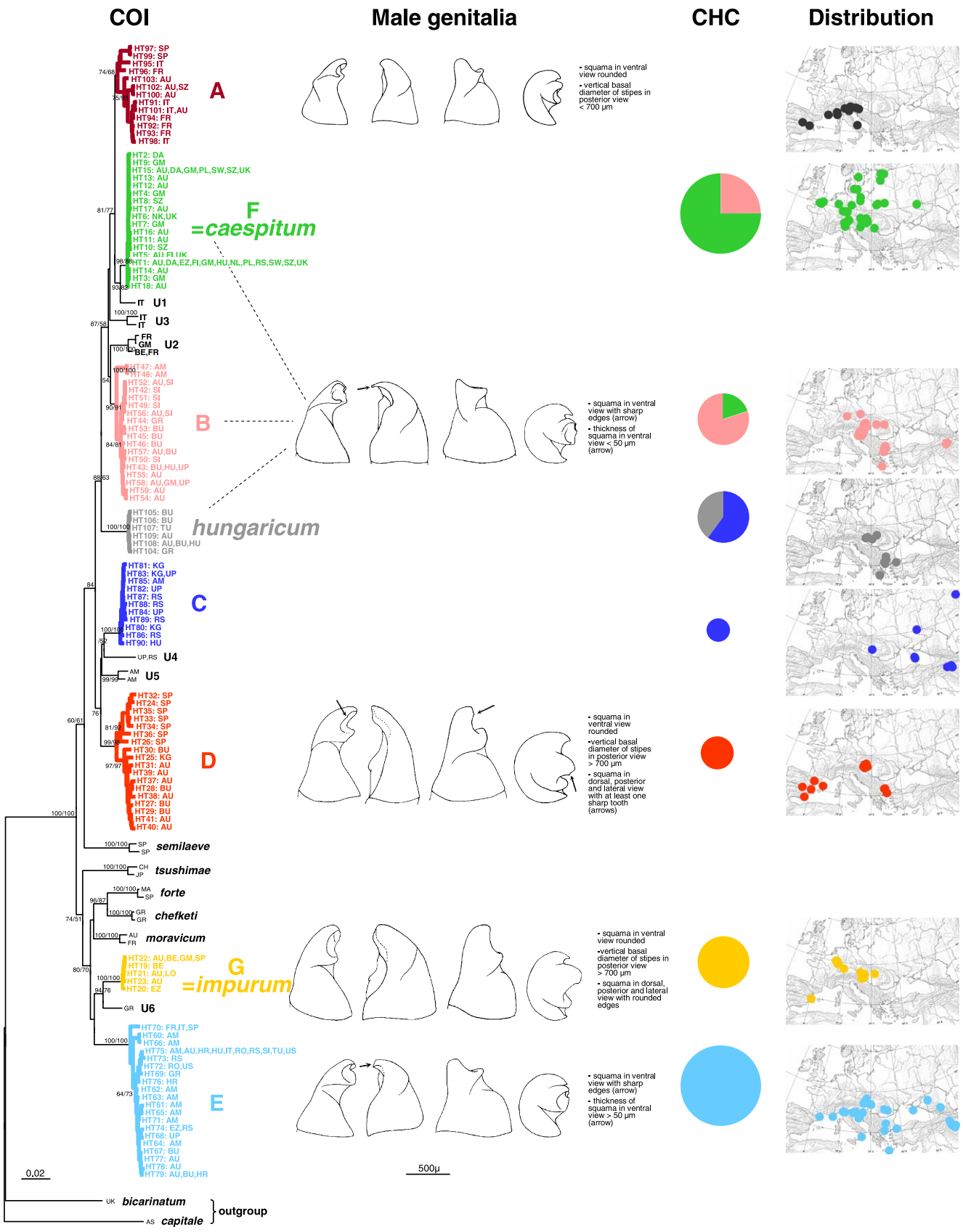
COI
Male genitalia
CHC
Distribution
A
F
=caespitum
B
hungaricum
C
D
G
=impurum
E
semilaeve
tsushimae
forte
chefketi
moravicum
bicarinatum
capitale
outgroup
U1
U2
U3
U4
U5
U6
- squama in ventral view rounded
- vertical basal diameter of stipes in posterior view < 700 µm
- squama in ventral view with sharp edges (arrow)
- thickness of squama in ventral view < 50 µm (arrow)
- squama in ventral view rounded
- vertical basal diameter of stipes in posterior view > 700 µm
- squama in dorsal, posterior and lateral view with at least one sharp tooth (arrows)
- squama in ventral view rounded
- vertical basal diameter of stipes in posterior view > 700 µm
- squama in dorsal, posterior and lateral view with rounded edges
- squama in ventral view with sharp edges (arrow)
- thickness of squama in ventral view > 50 µm (arrow)
0.02
500µ

Fig. 2. Majority rule consensus tree of the last 11,290 of both runs of 36,250 sampled trees, obtained from BMCMC of 1113 bp of the COI gene based on the GTR + I + G model. Tree broken down to haplotypes (HT) which are numbered as in Appendix C, online supplementary material. Posterior probability values >50 are given above branches, except at terminal branches. For country codes see Section 2.

All trees showed a polyphyletic origin of the *T. caespitum/impurum* complex (Figs. 1 and 2). Taxa E and G were grouped in one phylogenetic lineage (node support NJ/MP/BMCMC = 80/70/100) that includes *T. forte*, *T. chefketi*, and *T. moravicum* in all trees (Figs. 1 and 2). A, B, and F were grouped together in another lineage in all trees (NJ/MP/BMCMC = 87/73/100). Taxa C and D were grouped together in a further lineage (NJ/MP/BMCMC = 76/73/100), and the two taxa were attributed to a lineage containing also F, A, B, *T. hungaricum*, and *T. semilaeve* but with partly lower node support (NJ/MP/BMCMC = 60/61/100). *T. tsushimae* was positioned within the lineage of E and G

in the NJ tree (NJ = 74) and, with weaker support, in the MP tree (MP = 51). The BMCMC tree placed *T. tsushimae* basally within the ingroup.

In a NJ tree based only on the first and second positions of the COI data (tree not shown), taxa F, A, B, C, D, *T. hungaricum*, and *T. semilaeve* formed one substructured lineage (node support = 94) and taxa G, E, *T. forte*, *T. chefketi*, and *T. moravicum* another one (node support = 89). *T. tsushimae* was attributed to the latter lineage with weak support (58).

COII sequencing resulted in 20 haplotypes within the samples determined as *T. caespitum* or *T. impurum* prior to our analyses. The COII data set revealed no saturation of substitutions. As the partition homogeneity test detected no significant incongruence between the COI and COII data sets ($P = 0.99$), we combined COI and COII, which yielded 34 haplotypes of *T. caespitum*/*T. impurum*. NJ, MP, and BMCMC trees based on COII only (trees not shown) and of COI + COII (Fig. 3) were congruent in topology and very similar to the COI pattern. All taxa defined on the

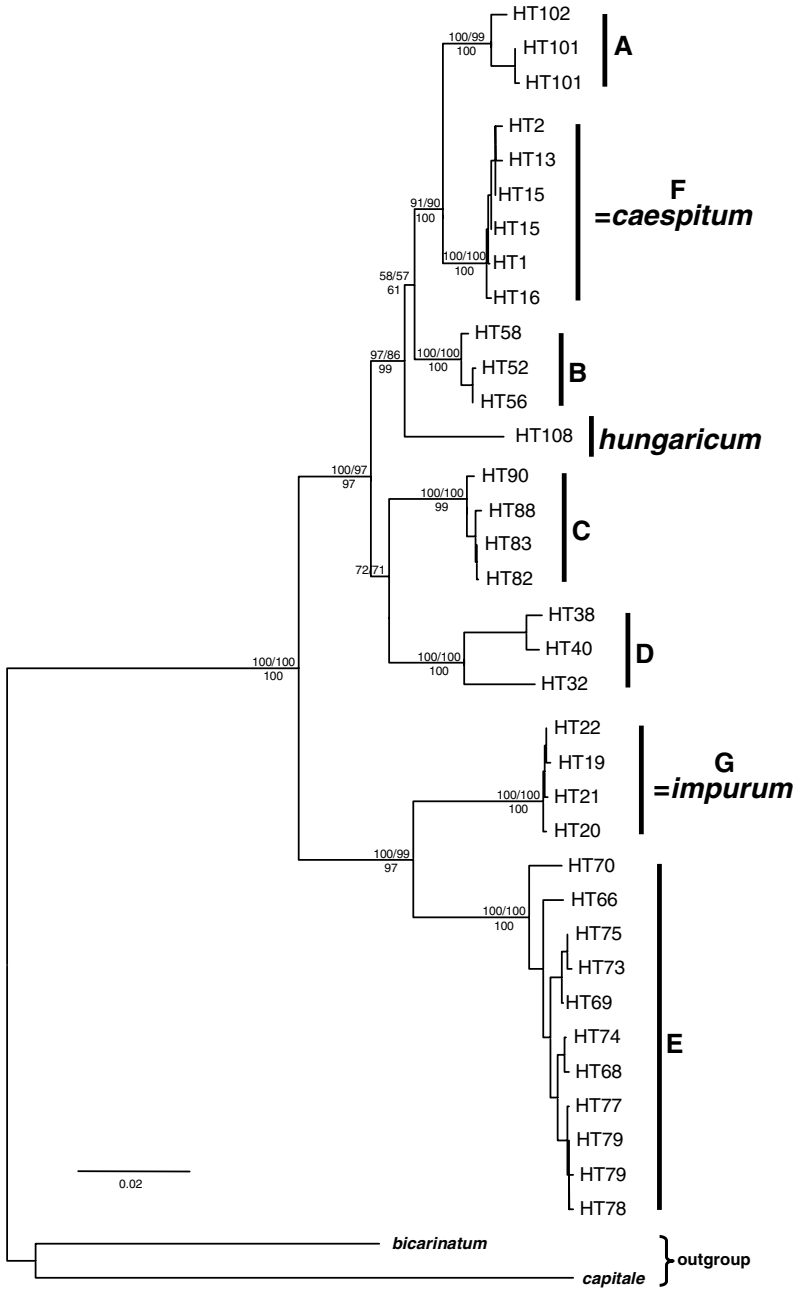

Fig. 3. Phylogenetic tree based on NJ calculated with the Tamura-Nei algorithm of a total of 1567 bp of the combined COI + COII data set, broken down to haplotypes (HT). Haplotype numbers correspond with COI haplotype numbers. HT15, HT79, and HT101 of COI each resulted in two different haplotypes of COI + COII. The included samples are indicated in Appendix A, online supplementary material. The scale bar denotes 0.02 substitutions / site. NJ bootstrap values >50 are given at nodes (except at terminal nodes) above branches, MP bootstrap values are given above branches, after forward slashes, BMCMC posterior probability values >50 of the BMCMC are given below branches.

basis of the COI tree formed distinct units again, supported by high node support values (99–100). Polyphyly was also corroborated with high node support (97–100).

3.2. Worker morphometry

A total of 422 workers from 128 nests of *T. hungaricum* and taxa A–G were analyzed morphometrically. DA ordination of 19 variables (Appendix D) of single workers according to the mtDNA hypotheses resulted in a 16.5% error rate. Bootstrapping (1000 replicates) of the calculation of a discriminant function on the whole data set minus one randomly selected worker, and subsequent classification of this single worker, resulted in 20% error. The discrimination appears robust enough to produce similar results when applied to data other than ours. To test whether the DA reveals an underlying phyletic pattern, we established a fictitious set of 8 species and assigned each individual worker randomly to one of these species, with the only demand that nestmates must be conspecific. Attributions by the pooled covariance matrix and the 19 variables were inconsistent in 74.6% of the cases, which was taken as an argument in favor of a phyletic background of the mtDNA hypotheses. In another DA we ordinated nest means by 12 variables (Appendix D), which resulted in a 7.0% error rate. At species level, the probabilities of consistent classification of nest means ($P \geqslant 0.95$) were: *T. hungaricum* 87.5% ($n = 16$); A 68.8% ($n = 16$); B 6.7% ($n = 15$); C 100% ($n = 12$); D 91.7% ($n = 12$); E 91.3% ($n = 23$); F 22.2% ($n = 18$); G 62.5% ($n = 16$). For some taxa, especially B and F, the set of morphometric characters did not provide a clear-cut discrimination and should not be used as the sole approach, while for other taxa, especially *T. hungaricum* and C, D, and E, the discrimination was trustworthy. Lumping taxa B and F to (B + F) after DA lowered the inconsistent classifications to 12.6% (single workers) and 3.1% (nest means).

3.3. Male genitalia

The genitalia of 97 males from 42 nests were analyzed: *T. hungaricum* 19 males (9 nests), A 9 males (5 nests), B 10 males (4 nests), D 5 males (3 nests), E 9 males (3 nests), F 31 males (13 nests), and G 14 males (5 nests). Samples of taxon C contained no males. For taxa A, D, E, and G, differential characteristics were found, with low variation within the taxa. No characters were detected for the discrimination between *T. hungaricum* and taxa B and F, but these three taxa could be distinguished from the rest. Characters of the squama and the stipes are compiled in Fig. 1.

3.4. Geographical distribution

Being aware that some taxa and regions were scantily sampled, we put forward a tentative sketch of distribution (Fig. 1). *T. hungaricum* was recorded in the Pannonic–Balkanic region, ranging from AU eastwards and southwards to 38° N. Taxon A was restricted to the alpine zone above 1300 m a.s.l. and showed a disjunct distribution in Iberia, the Cevennes, the Apennines, and the Western and Central Alps. Taxon B occurred in the Pannonic–Balkanic region and, with a large gap, in the Caucasus. Northern and Western limits were at 51°N and 9°E. The scarce data of taxon C indicated an eastern distribution area, from HU eastwards to KG, 40°–54°N. Taxon D displayed a disjunct distribution on the Iberian Peninsula, in Central Europe, the Balkans and KG, south of 49°N, from 8°W to at least 76°E. Taxon E showed a Ponto–Mediterranean–Caucasian distribution with a northern limit at 49°N. This taxon was absent from most of Iberia (one record from La Platera immediately south of the Pyrenees). Taxon F was found to be chiefly European north of 46°N, ranging from England to the North Caucasus (RS), northwards to South SW. Taxon G occurred in Central and NW Europe at 40–50°N and from 1°W −20°E, with one isolated record on the Iberian Peninsula.

The ranges of taxa A–G and *T. hungaricum* met in Central Europe. Taxa E and F overlapped in a Central European zone with a maximum north-south extension of 300 km. Syntopic occurrence was recorded in 11 combinations (AU, AM, EZ, HU, and RS; Appendix A): *T. hungaricum* with B, C, and F; B with D and F; C with E and F; D with E and F; E with F; F with G.

4. Discussion

4.1. Mitochondrial DNA

The COI stretch which revealed the existence of 13 lineages within *T. caespitum* and *T. impurum* has proven to resolve entities at the species level in *Cardiocondyla*, *Cataglyphis*, *Lasius*, *Messor*, *Myrmica*, *Solenopsis*, and *Tetramorium* ants (Heinze et al., 2005; Knaden et al., 2005; Ross and Shoemaker, 2005; Savolainen and Vepsäläinen, 2003; Schlick-Steiner et al., 2006; Steiner et al., 2004, 2005a,b, 2006). The fact that the minimum COI variation among the present *Tetramorium* taxa is partly exceeded by maximum sequence divergence within taxa (e.g., 1.6% between A and F vs. 2.9% within D) is paralleled by morphologically well-separated *Formica* ant species that turned out to be highly similar in terms of mtDNA (Goropashnaya et al., 2004). While this poses a conceptual problem to Hendry et al. (2000), it is regarded a normal phenomenon in a transitional stage of speciation by Avise and Walker (2000). COII sequencing confirmed the COI results. Below, with the results of other methods, we discuss the hypothesis that the mtDNA entities A–G represent species.

4.2. Morphology

Discrimination between *T. hungaricum* and taxa A–G required 19 morphometric characters for single workers and 12 for nest means. This is an exceptionally high demand for differential diagnosis compared with other DA

studies. We know of only one example with more characters: the discrimination of two mite species based on 56 morphological traits (Klimov et al., 2004). In other species groups of ants the highest number of characters used is ten (Seifert, 2003). To our knowledge, however, DA has been applied only to discriminate two taxa, while we had to cope with eight. The number of required characters and the relatively low probability values for some taxa of our study indicate a high morphological similarity of the workers.

The low values of mtDNA divergence between taxa B and F parallel both worker morphology and male genitalia. While discrimination based on worker morphology is difficult but possible, no differential characters in the male genitalia of the two taxa are evident. This does not challenge the mtDNA results because not even *T. hungaricum* can be distinguished from B and F by male genitalia, although this species is well separated by mtDNA and worker morphometry. Taxa A and F, on the other hand, differ distinctly in male genitalia, despite very low mtDNA divergence. On the whole, worker morphometry and male genitalia support the mtDNA-derived hypothesis.

4.3. Cuticular hydrocarbons

Steiner et al. (2002) classified GC–MS data of cuticular hydrocarbons (CHC) of *Tetramorium* samples by Self-Organizing Maps, a neural network algorithm based on unsupervised training, into seven entities. That study included samples which had been morphologically determined as *T. caespitum* (then comprising *T. hungaricum* according to Seifert, 1996) and *T. impurum*. No samples of taxon A were available then, and because all the material came from Central Europe, geographical variation may have been underestimated. For the present study, 42 of the nest samples in Steiner et al. (2002) were DNA-extracted. The configuration of the mtDNA results largely corresponds with the CHC clusters: only one sixth of the samples deviate (Fig. 1). The alternate distribution of B and F samples in the neural network corresponds with worker morphometry and male genitalia morphology. Thus, the results of Steiner et al. (2002) roughly match the DNA-derived phyletic pattern put forward in the present study.

4.4. Combining evidence and neotype designation

Sanetra and Buschinger (2000) studied allozyme patterns of 14 Palearctic *Tetramorium* species and found the highest intraspecific variability in *T. caespitum* and *T. impurum*. In our approach, every single method revealed enormous variation within the *T. caespitum/impurum* complex. While the results of the different methods were largely consistent, some conflicts arose in detail. Topologies of COI, COII, and COI + COII trees are congruent. Worker-morphometry-based discrimination of the seven taxa studied in depth resulted in an error of 16.5 and 7.0% for single individuals and nest means, respectively, as compared with the mtDNA results. Male genitalia of four of the seven taxa could be distinguished without exception, but no discriminating characters could be found for the remaining three taxa. CHC-based classification deviated in 16.7% of the analyzed samples. Possible explanations for conflicts include single cases of incomplete lineage sorting of ancestral polymorphisms during successive rounds of speciation reflected in mtDNA (Pamilo and Nei, 1988) and environmental influences on cuticular hydrocarbon profiles (e.g., Gamboa et al., 1991). However, the combined evidence of molecular phylogeny, worker morphometry, male genitalia morphology, CHC chemistry, and biogeography overcomes the deficits of single disciplines. Syntopic occurrence (taxa B and F; C and D) provides an additional argument for systematic decisions. These pairs exhibit a minimum COI sequence divergence of less then 4% (Fig. 1, Appendix C), but conjoint occurrence suggests full species status (Mallet, 1995). In the case of B and F, this argument preponderates even the weak separation by morphological and cuticular hydrocarbon characters. Summing up, we confirm the species status of *T. hungaricum* (see Appendix E for a morphological and molecular characterization) and regard the seven entities A–G as full species.

Which of the species A–G is *T. caespitum* (L.) and which is *T. impurum* (Foerster, 1850)? The type material is lost (*T. caespitum*: Bolton, 1979; confirmed by M. Fitton, Linnaean Society collection, London, pers. comm.; *T. impurum*: destroyed at the Zoological Museum, Berlin, B. Seifert, unpubl.). Thus, neotypes can be designated under the terms of Art. 75 of ICZN 1999. The descriptions of *T. caespitum* and *T. impurum* are too vague for identification. According to Linnaeus (1758) *T. caespitum* "habitat in Europae tuberibus". Although there is no guarantee that Linnaeus' type specimen came from Sweden, we equate species F with *T. caespitum* because F is the only species (including taxa U1–U6) that occurs up to Scandinavia. We designate neotype material from SW and redescribe *T. caespitum* in Appendix E. This decision serves taxonomic stability, because (1) based on male genitalia morphology, species F or G had probably been taken for "*caespitum*" by Kutter (1977); and because (2) the characterization of the worker of "*caespitum*" in Seifert (1996), mainly based on the structure of the petiolar and postpetiolar node, and, as a tendency, in Agosti and Collingwood (1987), based on overall color, applies to species F.

In the case of *T. impurum*, clear information on the type locality is available: "am Lousberg bei Aachen" in Western GM, at the Belgian border. Species F and G have been recorded from this area, as has been U2, one of the six lineages disregarded due to insufficient sample sizes (Appendix A). U2, however, is no *T. impurum* candidate because it corresponds to what has been assigned to "*caespitum*" in the past (structure of petiolar and postpetiolar node, overall color). Since the original habitat at the type locality—now situated in the center of the city of Aachen—has been completely destroyed by conversion into a settlement area (B. Seifert, unpubl. data), we designate neotype material, representing our species G, from the vicinity of Brussels, c. 100 km from the original type locality, and redescribe

T. impurum in Appendix E. This decision serves taxonomic stability, because (1) based on male genitalia morphology, species G had probably been taken for "*impurum*" by Kutter (1977); and because (2) the characterization of the worker of "*impurum*" in Seifert (1996), mainly based on the structure of the petiolar and postpetiolar node, and, as a tendency, in Agosti and Collingwood (1987), based on overall color, best applies to workers of species G.

Our neotype designations of *T. caespitum* and *T. impurum* are in line with the principle of priority of the ICZN. The oldest name available for a *Tetramorium* species is *caespitum* (L.). *Formica fusca* Leach, 1825 is a junior primary homonym of *Formica fusca* Linnaeus, 1758. The homonymy is unresolved (Bolton, 1995), but the species name was never used in combination with *Tetramorium* except by Dalla Torre (1893) who listed it as a junior synonym of *T. caespitum*: this, however, is not "use of a name" from the perspective of the ICZN, Art. 23.9.5. The next younger name—*fuscula* (Nylander, 1846), coined for a Finnish ant—was synonymized with *caespitum* by Smith (1851), and rightly so, as our biogeographical data suggest. The fourth name in chronological order is *impurum* (Foerster, 1850). Thus, our neotype designations serve taxonomic stability and will not be touched by future revisions.

A taxonomic decision on species A–E would require the revision of about 50 names which come into question for Palearctic *Tetramorium* species (Bolton, 1995). We therefore content ourselves with the code letters and characterize these species in Appendix E.

For the mtDNA entities U1–U6 we likewise suppose species status. In the case of U4, this is supported by a characteristic, deviating allozyme pattern (M. Sanetra, unpubl. data).

4.5. Evolutionary interpretation of morphological characters and species discrimination

All mtDNA analyses suggest two strongly diverging lineages (Figs. 1–3). One lineage includes *T. impurum*, species E, *T. forte*, *T. chefketi*, and *T. moravicum* (the latter three with clear morphological differences to *T. impurum*), and perhaps *T. tsushimae*, which is positioned differently by different methods and may even be a sister-group of the two lineages. The other lineage includes *T. caespitum*, species A, B, C, D, *T. hungaricum*, and though less supported, *T. semilaeve*, a species with workers morphologically similar to *T. hungaricum*. The two lineages appear reliable since they can be reproduced when only the first two nucleotides of each codon are taken into account. These are functionally more constrained (Simon et al., 1994), and thus more conservative, and indicative of basal furcations of a tree. Nevertheless, the evolutionary interpretation remains preliminary because only mitochondrial markers have been analyzed.

In our trees an early furcation separates the *T. caespitum* and the *T. impurum* clusters. This contrasts with the previously assumed sister-species relationship (e.g., Sanetra et al., 1999; Sanetra and Buschinger, 2000; in these works, however, the majority of "*T. impurum*" samples came from alpine habitats and probably represented species A which, according to our mtDNA data, is actually closely related with *T. caespitum*). The fact that some species of different lineages have very similar worker morphologies, whereas each lineage embeds morphologically clearly differing species, might reflect plesiomorphy or convergent evolution, or a combination of the two. Fig. 4 visualizes morphological worker similarity of *T. caespitum*, *T. impurum*, and species A–E, which are connected in accordance with the branching pattern of the NJ tree (Fig. 1) and mapped by EPCA. Species further to the left are smaller for most metrics. The distance between two species depicts overall worker similarity. Branch crossings illustrate disparities between morphology and mtDNA. Either worker morphology similar to *T. caespitum* represented the ancestral morphology of the two lineages, or convergent evolution led to surprisingly high similarities. Male genitalia pose a comparable problem. The fact that the genitalia of *T. hungaricum*, *T. caespi-*

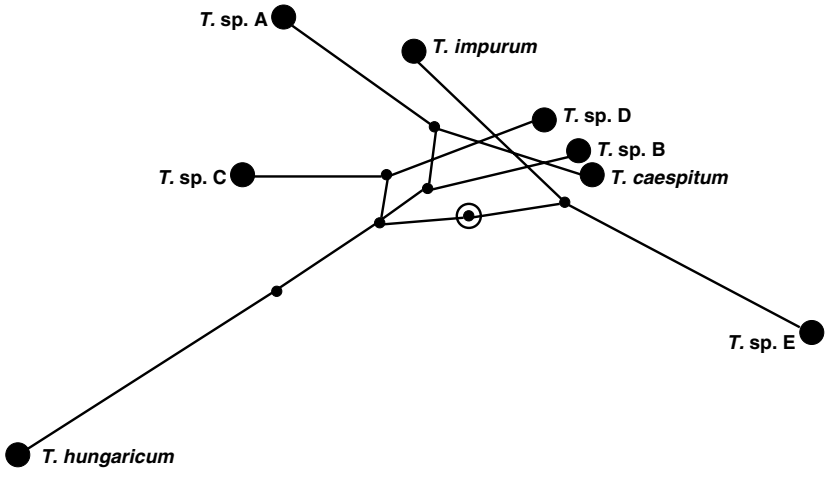

Fig. 4. COI NJ tree (Fig. 1) mapped into morphological space by evolutionary principle component analysis (all morphometric characters of workers included, Appendix B, online supplementary material, Appendix D). Taxa and nodes are indicated by dots, the root by an encircled dot.

tum, and species B cannot be distinguished might indicate the evolutionary basal situation, at least within their lineage. On the other hand, the genitalia of species A and *T. impurum* are highly similar, mainly differing in size.

Visualization of the mtDNA phylogeny in the worker morphological space provided by EPCA helps explain why the diversity behind *T. caespitum* and *T. impurum* had hitherto been ignored and why phylogenetically distant species have frequently been confused (compare Appendix A). The identification of *T. caespitum*, *T. impurum*, and the species A–E remains difficult. Two rules may be helpful: samples from Scandinavia should be *T. caespitum*, and any Central European *Tetramorium* from altitudes above 1300 m can be assigned to species A with some certainty, especially if the colony is polygynous (Steiner et al., 2003).

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Appendix D

Morphometric comparison of the workers of *Tetramorium hungaricum* and taxa A–G. Upper line: arithmetic mean ± 1 SD, lower line, in []: minimum and maximum values, n = number of measured specimens. Characters used in discriminant analysis (DA) of single workers and nest means indicated by +. Abbreviations of morphometric characters as in Appendix B, online supplementary material. All values are given as μm, except angles α, β, γ, δ, and ε which are given as °.

	hungaricum	A	B	C	D	E	F = *caespitum*	G = *impurum*	DA	
	(n = 48)	(n = 48)	(n = 71)	(n = 48)	(n = 36)	(n = 69)	(n = 54)	(n = 48)	Single workers	Nest means
α	73 ± 4	71 ± 5	74 ± 4	72 ± 4	75 ± 5	77 ± 5	74 ± 5	70 ± 4		
	[65, 82]	[60, 84]	[68, 84]	[62, 83]	[67, 91]	[68, 89]	[66, 89]	[62, 79]		
β	56 ± 2	53 ± 2	53 ± 2	56 ± 3	54 ± 3	53 ± 2	51 ± 2	52 ± 2		+
	[51, 61]	[48, 58]	[46, 57]	[50, 61]	[49, 60]	[48, 58]	[46, 56]	[47, 58]		
CL	675 ± 55	704 ± 46	767 ± 37	705 ± 44	761 ± 44	827 ± 74	767 ± 59	739 ± 41	+	
	[594, 842]	[620, 793]	[698, 859]	[616, 812]	[682, 855]	[651, 984]	[656, 981]	[634, 828]		
CW	666 ± 53	701 ± 50	759 ± 39	701 ± 46	744 ± 44	816 ± 76	765 ± 63	732 ± 44	+	+
	[583, 840]	[615, 797]	[689, 856]	[621, 799]	[670, 826]	[662, 977]	[624, 985]	[640, 836]		
dCV	31 ± 18	21 ± 3	22 ± 3	22 ± 2	22 ± 3	24 ± 2	23 ± 2	21 ± 2	+	
	[17, 135]	[16, 28]	[15, 30]	[18, 29]	[16, 27]	[19, 29]	[19, 29]	[15, 27]		
δ	90 ± 4	93 ± 4	92 ± 3	93 ± 3	91 ± 3	90 ± 4	92 ± 3	94 ± 4		+
	[82, 99]	[87, 101]	[86, 98]	[88, 99]	[83, 98]	[82, 98]	[83, 100]	[86, 103]		
EL	143 ± 9	137 ± 11	150 ± 9	138 ± 9	152 ± 10	167 ± 16	150 ± 12	139 ± 10	+	+
	[124, 170]	[115, 159]	[132, 174]	[122, 162]	[131, 170]	[129, 199]	[122, 189]	[120, 160]		
ε	74 ± 2	78 ± 3	79 ± 3	74 ± 3	78 ± 3	78 ± 5	81 ± 3	79 ± 3	+	
	[70, 83]	[73, 86]	[72, 86]	[68, 79]	[71, 86]	[70, 84]	[72, 87]	[73, 85]		
EW	107 ± 7	103 ± 7	112 ± 7	106 ± 8	115 ± 8	127 ± 13	112 ± 10	105 ± 8	+	
	[94, 129]	[92, 122]	[98, 127]	[92, 124]	[100, 129]	[100, 156]	[92, 138]	[90, 121]		
FL	256 ± 23	277 ± 23	299 ± 15	273 ± 20	298 ± 21	315 ± 30	304 ± 26	284 ± 17		
	[219, 335]	[233, 329]	[272, 336]	[231, 322]	[259, 346]	[247, 384]	[252, 396]	[250, 324]		
γ	67 ± 3	65 ± 3	63 ± 3	66 ± 3	61 ± 4	61 ± 3	62 ± 3	65 ± 4		
	[56, 71]	[58, 73]	[54, 70]	[58, 72]	[55, 68]	[54, 70]	[54, 71]	[55, 75]		
ML	755 ± 78	833 ± 66	900 ± 51	819 ± 62	903 ± 65	953 ± 99	905 ± 80	853 ± 56	+	+
	[639, 1005]	[718, 969]	[822, 1022]	[701, 962]	[775, 1019]	[757, 1180]	[762, 1192]	[739, 984]		
MPPL	217 ± 21	246 ± 18	266 ± 15	235 ± 17	263 ± 19	280 ± 27	271 ± 23	254 ± 17	+	
	[184, 286]	[127, 280]	[243, 303]	[200, 269]	[224, 298]	[224, 357]	[229, 341]	[221, 301]		
MPSP	283 ± 31	320 ± 30	338 ± 22	313 ± 28	332 ± 29	348 ± 41	338 ± 33	333 ± 27	+	
	[238, 390]	[270, 389]	[290, 390]	[252, 370]	[287, 393]	[274, 458]	[258, 456]	[276, 418]		
MPST	172 ± 17	190 ± 17	204 ± 11	189 ± 15	200 ± 14	216 ± 23	205 ± 19	197 ± 14	+	
	[146, 228]	[162, 224]	[179, 228]	[163, 223]	[175, 229]	[169, 274]	[167, 265]	[167, 236]		
MW	429 ± 38	456 ± 36	499 ± 29	450 ± 33	488 ± 34	517 ± 55	502 ± 45	468 ± 30	+	
	[375, 554]	[402, 524]	[456, 584]	[388, 527]	[427, 572]	[415, 660]	[415, 670]	[401, 527]		
PEH	233 ± 22	264 ± 19	277 ± 15	254 ± 17	278 ± 20	291 ± 31	278 ± 23	271 ± 16	+	+
	[206, 309]	[235, 306]	[251, 318]	[217, 283]	[242, 312]	[229, 365]	[236, 361]	[238, 316]		
PENL	160 ± 16	181 ± 13	198 ± 12	178 ± 11	186 ± 11	202 ± 19	198 ± 16	192 ± 13	+	+
	[137, 211]	[154, 206]	[175, 233]	[151, 203]	[165, 206]	[163, 250]	[157, 245]	[160, 229]		

(*continued on next page*)

Appendix D (*continued*)

	hungaricum	A	B	C	D	E	F = *caespitum*	G = *impurum*	DA	
	($n = 48$)	($n = 48$)	($n = 71$)	($n = 48$)	($n = 36$)	($n = 69$)	($n = 54$)	($n = 48$)	Single workers	Nest means
PEW	209 ± 21	240 ± 20	249 ± 16	228 ± 18	245 ± 19	263 ± 31	249 ± 24	240 ± 17		
	[171, 274]	[208, 294]	[225, 294]	[188, 278]	[208, 279]	[207, 330]	[201, 327]	[196, 282]		
PLSP	181 ± 20	192 ± 19	199 ± 15	194 ± 19	199 ± 21	207 ± 27	195 ± 21	197 ± 18		
	[141, 241]	[58, 233]	[165, 244]	[148, 232]	[165, 245]	[158, 279]	[147, 260]	[163, 235]		
PLST	187 ± 19	200 ± 16	216 ± 13	202 ± 15	219 ± 19	229 ± 25	212 ± 20	203 ± 14		
	[151, 253]	[175, 232]	[192, 243]	[174, 246]	[181, 266]	[181, 288]	[173, 274]	[174, 235]		
PnHL	164 ± 22	172 ± 25	211 ± 21	170 ± 21	195 ± 17	218 ± 25	212 ± 20	186 ± 21	+	+
	[136, 232]	[119, 235]	[157, 251]	[133, 214]	[160, 237]	[162, 272]	[154, 250]	[141, 228]		
PoOc	264 ± 26	281 ± 17	307 ± 16	275 ± 17	297 ± 17	319 ± 27	306 ± 23	293 ± 16		
	[231, 345]	[246, 314]	[281, 341]	[236, 309]	[272, 334]	[256, 378]	[256, 375]	[244, 320]		
PosSPl	274 ± 29	306 ± 28	323 ± 22	299 ± 27	319 ± 28	334 ± 39	323 ± 31	319 ± 27		
	[232, 371]	[255, 369]	[271, 374]	[236, 358]	[273, 375]	[262, 445]	[253, 428]	[255, 403]		
PosSPu	54 ± 15	51 ± 15	60 ± 13	47 ± 16	47 ± 17	72 ± 19	62 ± 16	45 ± 16	+	+
	[19, 93]	[16, 88]	[30, 98]	[18, 84]	[25, 98]	[34, 119]	[25, 95]	[9, 77]		
PPH	237 ± 24	273 ± 23	287 ± 15	265 ± 20	288 ± 22	299 ± 30	289 ± 23	275 ± 18		
	[203, 319]	[235, 325]	[264, 327]	[231, 313]	[240, 342]	[242, 372]	[247, 368]	[224, 324]		
PPW	263 ± 25	300 ± 22	320 ± 20	293 ± 23	312 ± 23	330 ± 37	319 ± 28	306 ± 18	+	+
	[217, 342]	{263, 343]	[284, 385]	[254, 356]	[280, 365]	[267, 426]	[274, 413]	[253, 345]		
PreOc	161 ± 14	175 ± 14	188 ± 10	175 ± 13	186 ± 13	204 ± 21	188 ± 16	186 ± 13		
	[141, 210]	[149, 200]	[170, 215]	[152, 203]	[165, 213]	[167, 251]	[158, 226]	[155, 222]		
SLd	512 ± 46	540 ± 44	596 ± 35	542 ± 37	596 ± 41	645 ± 61	594 ± 44	575 ± 37	+	
	[442, 669]	[465, 628]	[534, 679]	[481, 620]	[509, 667]	[492, 772]	[498, 714]	[486, 634]		
SPBA	171 ± 18	197 ± 19	207 ± 15	184 ± 17	201 ± 20	216 ± 29	204 ± 23	203 ± 19		
	[147, 227]	[160, 241]	[174, 235]	[152, 218]	[166, 238]	[158, 299]	[158, 296]	[164, 265]		
SPST	128 ± 17	148 ± 19	154 ± 14	144 ± 16	158 ± 18	157 ± 23	153 ± 18	154 ± 16		
	[96, 186]	[112, 197]	[127, 190]	[111, 179]	[118, 199]	[113, 218]	[113, 216]	[114, 205]		
SPWI	180 ± 19	213 ± 27	214 ± 17	198 ± 22	214 ± 27	218 ± 34	213 ± 25	217 ± 22	+	+
	[142, 236]	[154, 299]	[177, 258]	[154, 246]	[165, 274]	[150, 294]	[154, 315]	[160, 285]		
SWd	63 ± 6	69 ± 5	73 ± 4	66 ± 4	75 ± 5	76 ± 7	74 ± 6	70 ± 4	+	+
	[53, 82]	[61, 81]	[64, 85]	[58, 78]	[66, 87]	[63, 94]	[63, 86]	[61, 78]		

Appendix E

Redescriptions of *Tetramorium caespitum* and *T. impurum* and characterizations of *Tetramorium* spp. A–E.

Redescription of *Tetramorium caespitum* (L.): Neotype: worker labelled "Sweden: Floghult Bohuslan (58°58′N 11°25′E), 100 m a.s.l., 21.VI.2000, leg. C.A. Collingwood, #i132" and "NEOTYPE", kept in the Natural History Museum Vienna. In case of destruction or loss of the neotype specimen, a replacement neotype can be designated from a series of 8 workers from the same nest sample, having identical sample number, kept in the Natural History Museum Vienna, the Naturkundemuseum Görlitz, and the private collection of Schlick-Steiner and Steiner; additionally, 1 worker from the same nest sample in 99% ethanol, p.a. quality, having identical sample number plus the label "DNA voucher", is kept in the Natural History Museum Vienna. **Worker** (Morphometric characterization in Appendix D): Medium sized (CL and CW medium). Propodeum rather flat (PosSPl and PosSPu relative to CL + CW small). Tips of propodeal spines positioned rather far caudally (γ small, PLSP relative to CL + CW small). Anteroventral corner of the metapleuron seen in lateral view positioned rather frontally (ε large, MPPL relative to CL + CW large). Male (Fig. 1): Squama in ventral view with sharp edges, thickness of squama in ventral view <50 μm. No differences found to *Tetramorium hungaricum* and *T.* sp. B. mtDNA: 18 haplotypes of COI known (Appendix C, online supplementary material; for GenBank accession numbers see Appendix A, online supplementary material), COI GenBank accession number of worker (completely destroyed for DNA extraction) from neotype colony: AY909376.

Redescription of *Tetramorium impurum* (Förster, 1850): Neotype: worker labelled "Belgium: Mirwart vic. Saint-Hubert (50°02′N 05°16′E), 360 m a.s.l., 4.VIII.2000, leg. Y. Roisin, #i73" and "NEOTYPE", kept in the Natural History Museum Vienna. In case of destruction or loss of the neotype specimen, a replacement neotype can be designated from a series of 7 workers from the same nest sample, having identical sample number, kept in the Natural History Museum Vienna, the Naturkundemuseum Görlitz, and the private collection of Schlick-Steiner and Steiner; additionally, 1 worker from the same nest sample in 99% ethanol, p.a. quality, having identical sample number plus the label "DNA voucher", is kept in the Natural History Museum Vienna. Worker (Morphometric characterization in Appendix D): Medium sized (CL and CW medium). Eye short and narrow (EL and EW small). Mesosoma rather short (ML relative to CL+CW small). Propodeal spines long (α small, SPST relative to CL + CW large), diverging rather strongly (SPWI relative to CL + CW large). Tip of propodeal spine positioned rather dorsally (MPSP and PosSPl relative to CL + CW large). Propodeum ascends only slightly frontal of the spine (PosSPu relative to

CL + CW small). **Male** (Fig. 1): Vertical basal diameter of stipes in posterior view >700 μm. Squama in dorsal, posterior, and lateral view with rounded edges. mtDNA: five haplotypes of COI known (Appendix C; for GenBank accession numbers see Appendix A), COI Genbank accession number of worker (completely destroyed for DNA extraction) from neotype colony: AY909369.

Characterization of *Tetramorium hungaricum*: Worker (Morphometric characterization in Appendix D): Small sized (CL and CW small). Carinae on vertex few (dCV large) and low. Hairs on pronotal corners rather short (PnHL small). Propodeum rather flat (PosSPl and PosSPu relative to CL + CW small). Propodeal spines short (SPST relative to CL + CW small). Tip of propodeal spine positioned rather ventrally (MPSP and PosSPl relative to CL + CW small) and frontally (γ large). Anteroventral corner of the metapleuron seen in lateral view positioned rather caudally (ε small, MPPL relative to CL + CW small). **Male** (Fig. 1): squama in ventral view with sharp edges, thickness of squama in ventral view <50 μm. No differences found to *Tetramorium caespitum* and *T.* sp. B. mtDNA: six haplotypes of COI known (Appendix E; for GenBank accession numbers see Appendix A).

Characterization of *Tetramorium* sp. A: Worker (Morphometric characterization in Appendix D): Small sized (CL and CW small). Hairs on pronotal corners rather short (PnHL small). Eye short and narrow (EL and EW small). Petiolar node rather wide (PEW relative to CL + CW large). Propodeal spines diverging rather strongly (SPWI relative to CL + CW large). Postpetiolar node rather high (PPH relative to CL + CW large). Male (Fig. 1): Squama in ventral view more or less rounded. Vertical basal diameter of stipes in posterior view <700 μm. mtDNA: 13 haplotypes of COI known (Appendix C; for GenBank accession numbers see Appendix A).

Characterization of *Tetramorium* sp. B: Worker (Morphometric characterization in Appendix D): Medium sized (CL and CW medium). Highly similar to *Tetramorium caespitum*, but propodeum higher (PosSPl and PosSPu relative to CL + CW larger), tip of propodeal spine positioned less caudally (γ larger, PLSP relative to CL + CW larger), anteroventral corner of the metapleuronseen in lateral view positioned less frontally (ε smaller, MPPL relative to CL + CW smaller). Male (Fig. 1): squama in ventral view with sharp edges, thickness of squama in ventral view <50 μm. No differences found to *Tetramorium caespitum* and *T. hungaricum*. mtDNA: 18 haplotypes of COI known (Appendix C; for GenBank accession numbers see Appendix A).

Characterization of *Tetramorium* sp. C: Worker (Morphometric characterization in Appendix D): Small sized (CL and CW small). Propodeum rather flat (PosSPl and PosSPu relative to CL + CW small). Eye short and narrow (EL and EW small). Hairs on pronotal corners rather short (PnHL small). Petiolar node short (PENL relative to CL + CW small). Distance between basal outer margin of tips rather short (SPBA relative to CL + CW small). Tip of propodeal spine positioned rather frontally (γ large, PLSP relative to CL + CW large). Anteroventral corner of the metapleuron seen in lateral view positioned rather caudally (ε small, MPPL relative to CL + CW small). Male unknown. mtDNA: 11 haplotypes of COI known (Appendix C; for GenBank accession numbers see Appendix A).

Characterization of *Tetramorium* sp. D: Worker (Morphometric characterization in Appendix D): Medium sized (CL and CW medium). Mesosoma rather long (ML relative to CL + CW large). Propodeum rather flat (PosSPl and PosSPu relative to CL + CW small). Distance between basal outer margin of tips rather short (SPBA relative to CL + CW small). Petiolar node rather short (PENL relative to CL + CW small). Postpetiolar node rather high (PPH relative to CL + CW large). Propodeal spines long (SPST relative to CL + CW large). Tip of propodeal spine positioned rather caudally (γ small). Propodeum ascends only slightly frontal of the spine (PosSPu relative to CL + CW small). Male (Fig. 1): squama in ventral view more or less rounded, in dorsal, posterior, and lateral view with at least one sharp edge. mtDNA: 19 haplotypes of COI known (Appendix C; for GenBank accession numbers see Appendix A).

Characterization of *Tetramorium* sp. E: Worker (Morphometric characterization in Appendix D): Large sized (CL and CW large). Propodeum rather flat (PosSPl and PosSPu relative to CL + CW small) but ascends considerably frontal of the spine (PosSPu relative to CL + CW large). Propodeal spines short (α large, SPST relative to CL + CW small). Tip of propodeal spine positioned rather ventrally (MPSP and PosSPl relative to CL + CW small) and caudally (γ large, PLSP relative to CL + CW small). Male (Fig. 1): Squama in ventral view with sharp edges, thickness of squama in ventral view >50 μm. mtDNA: 20 haplotypes of COI known (Appendix C; for GenBank accession numbers see Appendix A).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2006.03.005.

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