Org Divers Evol (2011) 11:107–134
DOI 10.1007/s13127-011-0045-3

ORIGINAL ARTICLE

ORGANISMS DIVERSITY & EVOLUTION

Mixed colonies and hybridisation of *Messor* harvester ant species (Hymenoptera: Formicidae)

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Received: 3 January 2010 / Accepted: 5 April 2011 / Published online: 15 May 2011

Abstract The Mediterranean harvester ant species *Messor minor*, *M.* cf. *wasmanni*, and *M. capitatus* can co-occur in the same habitat. In Italian populations, we encountered colonies that contained workers from more than one species as identified via standard morphology, as well as colonies with workers that appeared to be morphologically intermediate between species. This unusual finding required further analysis. We analysed such colonies using microsatellites, mitochondrial DNA and refined morphometrics, and a simple inference key for the colony-level interpretation of data from the three sources combined. We infer that *Messor minor* and *M.* cf. *wasmanni* engage in bidirectional interspecific gene flow. Hybrids between these two species are inferred to produce fertile offspring, which would indicate that barriers to hybridisation do not exist or can be completely overcome. This is unexpected, given that they are non-sister species and broadly sympatric in nature. Our findings also indicate the possible occurrence of hybrid-hybrid crosses, a phenomenon rarely observed in ants. We cautiously interpret the data at hand as in support of the interspecific gene flow considerably shaping the genetic makeup of populations, raising the question about a potential adaptive value of this hybridisation. *Messor capitatus* mixes with hybrids of the other two species, but we found no indication of hybridisation involving this species. We discuss various hypotheses on the causations of colony mixing and hybridisation in the three *Messor* species at the proximate and ultimate level.

Keywords Hybridisation · Bidirectional interspecific gene flow · Non-sister species · Inference key

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Electronic supplementary material The online version of this article (doi:10.1007/s13127-011-0045-3) contains supplementary material, which is available to authorized users.

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Introduction

Interspecific hybridisation has long been thought to occur in only a few animal species, but more recently as many as 10% of known animal species have been considered to hybridise (Mallet 2005). In most instances, however, hybridisation is not quantitatively relevant in the sense of interspecific gene flow. This is because it is rare on a per-individual basis, and because the vitality and fertility of hybrid offspring are frequently reduced (Mallet 2005).

Hybridisation research has become a fruitful field of evolutionary biology: Understanding the genomic "po-

rosity" (e.g. Gavrilets and Losos 2009) of species helps to address general questions of their emergence, separation and integrity (Coyne and Orr 2004). Closely related species are more likely to be involved in hybridisation because reproductive barriers may not yet have fully evolved (Mallet 2005). In contrast, natural hybridisation of non-sister species is generally considered to be rare (Dasmahapatra et al. 2007; but see, e.g., Schmidt and Sperling 2008; Steiner et al. 2010; Van der Have et al. 2011).

Here, we present a system of three Mediterranean harvester ants, *Messor minor* (André 1883), *M.* cf. *wasmanni* Krausse 1910 and *M. capitatus* (Latreille 1798)—for the rationale behind using these taxon names, see Appendix 1 below. In large parts of central and south Italy, these ants occur in sympatry in natural habitats and can build up co-occurring populations with high densities of colonies and individuals of each species (Baroni Urbani 1971; Solida et al. 2010; D. A. Grasso unpublished data). Our study was triggered by the finding of conspicuous colonies in some Italian habitats. Some of those colonies contained workers from more than one species as identified via standard morphology, and some colonies contained workers that appeared to be morphologically intermediate between *M. minor* and *M.* cf. *wasmanni* (D. A. Grasso unpubl.; B. Seifert unpubl.). We studied the situation in more detail, because ants have rarely been reported to have mixed colonies composed of more than one species (but see, e.g., Czechowski 2001; Hölldobler and Markl 1990), except for social-parasite colonies (Buschinger 2009). Moreover, we suspected that the 'intermediate' worker morphologies could possibly represent hybrids, which would be a remarkable finding given that the degree of morphological differentiation between the three species would suggest that they are not closely related to one another (see below).

Inferring phylogenetic relationships from levels of overall morphological similarity can be problematic, as sometimes revealed by later in-depth phylogenetic reconstructions (ant example: Maruyama et al. 2008). This also concerns distinction between sister and non-sister relationships on such basis: On the one hand, sister species separated for a long time and/or evolving under strong divergent selection could be considerably differentiated morphologically; on the other hand, non-sister species could be very similar morphologically, due to morphological convergence or stasis. However, more frequent than such difficult instances may be a pronounced morphological similarity of sister species. Some such species pairs were first treated explicitly as sister species based on morphological assessment, and later confirmed as such by molecular phylogenetics. An exemplary case is that of *Lasius turcicus* Santschi, 1921 and *L. neglectus* Van Loon, Boomsma & Andrasfalvy, 1990 (Seifert 2000; confirmed by Steiner et al. 2004).

Thus, the considerable degree of morphological differentiation of pure-species individuals of *M. capitatus*, *M. minor* and *M.* cf. *wasmanni* prompted us to hypothesise that these *Messor* species are non-sister species. Our assessment was based on material from several localities in the distribution range of these species, including from localities at which they occur in sympatry (B. Seifert unpubl.). (1) *Messor capitatus* is the species most different from the others. The strong depression of the straight dorsal propodeal profile below the level of the high, evenly curved promesonotum, the pronounced slenderness of the mesosoma and petiole, the posterior slope of the petiole node forming a very blunt angle or a shallow convexity, and a pronounced length of the scape and legs make the species readily identifiable. (2) *Messor* cf. *wasmanni* is significantly bigger than *M. minor*, has a clearly angled dorsolateral pronotum, a reduced posterior part of the psammophore, a much more protruding anterior scape-base corner, and the reddish mesosomal pigmentation has, in fresh material, a red-wine tinge. (3) *Messor minor* is significantly smaller than *M.* cf. *wasmanni*, has a fully rounded dorsolateral pronotum, a well-developed posterior part of the psammophore, a less protruding anterior scape-base corner, and the reddish mesosomal pigmentation has a strong yellowish component but never a red-wine tinge.

To test the morphology-based hypothesis that *Messor minor*, *M.* cf. *wasmanni* and *M. capitatus* are non-sister species, we reconstructed the molecular-phylogenetic relation of the three species using mitochondrial DNA (mtDNA). Moreover, we addressed the question of whether the makeup of conspicuous colonies of these *Messor* ants could indeed be explained by colony mixing and/or hybridisation. For this, we chose an integrative approach that takes advantage of the complementarity of genetic and phenotypic markers; such integrative approaches are most powerful in detecting and exploring hybridisation (Gaubert et al. 2005). Specifically, we combined microsatellites, mtDNA and refined morphometrics. Each of these data sources has particular advantages and disadvantages in tracing hybridisation. Microsatellites are nuclear DNA markers and therefore indicate F1 hybrids through the combination of alleles from each parental species in all loci (Helms Cahan and Keller 2003), but hybridisation may remain undetected by microsatellites if repeated backcrossing has occurred (cf. Goodman et al. 1999). mtDNA is maternally inherited and thus detects the species identity of the maternal species also in the case of hybridisation. Also, through disagreement with the results from nuclear DNA and/or morphology, mtDNA can unravel hybridisation when backcrossing with the paternal species has occurred, even if backcrossing has completely eliminated the nuclear genome of the maternal species from the hybrid (e.g. Schlick-Steiner et al. 2010; Seifert and Goropashnaya 2004); mtDNA alone cannot indicate hybridisation, however. Morphometrics facilitates a view of the nuclear

genome independent of molecular markers and has proven useful in detecting hybridisation in ants (Kulmuni et al. 2010; Seifert 1999, 2009; Seifert et al. 2010). Similar to microsatellites, morphometrics will fail to detect hybridisation in case of repeated backcrossing.

The overarching idea in addressing the question of possible colony mixing and/or hybridisation was to first use information from the three sources separately and then integrate it. In doing so, we used a simple inference key for the colony-level interpretation of the individual-level results from all disciplines combined. We discuss possible explanations for complex patterns that emerged in the colony-level inferences, and then compare our results with established patterns of colony mixing and hybridisation. We also discuss various hypotheses on the causation of colony mixing and hybridisation in the *Messor* species at the proximate and ultimate level, and consider how future research into this novel study system can solve open questions.

Material and methods

Sampling

Series of about 50 workers each were obtained from 33 native colonies of *Messor minor*, *M.* cf. *wasmanni* and *M. capitatus* at four sites in Italy (see also the Electronic Supplementary Material 1 in the online version of this paper). The sites and sampling dates were: site 1: Colle Verde (42.007°N, 12.621°E), August 2006; site 2: Rome (41.893°N, 12.548°E), October 2004; site 3: Castelporziano (41.893°N, 12.491°E), July 2005; site 4: Genzano di Lucania (40.854°N, 16.031°E), August 2005. Distance between any two colonies at a site was 10 m at the minimum. The colonies were estimated to contain several hundred to a few thousand workers. Workers were collected from one nest entrance per colony. *Messor* workers can engage in interspecific interference behaviour close to nest mounds (Grasso et al. 2004a), but this can be excluded as a potential error source in our sample, because interfering individuals behave in a typical, easily recognisable way and such behaviour was not observed during collecting of the workers analysed in this study. Thus, we can rule out that workers that did not indeed inhabit a particular nest were included in the sample from that nest. Voucher specimens are deposited at the Senckenberg Museum of Natural History, Görlitz, under the colony codes given in Electronic Supplementary Material 1.

DNA extraction

For molecular analyses, specimens were randomly chosen from the complete worker series, i.e. without identifying them morphologically. DNA of single individuals was extracted using the GenElute Mammalian DNA Extraction kit (Sigma; St. Louis, USA). DNA extraction from the samples from site 1 failed, despite repeated trials; DNA was extracted from a total of 84 workers and DNA quality was assessed by gel electrophoresis. Several extracts showed low DNA content and/or severe fragmentation.

Microsatellites

PCR was performed in a 15 µl reaction volume containing 1× reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each primer, 0.375 U Taq DNA polymerase (Sigma/Promega; Madison, USA), and about 60 ng template DNA. Forward primers were 5'-end labelled with a fluorescent dye, either 6-FAM, HEX or TET. Cycling conditions were 94°C for 30 s, 60°C for 1 min and 72°C for 45 s for 32 cycles, with an initial denaturation step at 94°C for 5 min and a final extension step at 68°C for 20 min. Loci and primers used are given in Table 1. Fragments were run on an ABI PRISM 310 genetic analyser (Applied Biosystems). GeneScan 500-TAMRA was used as internal size standard. GENESCAN and GENOTYPER software (Applied Biosystems) was used for fragment analysis data processing. A total of 78 workers was successfully genotyped (Electronic Supplementary Material 1). In six extracts, DNA concentration was too low for amplifying the complete set of microsatellites; thus, these extracts were skipped.

Table 1 Details on the microsatellites used: primer sequences, repeat motifs, and references or, for unpublished loci, GenBank accession numbers

Locus	Primer sequences (5'–3')	Repeat motif	Reference / GenBank acc.
Ms2A	CACGTAGGACGAACGTTG TAGAAATGGGTAGGCGTTCG	$(AG)_{20}$	Arthofer et al. (2005)
Ms2D	CGGCACGGAGACAATACTTC GCTGTTCGGCGAAAACTATC	$(TC)_4(TC)_{25}(TC)_4$	Arthofer et al. (2005)
Ms24B	CCTTTGCCGTGAAAATC ATCGATTATCGCCTGAGC	$(CT)_{12}G(TC)_3$	unpublished, EU441277
Ms13J	GGATCGTTCCCTCTTCGTT CAGGGATTTGCGTGACCTAT	$(CT)_{11}$	unpublished, EU441278

Analysis of multilocus genotypes allows inference of genetic ancestry without relying on prior information on sampling locations or species identity. We used a model-based Bayesian clustering algorithm implemented in STRUCTURE 2.3.3 (Pritchard et al. 2000) that identifies subgroups with distinctive allele frequencies. The program places specimens into K clusters, where K is established in advance but can be varied across runs. The program can be used to evaluate a series of possible values for K using posterior probabilities, but here we used $K=3$ in all runs, as this was the number of pure species among which we aimed to identify potential hybridisation via admixture in the STRUCTURE results (cf. McDevitt et al. 2009). As done in other hybridisation analyses (e.g. Becquet et al. 2007; Senn and Pemberton 2009), we used the admixture model with correlated allele frequencies. Using the recommendations of Pritchard et al. (2010), we empirically determined appropriate numbers of generations: We varied the number of generations for the burn-in and the data-collection phase from 10,000 / 90,000 to 100,000 / 900,000 to 1,000,000 / 9,000,000; based on the very good consistency of the values of summary statistics and estimated memberships of specimens into clusters across these three runs, we considered 10,000 generations in the burn-in and 90,000 generations in the data-collection phase as sufficient and used these to conduct 20 additional, independent runs. We calculated the similarity in the placement of specimens into clusters among each pair of the 20 runs according to Nordborg et al. (2005), using the R-script Structure-sum (Ehrich 2006), and found very high consistency across runs (mean similarity coefficient 0.9978±0.0006 standard deviation; a coefficient of 1 would arise from completely identical runs); we therefore randomly selected one of the 20 runs as the final result.

We considered it to be more robust not to make prior assumptions about the origin of specific alleles, and rather used the results from the STRUCTURE analysis to score the occurrence of alleles in the pure species (as did, e.g., Kulmuni et al. 2010; Senn and Pemberton 2009); thus, alleles were identified as diagnostic for a species if they occurred in non-admixed individuals of exclusively this species.

mtDNA

PCR was performed in a 25 µl reaction volume containing 1 × reaction buffer, 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM forward and reverse primers, 0.625 U Taq DNA polymerase (Sigma / Promega; Madison, USA), and 3 µl template DNA. PCR conditions were 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 to amplify an approx. 1580 bp long segment of the *cytochrome c oxidase subunit I* (*COI*) gene were LCO1490 (Folmer et al. 1994) and Pat (Simon et al. 1994), but for some samples the newly developed primer "PatMessor", 5'-aatctattg cacttttctgcca-3', was used instead of Pat. The primers were also used as sequencing primers. PCR of some samples showed poor amplification of the 1.5 kb product. For those samples we used for PCR the new internal primers "Messorfor2", 5'-cggctctctaggaataatttatgc-3', and "Messorre2", 5'-atwgcaataattatagttgc-3', in various combinations with the above primers. PCR products were purified using he QIAquick PCR purification kit (Qiagen; Hilden, Germany) and directly sequenced in both directions using the Big Dye termination reaction chemistry (Applied Biosystems; Foster City, USA). A total of 58 workers were sequenced successfully (Electronic Supplementary Material 1); 26 extracts were skipped due to severe fragmentation of the DNA (as evident from gel electrophoresis of the extracts) resulting in no amplification of the 1580 bp target region. Nuclear mitochondrial pseudogene (numt; Bensasson et al. 2001) sequences have been identified in ants (Beckenbach 2009), but we ruled out the presence of potential numts by alertness for multiple PCR bands and sequence double peaks, by checking Phred values via Sequence Scanner software (http://www.appliedbiosystems.com/sequencescanner), and, after sequence alignment (see below), by searching for stop codons, indels or other, uncommon mutation patterns. *COI* sequences have been deposited with GenBank (accession numbers EU441219–EU441276).

Other *Messor* species occurring in sympatry with *M. minor*, *M.* cf. *wasmanni* and *M. capitatus* were *Messor bouvieri* Bondroit and *M.* cf. *structor* (Latreille) (this includes '*M. structor* sp.A' and '*M. structor* sp.B'), and homologous *COI* sequences were added from GenBank (accessions DQ074323–DQ074325; DQ074330, DQ074338, DQ074341, DQ074343, DQ074348, DQ074356). The sequences added from GenBank were 1255 bp long and overlapped in 1213 bp with the stretch sequenced for the present study; we therefore used 1213 bp of *COI* for phylogenetic reconstruction. Sequences were aligned with default settings of the program Clustal X (version 1.83; Thompson et al. 1997). For phylogenetic inferences we used neighbour joining (NJ) based on distance according to Tamura and Nei (1993) as implemented in MEGA (version 2.1; Kumar et al. 2001), maximum parsimony (MP) as implemented in PAUP* (test version 4.0b3a; Swofford 1998), and maximum likelihood (ML) as implemented in RAxML (version 7.2.7; Stamatakis et al. 2008); the ML analyses were performed through the CIPRES Science Gateway V. 3.1 (http://www.phylo.org/sub_sections/portal/). The MP tree was generated with a heuristic search using tree bisection reconnection and a random-taxon-addition sequence with 10 replicates; MAXTREES was set to 1,000,000. Weighting of characters by the

maximum value of the rescaled consistency index was performed once. For the ML analysis, data were partitioned according to codon positions, and parameter estimation and branch length optimisation were done individually for each partition. GTR + Γ was used as model. For all trees, node support was calculated by 1000 bootstrap replicates (Felsenstein 1985). In the interpretation of the trees, we applied a node-support threshold of >75 (Pattengale et al. 2010; Soltis and Soltis 2003). As in the study by Schlick-Steiner et al. (2006), *Aphaenogaster iberica* Emery and the Nearctic species *Messor lobognathus* Andrews and *M. chamberlini* Wheeler were used as outgroups (GenBank accessions DQ074361–DQ074363). We have deposited the aligned data matrix with TreeBase (Study accession number S11446).

Morphometrics

Morphometric analysis was conducted on a separate set of individuals (244 workers) than for the preceding genetic analyses. This situation has the drawback that it cannot be excluded that apparent differences in the results between the disciplines might result from sampling artifacts. Unfortunately, however, subsequent analyses using the same set of individuals for all disciplines failed because DNA extractions from the gaster failed to amplify, either because of inhibition of the PCR by the gaster contents and/or DNA degradation.

Specimens for morphometrics were selected according to the following scheme. First, the external morphology of all workers was determined. Second, from colonies with workers that were considered to represent normal intraspecific variation, based on experience with intranidal variation in ants including in the genus *Messor*, workers were randomly selected. Third, from colonies with workers whose morphological variation surpassed what was considered as normal intraspecific variation, workers representing the extremes of the variation were selected. Measurements of 17 morphometric characters were made on mounted and dried specimens. Workers of *Messor* species are size polymorphic, and the morphometric primary data were therefore subjected to allometry corrections, by regression of character ratios against body size using the method of Seifert (2008); in doing so, we used cephalic size, the arithmetic mean of cephalic length and width, as a proxy for body size. Software SPSS 15.0 (SPSS Inc.) was used for all statistical analyses. For a detailed treatment of the morphometric procedure applied, see Appendix 2.

Inference key for colony-level interpretation of combined data

For a colony-by-colony interpretation of our multisource data regarding a potential shuffling of genomes at the individual and colony level, we designed a simple inference key (see Appendix 3). In detail, this key is designed for the interpretation of data for a set of individual workers from a given colony of eusocial male-haploid organisms that is suspected to contain genomes of more than one but a maximum of three species. It uses microsatellite, mtDNA and morphometric data. Based on the hereditary patterns established for the three data sources, the inference key classifies colonies based on five questions, as follows.

(i) What species are discernible in the colony?

(ii) How many individuals are involved in the workers' parenthood? The inference key accounts for male haploidy, in that in male-haploid, female-diploid organisms the maximum number of alleles per microsatellite consistent with a single mother mated once is three across all offspring, with at least one of the alleles occurring in all worker individuals, which are female (whereas for diploid organisms the corresponding value would be four, without additional constraints). If a single mtDNA haplotype has been retrieved from a colony, the presence of three alleles per microsatellite with none of these occurring in all individuals, or of more than three alleles per microsatellite, is used for the inference of either a single queen mated multiply or more than one queen per colony – no distinction is possible in these instances. The presence of more than one mtDNA haplotype is used to infer more than one queen.

(iii) Is there evidence of hybridisation, i.e. of gene flow across species? A colony is inferred to contain hybrids, if any one of the following three criteria applies to at least one individual from that colony: admixed microsatellite genotype, intraindividual combination of a pure-species microsatellite genotype of one species with a mtDNA haplotype of another species, hybrid phenotype.

(iv) In case of hybridisation, how far does it date back? F1 hybridisation is diagnosed against older hybridisation using the following rationales. With F1 hybrids, microsatellites, which are biparentally inherited nuclear DNA, should reveal a heterospecific 50:50 admixture, and morphology, which is nuclear encoded and thus likewise biparentally inherited, should be intermediate between species. With hybridisation older than F1, the hybrids should be more similar in microsatellites and morphology to any of the pure parental species than F1 hybrids would be. In the case of several or many backcrossings with the paternal species, both microsatellites and morphology would indicate the pure paternal species, but hybridisation older than F1 is then discernible via a mtDNA

haplotype of the maternal species (instances of a high number of backcrossings with the maternal species cannot be detected via our approach, i.e. colonies to which this potentially applies are classified as pure-species colonies in step *iii*).

(v) Likewise in case of hybridisation, is there information on its direction(s) concerning the identity of the parental species? mtDNA is used to infer the maternal species.

Results

Microsatellites and mtDNA

Analysis of the microsatellite data using the Bayesian clustering algorithm classified nine individuals, from five colonies, as hybrids of *M. minor* and *M.* cf. *wasmanni*, the remaining 69 as pure *M. minor*, *M.* cf. *wasmanni* or *M. capitatus* (Fig. 1a). In all three species, some microsatellite alleles diagnostic of the species were found in the sample (*M. minor*: 13, *M.* cf. *wasmanni*: 7, *M. capitatus*: 20; Table 2; but see Discussion for a critical treatment of diagnostic alleles). In three of the four loci (Ms24B, Ms2D, Ms13J; referred to as diagnostic loci below), *M. minor* and *M.* cf. *wasmanni* did not share any alleles; the fourth locus was monomorphic in these two species. The genotypes of the nine individuals inferred to be hybrids are given in Table 3. For the three individuals from colony M20, the data were consistent with F1 hybrids, as they had one allele each from both parental species in each diagnostic locus. For three other individuals (the 1st individual from M3, the one individual from M16, and the one from M61), the data were consistent with exclusively backcrossing with one of the parental species after the initial hybridisation, as these individuals were homozygous for one parental species in two diagnostic loci and had alleles from both parental species in the third diagnostic locus. The remaining three individuals (the 2nd and 3rd individuals from M3, the one individual from M40) were homozygous for *M.* cf. *wasmanni* in locus Ms24B but homozygous for *M. minor* in Ms2D and Ms13J; this pattern indicates hybrid-hybrid crosses (but backcrossing could have occurred in addition).

In the *COI* phylogeny (Fig. 1b), the haplotypes corresponding to *M. minor*, *M.* cf. *wasmanni* and *M. capitatus* formed three well-supported crown groups (bootstrap support 96–100), with the exception of *M. minor* in the ML analyses (bootstrap support<75). The trees also revealed that *M. minor* and *M.* cf. *wasmanni* are non-sister species (bootstrap support for monophyly of *M. minor*+*M. bouvieri* 86–99), and that *M. minor* and *M.* cf. *wasmanni* both are non-sister to *M. capitatus* (bootstrap support for monophyly of *M. minor*+*M. bouvieri*+*M.* cf. *wasmanni* 86–100). *Messor minor* and *M.* cf. *wasmanni* were retrieved as more closely related to one another than either was to *M. capitatus*.

Morphometrics

The primary data for the 17 morphometric characters and ratios calculated are given in Electronic Supplementary Material 2. A qualitative (i.e. non-morphometric) assessment (for details, see Appendix 2) was used to form a-priori hypotheses on the species identity for all 244 individuals measured: 60 workers were hypothesised to represent *Messor capitatus*; out of the remaining individuals, 48 were identified as typical *M. minor*, 66 as typical *M.* cf. *wasmanni*, and 70 as intermediate between the latter two species. No individuals were morphologically intermediate between *M. capitatus* and either of the two other species. A first allometry correction done over all species (Appendix 2; corrected data in Table 4) reduced the standard deviation and variance in the average of all characters and all distinguished entities to 70.1% and 49.2%, respectively. Consequently, morphological differences independent of individual size became more apparent for *M. minor*, *M.* cf. *wasmanni* and *M. capitatus*. For example, the data in Table 4 show that *M. minor* has the shortest head of all entities, and the longest eye and gular setae, *M.* cf. *wasmanni* the shortest eye, mid femur and shortest mesosoma, and *M. capitatus* the longest head, scape, mid-femur and mesosoma, the least angular posterior profile of the petiolar node, the lowest metapleuro-propodeal height, and the shortest gular setae of all species.

The allometry-corrected data were then used in a 2-class discriminant analysis with *M. capitatus* as one class and all other forms as the second class, using discriminant function D(1) (Appendix 2). Classification using D(1) confirmed the distinctness of all 60 workers hypothesised a priori to represent *M. capitatus* from the collective cluster of 184 specimens of *M. minor*, *M.* cf. *wasmanni,* and the supposed intermediates.

Fig. 1 Multisource data on *Messor minor*, hybrids of *M. minor* and ▶ *M.* cf. *wasmanni* (*mw*, *mmw*), *M.* cf. *wasmanni*, and *M. capitatus*; identities of samples are colour-coded (for site and colony codes, see Electronic Supplementary Material 1). **a** Assignment of microsatellite data of individuals (vertical bars) to three classes using a Bayesian clustering algorithm, illustrated in order of geographic origins (DNA extraction from site-1 samples failed) and colonies genotyped. **b** mtDNA phylogeny, including two additional *Messor* species; bootstrap values >75 shown at nodes (dashes, values ≤75) as NJ/MP/ML. **c** Classification of individuals hypothesised a priori on morphological grounds as *M. minor*, *M.* cf. *wasmanni* or hybrids, applying discriminant function D(2) to allometry-corrected morphometric data

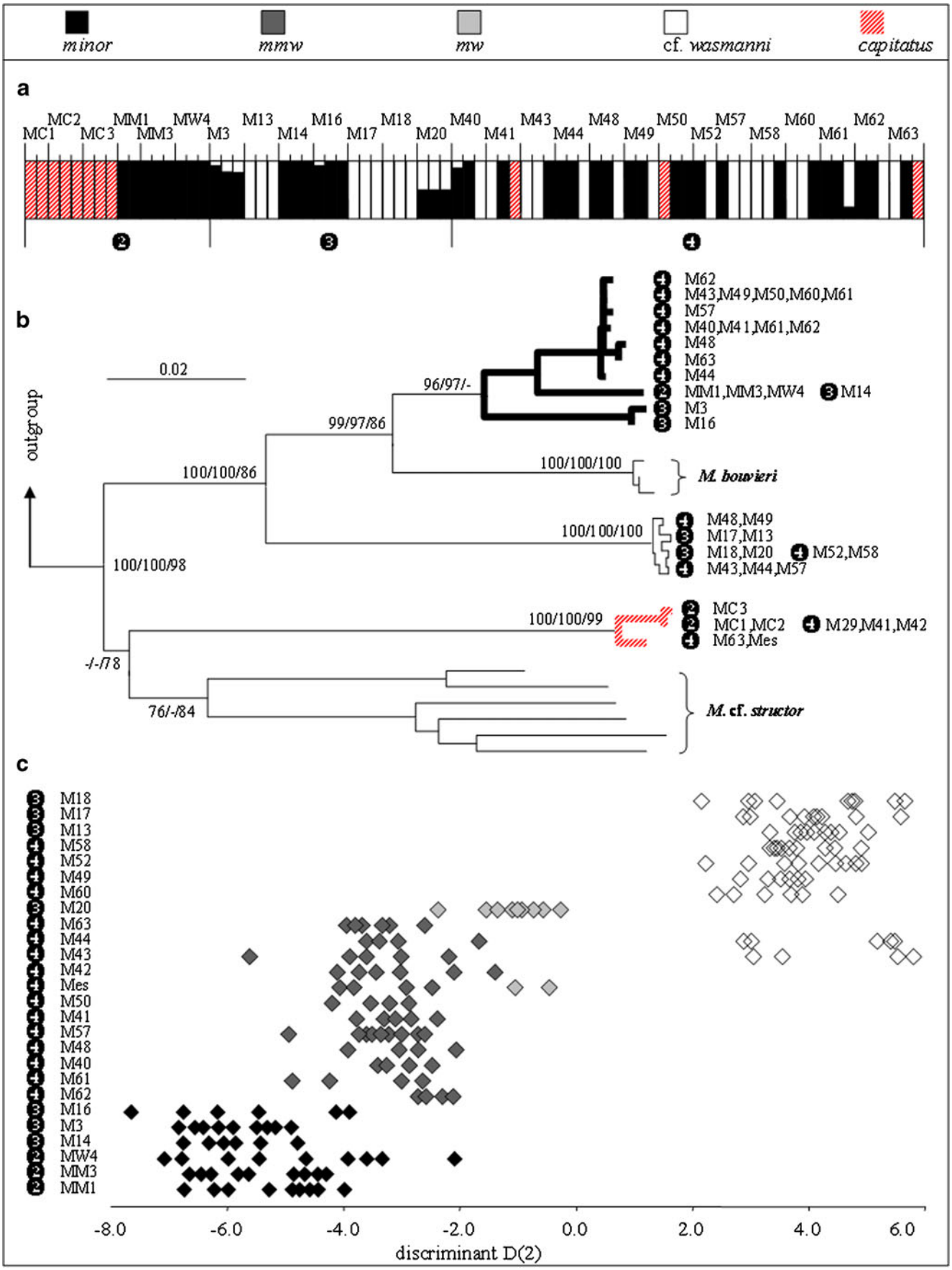

minor
mmw
mw
cf. wasmanni
capitatus
a
MC2 MM1 MW4 M13 M16 M18 M40 M43 M48 M50 M57 M60 M62
MC1 MC3 MM3 M3 M14 M17 M20 M41 M44 M49 M52 M58 M61 M63
2
3
4
b
0.02
outgroup
96/97/-
99/97/86
100/100/86
100/100/100
100/100/98
100/100/99
-/-/78
76/-/84
M62
M43,M49,M50,M60,M61
M57
M40,M41,M61,M62
M48
M63
M44
MM1,MM3,MW4
M14
M3
M16
M. bouvieri
M48,M49
M17,M13
M18,M20
M52,M58
M43,M44,M57
MC3
MC1,MC2
M29,M41,M42
M63,Mes
M. cf. structor
c
M18
M17
M13
M58
M52
M49
M60
M20
M63
M44
M43
M42
Mes
M50
M41
M57
M48
M40
M61
M62
M16
M3
M14
MW4
MM3
MM1
-8.0
-6.0
-4.0
-2.0
0.0
2.0
4.0
6.0
discriminant D(2)

Table 2 Alleles (and misamplifications) recorded for the four microsatellites analysed and allocated to *Messor minor*, hybrids of *M. minor* and *M.* cf. *wasmanni* (as inferred from Fig. 1a), *M.* cf. *wasmanni* and *M. capitatus*. Numbers in boldface: frequencies of alleles diagnostic in any of the species; n: number of individuals analysed

Microsatellite	Allele	*M. minor* n=33	Hybrid n=9	*M.* cf. *wasmanni* n=25	*M. capitatus* n=11
Ms24B	212	**9**			
	214	**47**	5		
	216		2	**2**	
	218		11	**40**	
	223				**3**
	232				**3**
	238				**2**
	250				**3**
	252				**2**
	262				**1**
	264				**2**
	272				**2**
	misamplifications	10	0	8	4
Ms2A	170	66	18	48	
	173				**21**
	187				**1**
	misamplifications	0	0	2	0
Ms2D	192				**8**
	194				**14**
	200		4	**50**	
	202	**10**	1		
	204	**10**	6		
	206	**5**			
	208	**5**	1		
	210	**8**	2		
	214	**2**	1		
	216	**20**	2		
	218	**4**	1		
	misamplifications	2	0	0	0
Ms13J	156	**38**	10		
	157	**19**	3		
	158	**9**			
	160			**13**	
	161		3	**11**	
	162		2	**21**	
	163			**5**	
	178				**1**
	179				**1**
	180				**1**
	181				**3**
	183				**4**
	184				**6**
	185				**1**
	186				**5**
	misamplifications	0	0	0	0

To give a more precise description of allometries for the *M. minor* - *M.* cf. *wasmanni* - intermediates cluster, the strongly deviating *M. capitatus* workers were excluded from further analysis. A second allometry correction of the

Table 3 Microsatellite alleles of the nine individuals inferred to be hybrids of *Messor minor* and *M.* cf. *wasmanni* based on microsatellite admixture using a Bayesian clustering algorithm. Colony codes as in Electronic Supplementary Material 1; numbering of individuals ("ind.") as in Figs. 1a, 2 (*left* to *right*). Diagnostic alleles shown in boldface: white on black background = diagnostic in *M. minor*; black on white background = diagnostic in *M.* cf. *wasmanni*

Colony	M3	M3	M3	M16	M20	M20	M20	M40	M61
Microsatellite	1st ind.	2nd ind.	3rd ind.	1st ind.	1st ind.	2nd ind.	3rd ind.	1st ind.	3rd ind.
Ms24B	**214**	**218**	**218**	**214**	**214**	**214**	**214**	**216**	**218**
	218	**218**	**218**	**218**	**218**	**218**	**218**	**216**	**218**
Ms2A	170	170	170	170	170	170	170	170	170
	170	170	170	170	170	170	170	170	170
Ms2D	**204**	**204**	**204**	**208**	**200**	**200**	**200**	**216**	**200**
	210	**210**	**214**	**218**	**204**	**204**	**204**	**216**	**202**
Ms13J	**156**	**156**	**156**	**156**	**156**	**156**	**156**	**156**	**162**
	157	**157**	**157**	**156**	**161**	**161**	**161**	**156**	**162**

uncorrected primary data was then calculated for the remaining 184 individuals using only the species-specific functions for typical *M.* cf. *wasmanni* and *M. minor* workers.

After the second allometry correction, the 70 workers with morphology assessed as intermediate between *M. minor* and *M.* cf. *wasmanni* were hypothesised to represent either *mw* (F1 hybrids) or *mmw* (hybrids backcrossed with *M. minor*). The individuals were grouped based on (*i*) the overall visual perception of angularity of the pronotum, petiole shape, general colouration, and sculpture of the entire body, and (*ii*) the values of corrected morphometric data relative to those for the specimens typical of the parental species. Subsequently, the data were used in a 2-class discriminant function, D(2) (Appendix 2), with *M. minor* and *M.* cf. *wasmanni* as classes; in other words, only the 114 specimens typical of *M. minor* or *M.* cf. *wasmanni* were allocated to classes a priori, while the data of the 70 intermediate specimens were run without using a-priori hypotheses. D(2) can be interpreted as describing a vector between typical *M. minor* and *M.* cf. *wasmanni* specimens (cf. Costedoat et al. 2005). The classification result from using D(2) for the 114 workers hypothesised to represent the parental species was in line with the a-priori hypotheses for all specimens. The remaining 70 individuals from 13 colonies were positioned fully intermediately between the parental species when sample means were considered. Their position along the vector also was in line with the a-priori hypotheses (not used as classes in D(2)) for the specimens of intermediate morphology (Fig. 1c): *mw* individuals fell exactly intermediate between those of the parental species, and *mmw* individuals fell between *M. minor* and *mw*. Concerning *mmw*, their plotting as a continuum between *M. minor* and (the hypothesised F1 hybrid) *mw* is in line with the inference of these individuals representing various stages in a sequence of repeated backcrossings of hybrids with *M. minor*.

Colony-level interpretation of combined data

Prior to colony-level interpretation of the individual-level results from all disciplines together, we reappraised whether the necessary precondition for using the inference key (Appendix 3) was met, namely that *M. minor*, *M.* cf. *wasmanni* and *M. capitatus* indeed are separate, well-diagnosable species. For *M. capitatus*, there was no reason to question its distinctiveness, as all three disciplines fully supported this for all its individuals. In contrast, microsatellites and morphometrics revealed some individuals to be intermediate between *M. minor* and *M.* cf. *wasmanni*. Considering microsatellites and morphometrics separately, we had inferred that these individuals represent hybrids between the two species. However, there would also be the alternative possibility that '*M. minor*' and '*M.* cf. *wasmanni*' do not represent separate species but intraspecific variation of a single species. Combining the results from all three data sources we refuted this alternative explanation, as the two species (*i*) were distinctive in worker morphology in all those colonies for which the combined data from all three sources suggested the same, pure species; (*ii*) each had diagnostic private alleles of microsatellites; and (*iii*) were supported as non-sister in the *COI* phylogeny, the intervening species being clearly recognised as separate species. The precondition for using the inference key was thus met.

We then applied the inference key (Appendix 3) to the multisource data as they are illustrated in Figs. 1 and 2; for a detailed treatment of the inferences for all colonies, see Appendix 4. Fourteen colonies were indicated to represent pure colonies of the three species (*M. minor*: M14, MM1, MM3, MW4; *M.* cf. *wasmanni*: M13, M17, M18; *M. capitatus*: M29, MC1, MC2, MC3, MC11, MC12, MC13). For these colonies, we found no incongruence with the inference of a single queen mated once: For colonies from which more than one worker had their mtDNA sequenced (colonies MC1, MC2, MC3, MM1, MM3, MW4), *COI*

Table 4 Morphometric data for *Messor minor*, *M.* cf. *wasmanni*, their hybrids *mmw* and *mw* as hypothesised on morphological grounds, and *M. capitatus*, after the first (over-all-species) allometry correction. Abbreviations of morphometric characters as in Appendix 2. Character PigC was not allometry corrected because of a strongly deviating trend in *M. capitatus* (see Appendix 2 for details). Data are given as arithmetic means±standard deviations, with minimum and maximum values in square brackets. Characters in which *M. minor* and *M.* cf. *wasmanni* are most different are shown in boldface. Hybrids are intermediate in all these key characters

	M. minor	mmw	mw	M. cf. wasmanni	M. capitatus
Characters, ratios	*n*=48	*n*=59	*n*=11	*n*=66	*n*=60
CS [μm]	1662±235	1757±172	1775±247	1862±298	1879±406
	[1235, 2267]	[1216, 1992]	[1459, 2344]	[1377, 2444]	[1212, 2786]
CL / $CW_{1.9}$	**0.964**±0.017	0.965±0.013	0.989±0.006	**0.990**±0.015	1.020±0.013
	[0.936, 1.014]	[0.930, 0.997]	[0.982, 1.006]	[0.965, 1.021]	[0.982, 1.047]
SL / $CS_{1.9}$	0.787±0.017	0.800±0.016	0.797±0.015	0.782±0.015	0.847±0.018
	[0.752, 0.821]	[0.770, 0.846]	[0.775, 0.829]	[0.748, 0.820]	[0.808, 0.896]
PoOc / $CL_{1.9}$	0.352±0.007	0.356±0.007	0.357±0.006	0.360±0.008	0.343±0.010
	[0.329, 0.366]	[0.341, 0.373]	[0.344, 0.367]	[0.345, 0.381]	[0.319, 0.362]
EL / $CS_{1.9}$	**0.199**±0.006	0.198±0.004	0.193±0.003	**0.178**±0.004	0.192±0.005
	[0.186, 0.213]	[0.184, 0.210]	[0.190, 0.198]	[0.167, 0.186]	[0.182, 0.204]
$LNdCV_{1.9}$	**3.37**±0.74	4.10±1.00	4.57±1.00	**5.92**±1.26	4.13±1.07
	[2.61, 5.51]	[2.73, 6.46]	[3.70, 7.20]	[3.56, 9.17]	[2.59, 6.83]
PigC	**27.1**±26.9	77.1±14.3	21.6±29.2	**90.5**±3.1	92.2±4.2
	[2, 92]	[30, 95]	[2, 90]	[80, 97]	[78, 98]
poGUHL / $CS_{1.9}$	**0.257**±0.031	0.235±0.027	0.195±0.022	**0.149**±0.025	0.131±0.020
	[0.195, 0.328]	[0.177, 0.305]	[0.158, 0.226]	[0.105, 0.213]	[0.095, 0.178]
ScBW / $CS_{1.9}$	**9.47**±0.29	9.23±0.34	9.74±0.37	**10.15**±0.32	10.09±0.51
	[8.86, 10.18]	[8.48, 10.08]	[8.95, 10.34]	[9.40, 10.81]	[8.14, 11.08]
ScBaC / $CS_{1.9}$	**3.99**±0.21	3.80±0.24	4.29±0.25	**4.61**±0.025	4.76±0.036
	[3.54, 4.58]	[3.23, 4.28]	[3.88, 4.77]	[4.12, 5.26]	[3.87, 5.40]
MW / $CS_{1.9}$	0.602±0.010	0.607±0.009	0.597±0.008	0.614±0.008	0.586±0.012
	[0.570, 0.632]	[0.583, 0.629]	[0.589, 0.614]	[0.597, 0.634]	[0.560, 0.615]
Fe2L / $CS_{1.9}$	**0.843**±0.018	0.843±0.013	0.822±0.019	**0.805**±0.016	0.872±0.019
	[0.799, 0.883]	[0.814, 0.883]	[0.794, 0.867]	[0.774, 0.846]	[0.823, 0.927]
STPLd / $CS_{1.9}$	0.306±0.008	0.308±0.006	0.304±0.007	0.312±0.006	0.299±0.007
	[0.290, 0.333]	[0.294, 0.324]	[0.297, 0.321]	[0.295, 0.329]	[0.284, 0.314]
PnHaa / $CS_{1.9}$	0.447±0.017	0.449±0.016	0.448±0.013	0.453±0.012	0.447±0.018
	[0.391, 0.475]	[0.408, 0.482]	[0.425, 0.461]	[0.427, 0.478]	[0.405, 0.483]
PrHaa / $CS_{1.9}$	0.356±0.014	0.355±0.014	0.343±0.007	0.349±0.013	0.328±0.013
	[0.324, 0.391]	[0.322, 0.390]	[0.334, 0.358]	[0.309, 0.371]	[0.293, 0.357]
ML / $CS_{1.9}$	**1.208**±0.019	1.204±0.017	1.195±0.015	**1.186**±0.022	1.224±0.024
	[1.158, 1.272]	[1.168, 1.237]	[1.177, 1.222]	[1.129, 1.242]	[1.159, 1.278]
$PEApo_{1.9}$	147.3±7.8	140.8±4.4	139.5±5.6	139.2±5.7	161.4±9.4
	[134, 173]	[129, 148]	[128, 148]	[127, 155]	[145, 180]

revealed a single haplotype, and for colonies for which microsatellite data were available (same as before, plus M13, M14, M17, M18), each locus had a maximum of three alleles, at least one of them occurring in all genotyped colony members. We did not infer the presence of mixed colonies containing just pure species. This is potentially problematic (see Discussion for details) because of our failure to use the same individuals in the molecular and the morphological analyses; i.e., it is based on the assumption that we would have detected the presence of more than one pure species in a colony in our morphological analysis, given that all workers of each colony (remaining after the molecular analysis) were examined morphologically.

Fourteen colonies (M3, M16, M20, M40, M43, M44, M48, M49, M52, M57, M58, M60, M61, M62) were inferred to contain hybrids of *M. minor* and *M.* cf. *wasmanni*. No evidence of the presence of *M. capitatus* was found for these colonies. The female lineages of these

colonies were not restricted to one of the species. In detail, the number of colonies for which we detected a single species in the female lineages was six for *M. minor* (M3, M16, M40, M60, M61, M62) and three for *M.* cf. *wasmanni* (M20, M52, M58), and the number of colonies for which we detected both species in the female lineages was five (M43, M44, M48, M49, M57). The age of hybridisation was inferred to range from F1 hybrids in one colony (M20) to older hybridisations in the remaining 13 colonies. The workers from three of the 14 colonies inferred to contain hybrids were inferred to stem from one queen per colony (M3, M16, M20), those from four colonies to stem from either one queen mated multiply or from more than one queen per colony (M40, M52, M58, M60), and those from seven colonies to stem from more than one queen per colony (M43, M44, M48, M49, M57, M61, M62). In the latter case, the female lineages were of the same species in two colonies (M61, M62) but of different species in the remaining five colonies.

Finally, five colonies were inferred to contain workers of pure *M. capitatus*, in addition to hybrids of *M. minor* and *M.* cf. *wasmanni* (M41, M42, M50, M63, Mes). In hybrids of three of these colonies we detected *M. minor* in the female lineages (M41, M50, M63), but for hybrids of the two other colonies no mtDNA data could be obtained.

We cannot rule out that colonies that were inferred to contain hybrids — with the likely exception of the F1 colony M20—possibly also contained either of the pure parental species, in addition to hybrids because different individuals were used for the molecular and the morphometric analyses. Taking into account that different genotypes and phenotypes can occur across offspring of the same hybrid queen, in the extreme case including ones indistinguishable from those of pure-species individuals, solely hybridisation was inferable for those colonies (for details, see Discussion below). In addition, our sampling may have been insufficient, because just 50 workers per colony were collected while colonies were estimated to contain considerably more workers.

Discussion

The three harvester-ant species *Messor minor*, *M.* cf. *wasmanni* and *M. capitatus* represent a novel study system for research into colony mixing and hybridisation in ants. In the following, we first critically assess our microsatellite, mtDNA and morphometrics data as a basis for our inferences of hybridisation, and discuss possible explanations for complex patterns in the colony-level inferences. Then we discuss the finding of mixed colonies in the context of findings from other ants, and highlight four traits of the inferred hybridisation. We conclude with a discussion of various hypotheses on the causation of colony mixing and hybridisation, and of open questions that remain to be addressed for validation of the hypotheses.

Assessing the data basis for our inferences of hybridisation

Concerning the microsatellite data, there are two aspects that should be critically assessed. Firstly, our finding of exclusively species-diagnostic alleles in loci Ms24B, Ms2D, and Ms13J needs to be treated with caution. For locus Ms24B, this is because 11 out of 78 individuals misamplified, which could have exacerbated any failure to detect alleles considered as diagnostic for one species in the other because of sampling artifacts. The likelihood of a false assessment in both cases is increased by the absence of gaps in allele lengths between species, especially between *Messor minor* and *M.* cf. *wasmanni* (Table 2). Out of the nine individuals from five colonies inferred to be hybrids based on microsatellites (Table 3), five individuals from three colonies (M3: 1st to 3rd individual; M16: 1st; M40: 1st) only carry diagnostic alleles of both *M. minor* and *M.* cf. *wasmanni* if the Ms24B alleles are indeed diagnostic. For two of these colonies (M3, M16), microsatellite admixture was the sole basis for inferring them to contain hybrids. Generally, for all four loci, analysis of more individuals might have undermined the diagnostic status of alleles. In fact, sharing of alleles among species is even likely at large sample sizes, due to ancestral hybridisation, ancestral polymorphism, or convergent mutations (Senn and Pemberton 2009). Thus, the use of diagnostic loci is inherently problematic: Alleles should never be treated as absolutely diagnostic, and the most that can be said is that the actual allele frequency is likely to be less than some small value (Goodman et al. 1999). We cannot evaluate how future findings (ideally also for a greater number of loci) would impact the microsatellite data and thus the inferences, but especially individuals inferred to be hybrids from the admixture results could then possibly be inferred not to be hybrids. On the other hand, Bayesian clustering algorithms generate membership coefficients for individuals by taking allele frequencies and linkage disequilibrium between alleles into account; thus, hybridisation can be inferred via such analysis also in the absence of diagnostic loci (Pritchard et al. 2010; Seifert et al. 2010; Senn and Pemberton 2009). In such instances, many of the individuals inferred to be hybrids can then carry alleles that are shared by pure-species individuals of both hybridising species (Senn and Pemberton 2009).

Secondly, the lowest membership coefficient we used for the interpretation of an individual as hybrid via the admixture analysis of microsatellites was 3.7% (Fig. 2: M16, 1st individual). Use of a low cut-off level for membership coefficients increases the risk of identifying

pure individuals as hybrids. However, other studies have used even lower values for the inference of hybridisation (e.g. McDevitt et al. 2009). Low cut-off values are considered justified, if the biological significance of low membership portions is indicated by individuals that are pure in microsatellites but have a mtDNA haplotype of the other species (cf. McDevitt et al. 2009), as is the case in our data (Fig. 2: e.g. M62, 3rd individual).

Concerning the mtDNA-based inferences, it needs to be mentioned that we cannot completely exclude incomplete lineage sorting for *COI*. In particular, for the two colonies (M52, M58) for which we have inferred hybridisation based on the combination of a *M.* cf. *wasmanni COI* haplotype but exclusively *M. minor* microsatellite alleles within one individual each, this could mean that these intraindividual incongruences actually represent not hybridisation but ancestral *COI* polymorphism in *M. minor* pure-species individuals. However, we consider incomplete lineage sorting as very unlikely here, because the *COI* haplotype found in the two individuals from M52 and M58 also was found in, for example, M18. Colony M18 has been inferred to be a pure-species *M.* cf. *wasmanni* colony (Fig. 2), and we consider it unlikely that no mutation has occurred after the split between *M.* cf. *wasmanni* and the ancestor of *M. bouvieri* and *M. minor* (Fig. 1b) in the 1213 base pairs analysed.

Concerning the morphometrics approach we have used for inferring hybridisation, we note that the results from morphometric analysis with the same methodology as used here were confirmed for >95% of the colonies by multilocus molecular evidence in another study of ant hybridisation (Seifert et al. 2010). However, our inferences are based on a smaller sample size, and assessing more colonies could reveal the variation within the pure species to be greater than our current data suggest. This could then lead to the inference of hybridisation for fewer colonies than at the moment; for nine of the 19 colonies inferred to contain hybrids (M41, M42, M43, M44, M48, M50, M57, M63, Mes), this inference is based on the morphometrics results alone. On the other hand, our morphometrics-based inferences for these nine colonies are lent weight by the four colonies (M20, M40, M61, M62) for which the inference of hybridisation via morphometrics is congruent with other evidence (without relying on microsatellite Ms24B).

In conclusion, we consider this inference as relatively firm for six of the 19 colonies inferred to contain hybrids: M20, M61 (basis for inference: admixed microsatellites, without relying on locus Ms24B, and morphometrics); M40, M52, M58, M62 (exclusively microsatellite alleles from one species, but a *COI* haplotype from the other species within individuals; for the latter two colonies additionally morphometrics). For eleven colonies, the inference is possibly more speculative: M41, M42, M43, M44, M48, M50, M57, M63, Mes (morphometrics alone); M3, M16 (admixed microsatellites, relying on Ms24B). For two colonies, the inference is definitely more speculative: M49, M60; here, we did not detect a hybrid individual via any of the three disciplines; for details on why we nevertheless consider hybridisation the most likely explanation, see below.

Fig. 2 Inferences for colonies (site and colony codes as in Electronic Supplementary Material 1) from applying an inference key (Appendices 2, 3) to microsatellite (top row within each colony), mtDNA (centre row), and morphometric (bottom row) data combined. Each coloured vertical bar represents a single worker analysed by the respective method; bars of different methods with identical horizontal position within a colony refer to the same worker. Properties depicted are: discernible species and hybrid identities (large discs, nuclear genomes; small inserted discs, mitochondrial genomes), minimum number of parents contributing to worker offspring, gene flow within and between species, and, in the latter case, age of hybridisation and its direction concerning identity of parental species. Grouped colonies share inferences

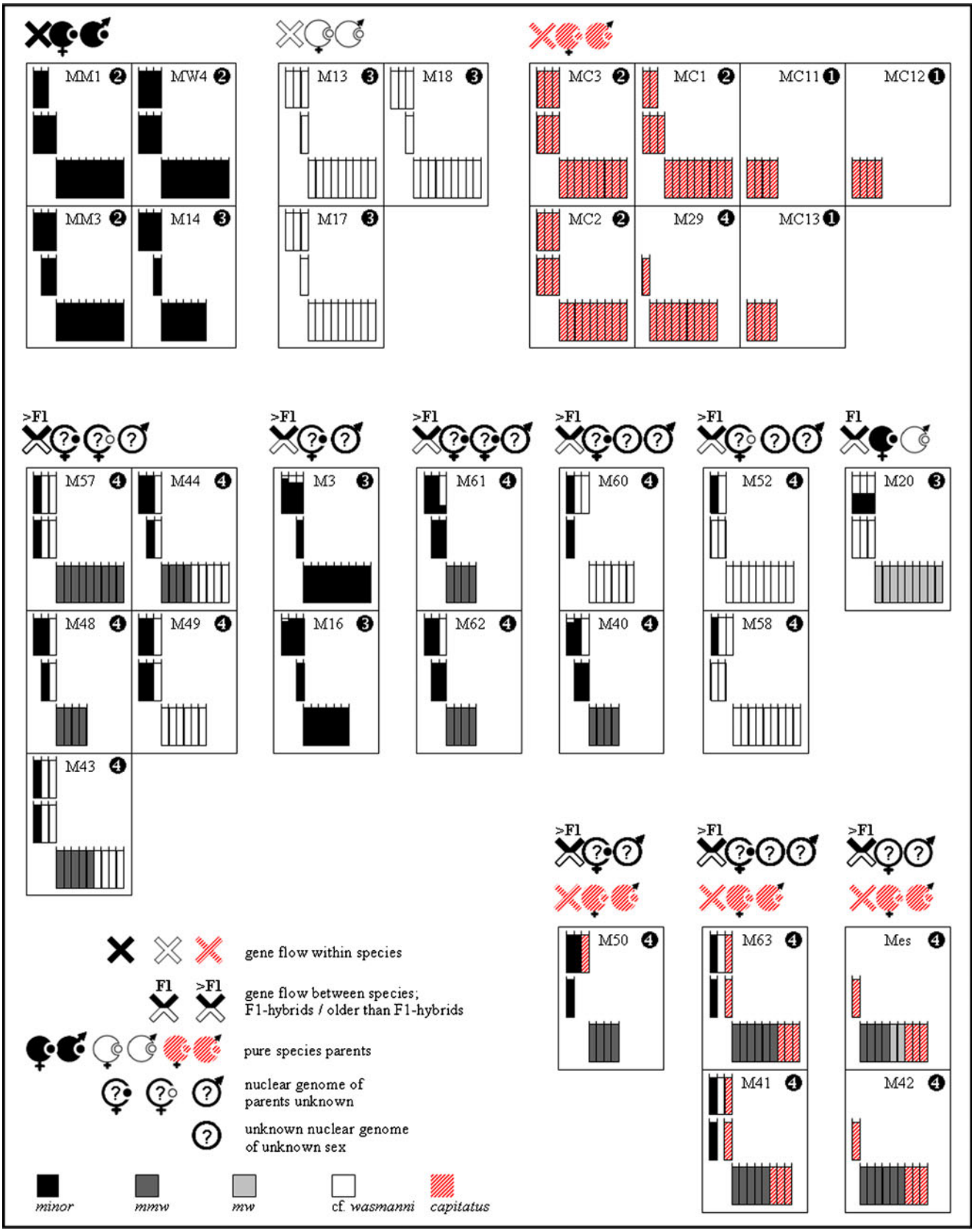
Possible explanations for complex patterns in the colony-level inferences

Here, we treat patterns in the colony-level inferences that are difficult to understand, and also discuss alternatives to our inferences. We have taken great care to avoid any confusion of samples, and therefore do not discuss the latter as a possible explanation.

Disparities between multilocus genotypes and phenotypes

In many colonies inferred to contain hybrids, disparities existed between multilocus genotypes and phenotypes: Either hybrid genotypes and pure-species phenotypes co-occurred in a colony, or vice versa. Genotype-phenotype disparities tend to arise in hybridisation studies and often remain unexplained (e.g. Seifert et al. 2010; Senn and Pemberton 2009). Five explanations could account for the discrepancies we have found, either individually or in combination.

(i) Non-representative sampling: We might have failed to detect the complete genotypic and phenotypic variation present in a colony. This could explain the detection of pure-species microsatellite genotypes of either or both parental species together with hybrid phenotypes (colonies M40, M41, M43, M44, M48, M50, M57, M61, M62, M63). It could also explain individuals identified as hybrids via the intraindividual combination of mtDNA of one species and a microsatellite genotype of the other species together with the pure-species phenotype of the species indicated by mtDNA (M52, M58). If the non-detected genotypes matching


MM1 2
MW4 2
MM3 2
M14 3
M13 3
M18 3
M17 3
MC3 2
MC1 2
MC11 1
MC12 1
MC2 2
M29 4
MC13 1
>F1
M57 4
M44 4
M48 4
M49 4
M43 4
>F1
M3 3
M16 3
>F1
M61 4
M62 4
>F1
M60 4
M40 4
>F1
M52 4
M58 4
F1
M20 3
>F1
M50 4
>F1
M63 4
M41 4
>F1
Mes 4
M42 4
gene flow within species
F1 >F1
gene flow between species;
F1-hybrids / older than F1-hybrids
pure species parents
nuclear genome of
parents unknown
unknown nuclear genome
of unknown sex
minor
mmw
mw
cf. wasmanni
capitatus

the detected phenotypes, and vice versa, were in fact present in the colonies, this could have been due to colony mixing involving one or more of the parental species, in addition to hybridisation and/or to more than two individuals being involved in the parenthood of the colony's workers. We cannot exclude such sampling artifacts, because we did not analyse all individuals under all disciplines. In particular, non-detection of phenotypes matching the detected genotypes may have occurred due to the removal of all individuals of these phenotypes when choosing individuals for the molecular analysis: This is supported by the presence of a pure-species *M. capitatus* genotype but failure to detect this species morphometrically in colony M50, which is explained best by this type of sampling artifact (given that hybridisation involving *M. capitatus* was not indicated in any instance).

(ii) PCR artifacts: Null alleles (Callen et al. 1993) and allelic dropout (Miller et al. 2002) can result in the appearance of individuals admixed in microsatellites as pure-species individuals, if one or more alleles from one of the species that were present were not amplified. This can be ruled out only if a pure-species microsatellite genotype was detected as heterozygous in all diagnostic loci. Here, this was not the case for any individual.

(iii) Poor hybrid detection via microsatellites due to low number of loci: Of our four microsatellites, three were diagnostic in *M. minor* and *M.* cf. *wasmanni*. Such low numbers of loci suffice to detect simple backcrosses and hybrid-hybrid crosses, but more loci would be needed to identify older backcrosses and hybrid-hybrid crosses with small genomic portions of one of the species. Consequently, we could have underestimated the number of hybrids via microsatellite admixture (cf. Randi 2008). Our detection of individuals pure in microsatellites but with a mtDNA haplotype of the other species lends weight to this explanation, and colonies with morphometrically but not genetically detected hybrids could be explained this way (M41, M43, M44, M48, M50, M57, M63).

(iv) Selection for hybrid phenotype: If the hybrid phenotype *mmw* confers some selective advantage, it could be fixed in hybrids sufficiently backcrossed to appear as pure-species individuals in their microsatellite genotypes (this could also apply when more loci are analysed). This could explain colonies in which hybrid phenotypes but not genotypes were detected (M41, M43, M44, M50, M48, M57, M63).

(v) Transmission ratio distortion (TRD): TRD, whereby the ratio of allelic segregation deviates from the ratio expected under Mendelian inheritance, can apply to coding and non-coding DNA, and has been observed from within populations to interspecific hybridisation (e.g. Koide et al. 2008; McMeniman and Barker 2005). It was posited also for *Formica* ants (Kulmuni et al. 2010). If in our case TRD affected either microsatellites or genes encoding worker morphology, in that in hybrids the transmission of alleles from one parental species is favoured over transmission from the other, individuals with apparently pure-species genotype or phenotype could in fact be hybrids. Our detection of hybrid microsatellite genotypes and phenotypes in the same or in other colonies is not necessarily in conflict with this explanation: TRD can result in complex patterns, including ones in which for the same set of loci Mendelian ratios are observed in offspring from some combinations of parental alleles, but TRD in others (McMeniman and Barker 2005).

Within-colony variation in genotype and/or phenotype

Strong variation across individuals of the same colony in genotype and/or phenotype regarding the contributions from the two parental species was another pattern we found in colonies inferred to contain hybrids. Concerning the genotype this applies to eleven colonies (M40, M41, M43, M44, M48, M52, M57, M58, M61, M62, M63; mostly with pure-species genotypes of both species), concerning the phenotype it applies to two colonies (M43, M44; hybrid and pure-species phenotype of one parental species); colonies M40 and M60, in which we did not detect a hybrid individual via any of the three disciplines, are treated separately in the next section. Increased variation in simple hybrids as compared to the parental species has been found in other studies as well (e.g. Grant and Grant 1994). The only ant example we know of is an unpublished *Cardiocondyla* crossbreeding experiment (K. Yamauchi pers. comm.): Worker offspring of an F1-hybrid queen mated with one paternal-species male had phenotypes ranging from that of the maternal species through intermediates to that of the paternal species; specific mechanisms remain unexplored. Four effects could explain the strong variation we found, individually or in combination.

(i) Non-representative sampling: Sampling artifacts could apply as discussed above. Within-colony variation could then be due to colonies being mixed of hybrids and either or both of the parental species. Alternatively, polyandry of hybrid queens could have occurred: If no recombination occurs across nuclear loci, offspring from an F1-hybrid queen mated with males of both pure species will include individuals resembling one parental species in nuclear loci and others resembling the other parental species, in addition to individuals having one allele each from both species in all loci.

Polyandry may not be very likely, because of the inference of monandry in the pure-species *M. minor* and *M.* cf. *wasmanni* colonies (Fig. 2), but it cannot be excluded. Genotypically, this situation could apply to individuals from five colonies (M40, M41, M52, M58, M63; concerning M40, it cannot apply to the 1st individual in Fig. 2 for which recombination is indicated, see Table 3): In each of these colonies, just one mtDNA haplotype but more than three alleles in at least one microsatellite were genotyped, and we may have failed to detect individuals with one allele each from both parental species in the three diagnostic loci, even though they were present in the colony. Phenotypically, this explanation could hold for two colonies (M43, M44): In these, we detected pure-species *M.* cf. *wasmanni* and hybrid phenotypes, and we would have failed to include the individuals with pure-species *M. minor* phenotype in the morphometric sample.

(ii) PCR artifacts: If such artifacts applied, workers with apparently homozygous pure-species microsatellite genotypes of different species but with a single mtDNA haplotype would in fact have been admixed; we would then have overestimated variation across these individuals. Such artifacts could apply to colonies M40, M41, M52, and M58.

(iii) Polygyny: In the colonies inferred to contain hybrids and strong variation across workers, the presence of more than one queen was either detected (M43, M44, M48, M57, M61, M62) or indicated as a possibility (M40, M41, M52, M58, M63; alternatively, one queen mated multiply). Presence of offspring from two hybrid queens in a colony, one genetically close to one species and mated with a male of this species, the other one genetically close to the other species and mated with a male of that species, could explain all instances of strong within-colony variation, genotypically (M40, M41, M43, M44, M48, M52, M57, M58, M61, M62, M63) and phenotypically (M43, 44).

(iv) TRD could explain those colonies with pure-species microsatellite genotypes of both parental species, but not intermediates that potentially contained polyandrous F1 queens (M41, M52, M58, M63; see above), if these queens each mated with males of both pure species.

Colonies M49 and M60

In colonies M49 and M60, we found pure-species genotypes of both *M. minor* and *M.* cf. *wasmanni*, but the phenotype of the latter only. We have inferred hybridisation, which certainly is speculative. Out of the above explanations, the following four should be considered here, the last three of which involve hybridisation. (*i*) Non-representative sampling: Failure to include in the morphometric analyses workers with the *M. minor* phenotype may have occurred; thus, both colonies could represent colony mixing involving the two pure species. We cannot rule out this explanation but consider it unlikely that the artifact applies to both colonies that would then be the only instances of pure-species colony mixing. (*ii*) PCR artifacts (the 1st and the 3rd worker of M49 and the 1st worker of M60, both in Fig. 2, were heterozygous in just one locus each, and the other three workers genotyped were not heterozygous in any locus). (*iii*) Poor hybrid detection via microsatellites due to low number of loci. (*iv*) TRD.

Colony mixing

Mixing of two or more ant species in the same colony has long been achieved experimentally, and has involved species from several genera (e.g. Carlin and Hölldobler 1983; Errard 1984). In nature, colony mixing has been considered as unusual, except in social parasites (Buschinger 2009), but has been observed occasionally in free-living genera, including *Formica*, *Pogonomyrmex* and *Temnothorax* (Czechowski 2001; Hölldobler and Markl 1990). We detected mixed colonies consisting of *M. capitatus* and hybrids of *M. minor* and *M.* cf. *wasmanni*. Also in certain instances of mixed colonies of other ants, colony mixing was reported or suspected to co-occur with hybridisation (Pusch et al. 2006b). Our finding raises the question about a potential causal connection between colony mixing and hybridisation in the *Messor* system. We did not infer the presence of mixed colonies consisting of pure *M. minor* and *M.* cf. *wasmanni* in our sample; this could be due either to a sample-size issue or to other sampling artifacts (see the preceding Discussion section for a treatment of colonies M49 and M60), or it could reflect a connection between colony mixing and hybridisation (see below). The lack of mixed colonies made up of hybrids of *M. minor* and *M.* cf. *wasmanni* and any of the pure parental species could likewise be due to our study design rather than to actual absence of such colony mixing in the sample (for details, see Results and the preceding Discussion section); as mentioned before, inferences for the F1-colony M20 are likely not affected by this problem. Sampling of more colonies and of more individuals per colony as well as analysis of the same individuals under all three disciplines will be needed to definitively settle these issues.

Four unexpected traits of hybridisation

There are four unexpected traits to our inferences concerning the hybridisation of *M. minor* and *M.* cf.

wasmanni. Firstly, whereas hybridisation of ant sister species is well documented (e.g. Pusch et al. 2006c), that of naturally sympatric non-sister species is less common (reported from *Temnothorax*, *Formica* and *Lasius*; Feldhaar et al. 2008; Kulmuni et al. 2010; Seifert 1999; Seifert and Goropashnaya 2004; Van der Have et al. 2011).

The second trait is our inference that the hybridisation results in bidirectional gene flow which extends beyond F1 hybrids, and thus in more than one hybrid morphology. This, again, is a phenomenon rarely observed in ants (Feldhaar et al. 2008; Van der Have et al. 2011). Such genetic introgression distinguishes the *Messor* case from hybrid-driven caste determination as established for *Pogonomyrmex* ants that results only in F1-hybrid workers (Helms Cahan and Keller 2003). Strategic hybridisation to overcome the lack of conspecific males when population densities are low, as suggested for *Acanthomyops* (Umphrey 2006) and *Chthonolasius* ants (Seifert 2006), would likewise result only in F1 hybrids; moreover, *M. minor* and *M.* cf. *wasmanni* are common species with high population densities (Baroni Urbani 1971; Solida et al. 2010; D. A. Grasso unpubl.). Hybrid speciation (Mallet 2007) would be expected to result in one specific hybrid, without genetic and morphological transitions to either parent, and the parental species and the hybrid would be expected to be spatially and/or ecoethologically separated (e.g. Gompert et al. 2006; Schwarz et al. 2005), rather than co-occurring in one spot as is the case at the present study's site 3.

The bidirectional gene flow we posit between non-sister species is significant from the evolutionary perspective because it indicates that prezygotic barriers are overcome and postzygotic ones have not formed, despite the relatively distant phylogenetic relation of the species. Concerning the postzygotic barriers, this is not surprising: They are generally believed to evolve more slowly than species diverge, although this is supported by few data so far (Coyne and Orr 2004; Turelli et al. 2001; but see Ross and Trager 1990 on introduced fire ants). Prezygotic barriers, in contrast, are known to be associated with ecological divergence (Funk et al. 2006) as well as to be important in speciation and in maintaining the separation of species. Accordingly, non-sister species occurring sympatrically are expected to be strongly isolated by prezygotic barriers (Coyne and Orr 2004). The absence of interspecific gene flow between *M. capitatus* and the remaining two species could reflect that *M. capitatus* is more distantly related to the other two than these are to one another, as suggested by the NJ and MP reconstructions (Fig. 1b): Hybridisation barriers could be fully evolved and thus prevent hybridisation.

The third trait is our inference that hybrid-hybrid crosses could occur in this system. This would be unexpected, because hybrid males have rarely been found in ants (Feldhaar et al. 2008). However, our inference involves uncertainty in that it relies on allele patterns for microsatellite Ms24B, which had a considerable misamplification rate (see above). If this uncertainty can be overcome in the future, in-depth analysis of the situation would be rewarding. For example, the in-depth analysis of *Formica polyctena* × *F. aquilonia* hybrid males led to the discovery of a novel, sex-specific pattern of transmission ratio distortion (Kulmuni et al. 2010).

The fourth trait is our inference that a considerable fraction of colonies contains hybrid individuals, and that the hybridisation thus shapes the genetic makeup of populations considerably. In contrast to hybrid zones of parapatric species, where such introgression is common (Barton 2001; ant example: Pusch et al. 2006a), these *Messor* ants are broadly sympatric. This unexpected inference of widespread introgression in populations of sympatric species raises the question about a selective advantage as opposed to a disadvantage of hybridisation in this system.

The how of colony mixing and hybridisation: hypotheses and open questions

This study establishes *that* colony mixing and probably hybridisation occur in this *Messor* system, but further work will be necessary to establish *how* these patterns arise. The latter question can be approached at two levels, corresponding to the proximate-ultimate dichotomy (Steiner et al. 2009). Answering the question at the proximate level necessitates drawing on the biology of *Messor minor*, *M.* cf. *wasmanni* and *M. capitatus*; in several respects, this will require further study, as insight into the behavioural ecology and reproductive biology of these ants is scarce (but see Grasso et al. 1999; 2002; 2004a; b; Harkness and Isham 1988; Solida et al. 2007, 2010). At the ultimate level, we will have to examine the long-term selection acting on colony mixing and hybridisation due to habitat variables and the presence of competing species.

At the proximate level, mixed colonies could occur via one or more of five mechanisms. (i) Raiding of heterospecific brood could occur, as established for *Pogonomyrmex* and *Messor* species (Hölldobler and Markl 1990; Rissing and Pollock 1991). For the *Messor* species under study, brood raiding was shown for incipient colonies in the laboratory (Grasso et al. 2004b), but the occurrence and frequency of brood raids under field conditions remain to be assessed.

(ii) Colony foundation through cooperation of queens, established for some *Messor* species (Grasso et al. 2002; Rissing and Pollock 1991), could occasionally involve more than one species (cf. Mori and Le Moli 1998; Schlick-Steiner et al. 2008), although this has not been reported for these species based on field data.

(iii) Young queens of one species could be adopted into established (and possibly queenless) colonies of another species. We have occasional observations potentially supporting this: After nuptial flights, newly mated queens of both *M. minor* and *M.* cf. *wasmanni* were observed digging next to well established con- and heterospecific colonies, including in the interspace of tunnels (Solida et al. 2010). Pursuing this possibility will also require assessing the degree of colony closure (the inaccessibility of a colony to non-members) in these ants.

(iv) Colony fusions or invasions could occur. This has been observed in *Temnothorax* ants (Pusch et al. 2006b), and achieved experimentally for *M. minor* and *M. capitatus* (Grasso et al. 2004a; b).

(v) Colony mixing could be directly caused by hybridisation; this is discussed under (*b*) below.

If hybridisation is indeed, as we suggest, not the exception in these ants, how might it occur? Investigating the proximate causes of hybridisation involves the following considerations. In ants, interspecific mating can be prevented by, among others, imprinted behaviour (Isingrini et al. 1985), and by the species specificity of contact pheromones (Beibl et al. 2007). In the hybridising *Messor* species, interspecific mating could occur via one or more of five mechanisms (a–e). (a) Hybridisation could occur during nuptial flight, by (single or multiple) mating of queens with exclusively heterospecific males, and not be causally connected to colony mixing. The opportunity for interspecific mating during nuptial flight could be considerable for *Messor minor* and *M.* cf. *wasmanni*, due to the frequent synchrony and similar flight altitudes of their nuptial flights (D. A. Grasso unpubl.). Given that we have posited the co-occurrence of colony mixing and hybridisation for those colonies that consist of *M. capitatus* and hybrids of *M. minor* and *M.* cf. *wasmanni*, support for this mechanism might arise if all other mechanisms are ruled out.

(b) Hybridisation could occur during nuptial flight and directly result also in colony mixing, namely through multiple mating of queens, with both con- and heterospecific males. Multiple mating of queens may be rather widespread in ants, and has been observed in *Messor ebeninus* Santschi, the only *Messor* species for which copulation observations are available (see review by Baer 2011). To assess this possibility, sperm from queen spermathecae needs to be analysed; however, the inference of a single queen mated once in the pure *M. minor* and the pure *M.* cf. *wasmanni* colonies tentatively indicates the absence of multiple mating in these ants in general. In any case colony M20, an F1 hybrid of *M. minor* and *M.* cf. *wasmanni*, would not be explicable under this mechanism, because no pure-species workers were found in this colony; the colonies comprising *M. capitatus* and hybrids of the other two species would not be explicable under this mechanism alone either.

(c) Hybridisation could occur during nuptial flight and might secondarily mediate colony mixing, in that heterospecifically mated queens or hybrid queens might need to invade pure-species *Messor* colonies (either of one of the parental species or of a species not involved in hybridisation) to successfully found a colony. The mixed colonies of *M. capitatus* and hybrids of *M. minor* and *M.* cf. *wasmanni* potentially support this mechanism. Colony M20, however, would not be explicable under this mechanism either, for the same reason as before. The other colonies containing hybrids cannot be used to validate this mechanism because, as mentioned above, we cannot rule out that some of the pure parental species co-occurred with hybrids in our sample.

(d) Hybridisation could occur during nuptial flight and might secondarily mediate colony mixing, in that hybrid colonies could be more likely than pure-species colonies to be raided or invaded by pure-species colonies. The colonies of *M. capitatus* plus hybrids support this mechanism, as does the inferred lack of mixed colonies of pure-species *M. minor* and *M.* cf. *wasmanni* in our data.

(e) Hybridisation might be socially mediated and reflect colony mixing. Here, interspecific mating could be mediated by the presence of both con- and heterospecific partners in the colony; this would jam the pheromone-based mate-recognition system (Feldhaar et al. 2008) or effect early behavioural imprinting and the aligning of contact pheromones, if such pheromone systems are fully developed at all in these species. If colony mixing is due to brood raiding, heterospecific sexuals would result from sexual brood brought into the colony among other brood. The inferred lack of mixed colonies made up of pure *M. minor* and *M.* cf. *wasmanni* in our data would be explicable under this mechanism, if colony mixing via one or more of mechanisms (i)–(iv) rarely occurs between these species but relatively frequently results in hybridisation when it does occur. Validating the possibility of socially mediated hybridisation requires studies that identify the likelihood of heterospecific mating by sexuals raised in mixed colonies as opposed to ones from colonies of the pure parental species.

In addition to a deeper investigation into the biology of the three *Messor* species, validating the various potential mechanisms to colony mixing and hybridisation will require analysing the frequencies of the various types of colonies – via larger samples per population and on a geographically larger scale. Analysis of the same individuals under all disciplines will be mandatory, in order to help distinguish colonies respectively comprising one, two or more pure species, hybrids, and hybrids plus pure species including any of the parental species of hybrids (the latter

could not be distinguished from hybrid colonies in this study). Also, analysis of workers of different ages in a colony would be useful, as has been performed for *Temnothorax* ants by Pusch et al. (2006a).

The question about the ultimate causations of colony mixing and hybridisation in this system is difficult to answer. This is because eusocial organisms such as ants present more levels of selection than other organisms, from individual to colony to population, and because selection is expected to interact and even be antagonistic between levels (Crozier and Pamilo 1996). An answer to the question about the ultimate causations will also require answering that about proximate causations. Nevertheless, some aspects can be discussed already.

The ultimate causations for colony mixing (those involving brood raiding or colony fusion) could be colony-level selection for territoriality (cf. Hölldobler and Markl 1990) and population-level selection for a K strategy (MacArthur and Wilson 1967) that reduces the likelihood of exceeding the habitat's carrying capacity. If brood raiding or colony invasion were to occur after hybridisation, by any of the parental species, this could represent selection against hybridisation at the population level. If, however, mixed colonies are due to cooperative colony foundation by queens of more than one species or to adoption of young queens into an established colony, then adaptiveness in the sense of, for example, increased efficiency in resource utilisation could be assumed at the colony level. This scenario would not be explicable by evolutionary theory that involves relatedness, but a similar paradox occurs in ants intraspecifically, i.e. supercolonies (reviewed by Steiner et al. 2009). Alternatively, if adoption of young heterospecific queens occurs, this could reflect an early stage of selection for socially parasitic behaviour. Here, the offspring of the young queen could capitalise on resources provided by the adopting colony. If colony mixing emerges directly from the mating of queens with con- and heterospecific males, the ultimate causation would likely be one for hybridisation.

The issue of selection for and against hybridisation has been debated intensely (e.g. Arnold 2006; Barton 2001; Mallet 2005). For most instances of hybridisation, a selective disadvantage would be expected for hybrids (Barton 2001). This could also be the case in the *Messor* system: Brood raiding or colony fusion could represent selection against hybridisation. At the same time, as gene flow between *Messor minor* and *M.* cf. *wasmanni* has been cautiously inferred to result in a considerable number of colonies that contain hybrids and occurs in a native system, its persistence could reflect some selective advantage. Thus, the parental species might benefit through increase of allele diversity, one generally accepted ultimate cause of hybridisation (Arnold 2006; Mallet 2005). Alternatively, hybrids themselves could have an adaptive advantage (see also the Discussion section entitled "Possible explanations for complex patterns in the colony-level inferences"), as was proposed for *Acanthomyops* ants (Umphrey 2006) – while hybrids are less fit than the parental species on average, some new gene combinations may be fitter than the parents, even in the parental environment (Barton 2001). One among the many possibilities of how a hybrid phenotype could be of adaptive value is resource utilization: The *Messor* species examined differ in the size of the seeds they use (Solida et al. 2007), and a certain seed size fraction could be more accessible to hybrids than to either parent; the frequency of pure-species and hybrid colonies could then result from the local selective regime (cf. Höfener et al. 1996; Seifert et al. 2010). Another scenario would be adaptiveness of hybrid colonies to unpredictable but recurrent environmental changes: This could be examined within the ecological-evolutionary framework of risk-spreading (Hopper 1999).

We have detected colony mixing and hybridisation in the *Messor* system presented here. We were unable to provide unambiguous proof of hybridisation for all colonies inferred to contain hybrids, but the evidence for colony mixing that involves pure *M. capitatus* is relatively firm, as is the evidence for hybridisation of *M. minor* and *M.* cf. *wasmanni* in the case of the one F1 colony and in that of five colonies with hybrids older than F1. We have thus laid the foundation for future studies on the genotypic and phenotypic mechanisms and the frequency of hybridisation in this system, as well as on potential causal connections between colony mixing and interspecific gene flow at the proximate and ultimate levels.

Acknowledgements Francesco Le Moli, doyen of research on the behavioural ecology of ants, passed away when the manuscript was at an early stage. Ross H. Crozier, pioneer of sociobiology in both theoretical and empirical genetic analysis, passed away when the manuscript was at the stage of revision. The remaining authors gratefully dedicate this paper to their memory. We thank Katsusuke Yamauchi for sharing unpublished data, Sandor Csősz, Heino Konrad, Susanne Krumböck, Karl Moder, Fabrizio Rigato, Andrea Stradner and Phil S. Ward for multiple support, and Michael Stachowitsch for a linguistic revision of the manuscript. Four anonymous referees and Editor-in-Chief Olaf R. P. Bininda-Emonds provided important input. RHC was supported by the Australian Research Council (DP0665890); BCS and FMS were supported by the Austrian Science Fund (J2639-B17, J2642-B17).

Appendices

Appendix 1 Allocation of species names

Allocation of species names is complicated for European *Messor* ants because of the lack of a recent taxonomic

revision. We applied the worker-morphology based taxonomic descriptions of *Messor minor* by André (1883) and of *M. capitatus* by Latreille (1798), and followed the naming tradition of Italian ant taxonomists, e.g. Fabrizio Rigato (pers. comm. 2008).

Some samples from our study system are morphologically identical with multiple specimens from Italian museum collections identified as "*Messor wasmanni* Krausse 1910". The corresponding description by Krausse (1910) is not interpretable, however, as it is merely a mention of stridulation differences from other species. The subsequent description by Krausse (1911) is very brief ("in Größe und Färbung dem *M. barbarus* sich nähernd, indes hinsichtlich des Pronotums quergestreift wie bei *M. barbarus minor* André", i.e. 'in size and colouration approaching *M. barbarus*, while the pronotum is transversely striated as in *M. barbarus minor* André'). No type specimens of *M. wasmanni* could be found (B. Seifert, unpubl.), which would make it desirable to designate a neotype from the type locality (Asuni, Sardinia). However, in Sardinia, four *Messor* species have been recorded (Baroni Urbani 1971) – *M. capitatus*, *M. minor*, *M. wasmanni*, and *M. structor* (Latreille 1798), to all of which the character of a transversely striated pronotum applies – complicating a neotype designation. *Messor wasmanni* might thus be considered as a nomen dubium at some point in the future.

At the same time, the samples mentioned are morphologically identical with the type specimens of *Messor concolor* Santschi 1927 (see Santschi 1927) that are preserved at the Museo Civico di Storia Naturale, Genova (S. Csősz pers. comm.). By application of ICZN (1999) Code Article 45.5, *M. concolor* Santschi is the valid name for *Messor barbarus* subsp. *semirufus* var. *concolor* Emery 1908. The pictorial information on type material in Emery (1908) – which also is the type material of *M. concolor* Santschi – matches the relevant samples of the present study (medium-length setae on gula from anterior margin backwards to level of posterior margin of eye, and much shorter setae posterior from that level). Conspecificity with *M. concolor* is further supported by the clustering of mitochondrial DNA sequences from the relevant present samples with four sequences deposited at GenBank (DQ074326–DQ074329) from specimens identified as *M. concolor* by comparison with the type material (Schlick-Steiner et al. 2006).

In conclusion, we refer to those samples as "*Messor* cf. *wasmanni*", thus acknowledging that at present it is uncertain whether the future will bring about that *M. wasmanni* is considered a nomen dubium, in which case the valid name may turn out to be *M. concolor*, or whether the name *M. wasmanni* will be conserved by a neotype designation.

Appendix 2 Morphometric procedure in detail

Morphometric analysis (244 workers) was performed without knowledge of the molecular genetic results. Specimens were selected from each colony after specimens for genetic analysis had been selected, applying the following rationale: Whenever a screening of all workers based on external morphology suggested a heterogeneity beyond what was considered to be normal intraspecific variation – on the basis of experience with intranidal variation in ants, including in the genus *Messor* – specimens representing the extreme ends of the variation within the colony were chosen.

All morphometric measurements were made on mounted and dried specimens, using a pin-holding stage that permitted full rotations around the X, Y and Z axes. A Wild M10 high-performance stereomicroscope equipped with a 1.6× planapochromatic front lens was used at magnifications of 160–320×. A Schott KL 1500 cold-light source equipped with two flexible, focally mounted light cables providing 30°-inclined light from variable directions, allowed sufficient illumination over the full magnification range and a clear visualization of silhouette lines. Two cold-light sources were used: the external Schott KL 2500 LCD and the coaxial polarised-light illuminator built into the microscope. Depending on what illumination regime was required, the two light sources were used either simultaneously or alternatively. This provided optimum resolution of any tiny structure and microsculpture at the highest magnifications. A Leica cross-scaled ocular micrometer with 120 gradation marks ranging over 65% of the visual field was used. To avoid parallax error, its measuring line was constantly kept vertical within the visual field. Mean measurement errors calculated were±0.6 μm for small and well-defined structures such as scape-base width (ScBW; for details, see list below), and±1.5 μm for larger structures that are difficult to position, such as cephalic length.

In the following, the 17 morphometric characters measured are listed, and crucial terms used are defined.

CL	Maximum cephalic length in median line; the head was carefully tilted to the position with the true maximum; excavations of occiput and/or clypeus reduced CL. Surface irregularities due to sculpture, carinae in particular, were considered by averaging between peaks and valleys of sculpture.
CS	Cephalic size; arithmetic mean of CL and CW.
CW	Maximum measurable cephalic width, irrespective of whether it occurred in front of, behind or across the eyes.

EL	Maximum length of eye. All structurally defined ommatidia, pigmented or not, were included.
Fe2L	Length of midfemur on extensor side; measured from distalmost point of femur to border between femur and trochantellus (see below). Equivalent measurements were possible with view on plane or edge of femur and intermediate viewing positions.
LNdCV	Natural logarithm of mean carinae distance on central vertex in μm; measured in dorsal view along a transversal line at level of eye centres and between imagined caudal prolongations of frontal carinae. The number of carinae/carinulae crossing this line was counted; those just touching the measuring line and those exactly at its endpoints were counted as 0.5.
Mesosomal longitudinal axis	From centre of metapleural gland orifice to lowest point of pronotal sclerite, in lateral view.
ML	Mesosoma length from transition point between pronotal neck shield and anterior pronotal slope to posterior end of metapleuron.
MW	Maximum pronotal width in dorsal view.
PEApo	Angle formed by dorsocaudal and caudal profile lines of petiolar node, calculated with tangens functions; in *M. capitatus* there is a continuous shallow convexity—here the calculated angle is formed by imaginary lines (a) from upper point of node to median point of convexity, and (b) from median point of convexity to lowest point of node slope.
PigC	Percentage of blackish pigmentation of head capsule, estimated in dorsal view and at low magnifications.
PNaa	Maximum pronotal height, measured perpendicular to mesosomal longitudinal axis.
poGuHL	Maximum length of gular setae with insertion points posterior to level of hind eye margin. The head was carefully adjusted, and parallax error was avoided. When the longest hair was exactly on the level of the hind eye margin, the mean was taken between this hair and the longest hair posterior to the level of the hind eye margin.
PoOc	Postocular distance; using the cross-scaled ocular micrometer, the head was adjusted to the measuring position of CL; caudal measuring point: median occipital margin as average between peaks and valleys of microsculpture; frontal measuring point: median head at level of posterior eye margin; the average of the left and right postocular distances was calculated.
PRaa	Maximum propodeometapleural height, measured perpendicular to mesosomal longitudinal axis.
ScBaC	Measured in same adjustment as ScBW: distance of anterior scape base corner from anterior margin of articular neck at its median portion; see Fig. 3.
ScBW	Maximum width of scape base from corner to corner, measured in dorsal view; see Fig. 3.
SL	Maximum straight-line scape length in dorsal view, excluding the articular condyle; the values for the two scapes were averaged.
STPLd	Maximum distance between the centre of the propodeal stigma and the posterior margin of the propodeal lobe, measured in dorso-dorso-caudo-lateral view; the specimen was carefully tilted to the position resulting in the true maximum of the distance. The region of the bulla glandulae metapleuralis was excluded.
Trochantellus	A narrow, inconspicuous segment between femur and trochanter, usually considered as part of the femur (Allaby 1999).

The first step of the analysis consisted of sorting out the individuals of *M. capitatus*. This species is easily separable from *M. minor* and *M.* cf. *wasmanni* by qualitative (i.e. non-morphometric) assessment based on (*i*) the strong depression of the straight dorsal propodeal profile below the level of the high, evenly curved promesonotum; (*ii*) the pronounced slenderness of mesosoma and petiole; (*iii*) the posterior slope of the petiole node forming a very blunt angle or a shallow convexity; and (*iv*) a pronounced length of the scape and the legs.

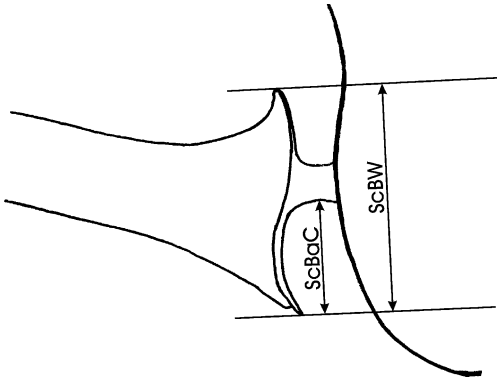


Fig. 3 Distances measured for morphometric characters ScBaC and ScBW

The second step was sorting out those individuals of *M. minor* and *M.* cf. *wasmanni* that showed typical species-specific character combinations, based on qualitative assessment. Typical specimens of *M.* cf. *wasmanni* have (*i*) a clearly angled dorsolateral pronotum; (*ii*) a reduced posterior part of the psammophore; (*iii*) a much more protruding anterior scape-base corner; and (*iv*) a red-wine tinge in the reddish mesosomal pigmentation (in fresh material). Typical *M. minor* specimens have (*i*) a significantly smaller body size than *M.* cf. *wasmanni*; (*ii*) a fully rounded dorsolateral pronotum; (*iii*) a well-developed posterior part of the psammophore; (*iv*) a less protruding anterior scape-base corner; and (*v*) a strong yellowish component in the reddish mesosomal pigmentation (and never a red-wine tinge).

These two steps resulted in 60 worker specimens determined as *M. capitatus,* 66 as typical *M.* cf. *wasmanni*, and 48 as typical *M. minor*. 70 specimens of either *M.* cf. *wasmanni* or *M. minor* showed intermediate character combinations suggesting a possible hybrid identity, and thus remained undetermined.

Workers of *Messor* species show strong allometric growth and very large variation in absolute body size. As a consequence, size differences between species can disguise body-size-independent shape differences or, alternatively, expose shape differences which are just a function of body-size differences. Here, allometries were described by linear regression of character ratios against body size. After first calculating species-specific functions only from the three sets of typical specimens, the parameters of these functions were averaged to give a single over-all-species description. This overall function was then used to predict character expressions for the assumption of each individual having the same cephalic size of, in our case, 1.9 mm (for details of the procedure, see Seifert 2008).

In detail, this removal of allometric variance was performed on the 174 typical specimens of the three species and the 70 intermediate specimens of either *M.* cf. *wasmanni* or *M. minor*, using the following corrections (the pigmentation character PigC was not corrected for allometry, because the within-species function for *M. capitatus* strongly deviated from those of the other two species):

$$\mathrm{PoOc/CL_{1.9}} = \mathrm{PoOc/CL/(0.0393{*}CS + 0.2779){*}0.3525}$$
$$\mathrm{LNdCV_{1.9}} = \mathrm{LNdCV/(-2.4192{*}CS + 9.0742){*}4.4778}$$
$$\mathrm{CL/CW_{1.9}} = \mathrm{CL/CW/(-0.0748{*}CS + 1.1347){*}0.9926}$$
$$\mathrm{SL/CS_{1.9}} = \mathrm{SL/CS/(-0.1204{*}CS + 1.0374){*}0.8086}$$
$$\mathrm{MW/CS_{1.9}} = \mathrm{MW/CS/(-0.0444{*}CS + 0.6865){*}0.6021}$$
$$\mathrm{Fe2L/CS_{1.9}} = \mathrm{Fe2L/CS/(-0.0906{*}CS + 1.0141){*}0.8419}$$
$$\mathrm{STPLd/CS_{1.9}} = \mathrm{STPLd/CS/(-0.0431{*}CS + 0.3883){*}0.3065}$$
$$\mathrm{PNHaa/CS_{1.9}} = \mathrm{PNHaa/CS/(-0.0308{*}CS + 0.5095){*}0.4510}$$
$$\mathrm{PRHaa/CS_{1.9}} = \mathrm{PrHaa/CS/(-0.0338{*}CS + 0.4085){*}0.3442}$$
$$\mathrm{ML/CS_{1.9}} = \mathrm{ML/CS/(-0.1461{*}CS + 1.4870){*}1.2094}$$
$$\mathrm{ScBW/CS_{1.9}} = \mathrm{ScBW/CS/(-1.400{*}CS + 12.549){*}9.889}$$
$$\mathrm{ScBAC/CS_{1.9}} = \mathrm{ScBAC/CS/(-0.4919{*}CS + 5.389){*}4.455}$$
$$\mathrm{EL/CS_{1.9}} = \mathrm{EL/CS/(-0.0297{*}CS + 0.2465){*}0.1900}$$
$$\mathrm{PoGUHL/CS_{1.9}} = \mathrm{PoGUHL/CS/(-0.0138{*}CS + 0.2040){*}0.1779}$$
$$\mathrm{PEApo_{1.9}} = \mathrm{PEApo/(-4.310{*}CS + 156.9){*}148.7}$$

The allometry-corrected data were then used in a 2-class discriminant analysis with *M. capitatus* as one class and all other forms as the second class, using the following discriminant function (given by SPSS):

$$\begin{aligned}\mathrm{D(1)} = {} & 5.186{*}\mathrm{CS} - 68.227{*}\mathrm{PoOc/CL_{1.9}} \\ & - 0.080{*}\mathrm{LNdCV_{1.9}} + 0.017{*}\mathrm{PigC} \\ & + 22.166{*}\mathrm{CL/CW_{1.9}} + 4.091{*}\mathrm{SL/CS_{1.9}} \\ & - 51.512{*}\mathrm{MW/CS_{1.9}} + 19.618{*}\mathrm{Fe2L/CS_{1.9}} \\ & - 23.356{*}\mathrm{STPLd/CS_{1.9}} + 7.018{*}\mathrm{PNHaa/CS_{1.9}} \\ & - 27.082{*}\mathrm{PRHaa/CS_{1.9}} + 16.477{*}\mathrm{ML/CS_{1.9}} \\ & - 1.299{*}\mathrm{ScBW/CS_{1.9}} + 1.784{*}\mathrm{ScBAC/CS_{1.9}} \\ & + 17.408{*}\mathrm{EL/CS_{1.9}} - 12.498{*}\mathrm{PoGUHL/CS_{1.9}} \\ & + 0.050{*}\mathrm{PEApo_{1.9}} - 6.906\end{aligned}$$

The classification using D(1) fully confirmed the distinctness of all 60 workers hypothesised a priori to represent *M. capitatus* from the collective cluster of 184 specimens of *M.* cf. *wasmanni, M. minor* and the supposed intermediates.

To give a more precise description of allometries for the collective cluster, the strongly deviating *M. capitatus* workers were excluded from further analysis. A second allometry correction was then calculated for the remaining

184 individuals, using only the species-specific functions for typical *M.* cf. *wasmanni* and *M. minor* workers, and CS=1.9 mm. If deviating from monophasic condition, these corrections were performed in a biphasic manner. The following corrections were used (here, the pigmentation character PigC was corrected for allometry, because the within-species functions for *M.* cf. *wasmanni* and *M. minor* did not strongly deviate from each other):

$$PoOc/CL_{1.9} = PoOc/CL/(0.0425*CS + 0.2763)*0.3566 - [\text{for } CS \leq 1.9]$$
$$PoOc/CL_{1.9} = PoOc/CL/(0.0327*CS + 0.2945)*0.3566 - [\text{for } CS > 1.9]$$
$$LNdCV_{1.9} = LNdCV/(-2.112*CS + 8.461)*4.623 - [\text{for } CS \leq 1.9]$$
$$LNdCV_{1.9} = LNdCV/(-0.751*CS + 6.050)*4.623 - [\text{for } CS > 1.9]$$
$$PigC_{1.9} = PigC/(-29.89*CS + 108.2)*51.4$$
$$CL/CW_{1.9} = CL/CW/(-0.0657*CS + 1.1030)*0.9780$$
$$SL/CS_{1.9} = SL/CS/(-0.1193*CS + 1.0102)*0.7835$$
$$MW/CS_{1.9} = MW/CS/(-0.0375*CS + 0.6806)*0.6094$$
$$Fe2L/CS_{1.9} = Fe2L/CS/(-0.0820*CS + 0.9807)*0.8250$$
$$STPLd/CS_{1.9} = STPLd/CS/(-0.0416*CS + 0.3889)*0.3098 - [\text{for } CS \leq 1.9]$$
$$STPLd/CS_{1.9} = STPLd/CS/(-0.0378*CS + 0.3812)*0.3098 - [\text{for } CS > 1.9]$$
$$PNHaa/CS_{1.9} = PNHaa/CS/(-0.0230*CS + 0.4948)*0.4511$$
$$PRHaa/CS_{1.9} = PrHaa/CS/(-0.0237*CS + 0.3997)*0.3546$$
$$ML/CS_{1.9} = ML/CS/(-0.1252*CS + 1.4387)*1.2008$$
$$ScBW/CS_{1.9} = ScBW/CS/(-1.316*CS + 12.302)*9.800$$
$$ScBAC/CS_{1.9} = ScBAC/CS/(-0.3934*CS + 5.068)*4.321$$
$$EL/CS_{1.9} = EL/CS/(-0.0296*CS + 0.2450)*0.1890 - [\text{for } CS \leq 1.9]$$
$$EL/CS_{1.9} = EL/CS/(-0.0215*CS + 0.2298)*0.1890 - [\text{for } CS > 1.9]$$
$$PoGUHL/CS_{1.9} = PoGUHL/CS/(-0.0043*CS + 0.2124)*0.2024 - [\text{for } CS \leq 1.9]$$
$$PoGUHL/CS_{1.9} = PoGUHL/CS/(-0.0427*CS + 0.2835)*0.2024 - [\text{for } CS > 1.9]$$
$$PEApo_{1.9} = PEApo/(0.689*CS + 142.8)*144.1$$

After these corrections, the 70 workers with morphology assessed as intermediate between *M.* cf. *wasmanni* and *M. minor* were hypothesised to represent either *mw* (F1 hybrids) or *mmw* (hybrids backcrossed with *M. minor*). This grouping was based (*i*) on the overall visual perception of angularity of the pronotum, petiole shape, general colouration, and sculpture of the entire body, and (*ii*) on the values of corrected morphometric data relative to those for the specimens typical of the parental species. Subsequently, data were computed in a 2-class discriminant function (with *M. minor* and *M.* cf. *wasmanni* as classes) in which only the 114 specimens typical of *M. minor* and *M.* cf. *wasmanni* were allocated to classes a priori, while the data of the 70 intermediate specimens were run without using a-priori hypotheses; the function given by SPSS was:

$$D(2) = 0.155*CS + 14.963*PoOc/CL_{1.9} + 0.271*LNdCV_{1.9} + 0.018*PigC_{1.9} + 32.031*$$
$$CL/CW_{1.9} + 22.590*SL/CS_{1.9} + 33.355*MW/CS_{1.9} - 35.615*Fe2L/CS_{1.9} + 67.915*STPLd/CS_{1.9} +$$
$$3.027*PNHaa/CS_{1.9} - 32.613*PRHaa/CS_{1.9} - 14.754*ML/CS_{1.9} + 0.270*ScBW/CS_{1.9} +$$
$$0.600*ScBAC/CS_{1.9} - 61.080*EL/CS_{1.9} - 18.636*PoGUHL/CS_{1.9} - 0.027*PEApo_{1.9} - 27.988$$

D(2) can be interpreted as describing a vector between typical *M. minor* and *M.* cf. *wasmanni* specimens (cf. Costedoat et al. 2005). The classification result from using D(2) for the 114 workers hypothesised to represent the parental species was in line with the a-priori hypothesis for all specimens. The remaining 70 individuals from 13 colonies, hypothesised to be hybrids or backcrosses of hybrids, were positioned fully intermediately between the parental species when sample means are considered. The position along the vector also was in line with the a-priori hypotheses (not used as classes in D(2)) for the specimens of intermediate morphology, with *mmw* individuals falling between *mw* and *M. minor*, and *mw* individuals falling between those of the parental species (Fig. 1c). This analysis did not provide a suggestion for backcrosses of hybrids with *M.* cf. *wasmanni.*

Appendix 3 Inference key for interpreting microsatellite, mtDNA and morphometric data of workers of eusocial male-haploids at the colony level

The inference key is designed for the interpretation of data on individual workers from a given colony suspected to contain genomes of more than one but of a maximum of three species, using microsatellite, mtDNA and morphometric data.

Based on the hereditary patterns established for the three data sources, the inference key classifies colonies using the following five questions. (*i*) What species are discernible in the colony? (*ii*) How many individuals are involved in the workers' parenthood? The inference key accounts for male haploidy, in that in male haploids the maximum number of alleles per microsatellite locus consistent with a single mother mated once is three across all offspring, with at least one of the alleles occurring in all individuals (whereas for diploid organisms the corresponding value would be four, without additional constraints). If a single mtDNA haplotype has been retrieved from a colony, the presence of three alleles per microsatellite with none of these occurring in all individuals, or of more than three alleles per microsatellite, is used for the inference of either a single queen mated multiply or more than one queen per colony – no distinction is possible in these instances. The presence of more than one mtDNA haplotype is used to infer more than one queen. (*iii*) Is there evidence of hybridisation, i.e. gene flow across species? A colony is inferred to contain hybrids, if any of three criteria apply to at least one individual from that colony: admixed microsatellite genotype, intraindividual combination of a pure-species microsatellite genotype of one species with a mtDNA haplotype of another species, hybrid phenotype. (*iv*) In case of hybridisation, how far does it date back? F1 hybridisation is diagnosed against older hybridisation using the following rationales. With F1 hybrids, microsatellites, which are biparentally inherited nuclear DNA, should reveal a heterospecific 50:50 admixture, and morphology, which is nuclear encoded and thus likewise biparentally inherited, should be intermediate between species. With hybridisation older than F1, the hybrids should be more similar in microsatellites and morphology to any of the pure parental species than F1 hybrids would be. In cases of several or many backcrossings with the paternal species, both microsatellites and morphology would indicate the pure paternal species, but hybridisation older than F1 is then discernible via a mtDNA haplotype of the maternal species (instances of a high number of backcrossings with the maternal species cannot be detected via our approach, i.e. colonies to which this potentially applies are classified as pure-species colonies in step *iii*). (*v*) Likewise in case of hybridisation, is there information on its direction(s) concerning the identity of the parental species? mtDNA is used to infer the maternal species.

The precondition for using the key is that the three methods have been established to be suitable for the species concerned, i.e. that (1) each species has alleles of the microsatellite loci not shared with the other species; (2) each species has distinct, species-specific mtDNA haplotypes; (3) the hybrid worker morphologies are morphometrically diagnosable, i.e., ideally, the F1 hybrid morphology plus morphologies intermediate between the F1-hybrid morphology and the two parental species can be differentiated.

Microsatellite data must be used to assign individuals to clusters (the number of clusters being identical to the number of species suspected of being involved) using the package STRUCTURE (Pritchard et al. 2000) or similar software. The admixture of individuals to different species is then classified in steps of 25%, as follows: 1–37.5%="25%"; 37.5–62.5%="50%"; 62.5–99%="75%". Mitochondrial DNA data may be used in the key only if there is no indication of paternal leakage plus recombination (e.g. Ciborowski et al. 2007, and references therein), as these would confuse the picture from the mtDNA data and thus lead to incorrect inferences using the key. In selecting individuals for the morphometric analyses, the extremes of variation should be included to cover the whole range of occurring morphologies.

In using the inference key, the following three notes should be borne in mind. (Note 1) The key is an approximation and views the species identities of hybrid worker offspring in steps of 25%. (Note 2) The key will fail in case of repeated backcrossings with the maternal species resulting in a hybrid colony that is indiscernible from a pure-species colony never involved in hybridisation. (Note 3) If, for any colony, data from one or two disciplines are lacking, we suggest – after merging the inferences of all the colonies with complete data – to carefully make minimum inferences using plausibility arguments. This involves comparing the colony in question with colonies with complete data.

Inference key

Step 1 View individuals separately

(A) In no individual is the presence of more than one species suggested by the disciplines combined (but more than one species can occur across individuals) .....Step 2

(B) In at least one individual, the presence of at least two species is suggested by the disciplines combined .....Step 4

Step 2 Across individuals

(A) All individuals are identical ..... **consistent with one species (identity discernible from any discipline)**; for information on the number of individuals involved in parenthood .....Step 3

(B) Individuals equal two species .....**consistent with two species (identities discernible from any discipline), no gene flow between species involved**; for information on the number of individuals involved in parenthood ..... Step 3, using the data of the two species separately

(C) Individuals equal three species ..... **consistent with three species (identities discernible from any discipline), no gene flow between species involved**; for information on the number of individuals involved in parenthood ..... Step 3, using the data of the three species separately

Step 3 Across individuals

(A) mtDNA reveals a single haplotype, and microsatellites in each locus reveal a maximum of three alleles, at least one of them occurring in all individuals ..... **consistent with one father and one mother**

(B) mtDNA reveals a single haplotype, microsatellites reveal more than three alleles in any locus, or up to three alleles, but none of them occurs in all individuals ..... **consistent with more than one father and/or more than one mother**

(C) (Only applicable if preceding step was step 5B): mtDNA reveals a single haplotype, and the alleles of all microsatellites suggest one pure species for some individuals, but the other pure species for other individuals ..... **consistent with more than one father and/or more than one mother**

(D) mtDNA reveals more than one haplotype, independently of the maximum number of alleles in each microsatellite locus..... **consistent with more than one mother, the minimum number of females involved equalling the number of haplotypes; no inference on the number of fathers**

Step 4 Across individuals

(A) Presence of two species is indicated ..... Step 5

(B) Presence of three species is indicated ..... Step 7

Step 5 Across individuals

(A) In each discipline, all individuals show the same pattern, microsatellites revealing 50:50 heterospecific admixture, and worker morphology being intermediate between species .....**consistent with F1 hybrids (mtDNA revealing the maternal species)**; for information on the number of individuals involved in parenthood ..... Step 3

(B) (Only applicable if the same individuals were used under all three disciplines): for some of the individuals, the presence of only one species (possibly of different identities across these individuals) is indicated by the disciplines combined; for these individuals ..... Step 2; for the remaining individuals decide between Step 5A and 5C

(C) Other ..... **consistent with hybridisation older than F1 (mtDNA revealing the maternal species)**; for information on the direction of hybridisation ..... Step 6; for information on the number of individuals involved in parenthood ..... Step 3

Step 6 Across individuals

(A) mtDNA suggests one species .....**consistent with all hybridisations in the colony's ancestry having started with females of one species (identity discernible through mtDNA)**

(B) mtDNA suggests two species ..... **consistent with the colony's ancestry including hybridisations that started with females of two species (identities discernible through mtDNA)**

(C) (Only applicable if preceding step was step 7B): mtDNA suggests three species ..... **consistent with the colony's ancestry including hybridisations that started with females of three species (identities discernible through mtDNA)**

Step 7 Across individuals

(A) For some individuals, microsatellites and morphometrics suggest the same single species, dentical with the species suggested by mtDNA ..... **consistent with one of the three species (identity discernible from any discipline) not being involved in interspecific gene flow;** for information on the number of individuals involved in the colony's parenthood concerning that species ..... Step 3; for information on the hybrid status concerning the other two species ..... Step 5

(B) Other ..... **consistent with the colony's ancestry involving hybridisations between all three species**; additional interpretations are difficult, but if one of the three species is revealed solely through a very small portion in microsatellite admixture ..... Step 5, adding to any interpretation that this species was involved at some point in the colony's ancestry, but was outcrossed subsequently; in any case: for information on the direction of hybridisation ..... Step 6

Appendix 4 Results of subjecting the combined data on the single colonies to the new inference key (Appendix 3)

For details, see also the Discussion section entitled "Possible explanations for complex patterns in the colony-level inferences".

M3: 1B→4A (*M. minor*, *M.* cf. *wasmanni*)→5C→6A (*M. minor*); →3A

M13: 1A→2A (*M.* cf. *wasmanni*)→3A

M14: 1A→2A (*M. minor*)→3A

M16: 1B→4A (*M. minor*, *M.* cf. *wasmanni*)→5C→6A (*M. minor*); →3A

M17: 1A→2A (*M.* cf. *wasmanni*)→3A

M18: 1A→2A (*M.* cf. *wasmanni*)→3A

M20: 1B→4A (*M. minor*, *M.* cf. *wasmanni*)→5A (*M.* cf. *wasmanni*)→3A

M29: key not applicable because no data on microsatellites; minimum inference: consistent with only *M. capitatus* present in the colony

M40: 1B→4A (*M. minor*, *M.* cf. *wasmanni*)→5C→6A (*M. minor*); →3B

M41: 1B→4B (*M. minor*, *M.* cf. *wasmanni, M. capitatus*)→7A (*M. capitatus*)→3A; →5C→6A (*M. minor*); →3C

M42: key not applicable because no data on microsatellites; minimum inferences: presence of *M. capitatus* and hybrids between *M. minor* and *M.* cf. *wasmanni*, no indication of more than one pair of parents, neither for *M. capitatus* nor for the hybrid of *M. minor* and *M.* cf. *wasmanni*

M43: 1B→4A (*M. minor*, *M.* cf. *wasmanni*)→5C→6B (*M. minor*, *M.* cf. *wasmanni*); →3D (two)

M44: 1B→4A (*M. minor*, *M.* cf. *wasmanni*)→5C→6B (*M. minor*, *M.* cf. *wasmanni*); →3D (two)

M48: 1B→4A (*M. minor*, *M.* cf. *wasmanni*)→5C→6B (*M. minor*, *M.* cf. *wasmanni*); →3D (two)

M49: 1B→4A (*M. minor*, *M.* cf. *wasmanni*)→5C→6B (*M. minor*, *M.* cf. *wasmanni*); →3D (two); this inference is based on the assumption that presence of both pure species in a colony would have resulted in their identification via the morphometric data for that colony, given that all workers remaining after molecular analysis were examined when selecting workers for morphometric analysis; here, however, all ca. 50 workers of the sample homogenously had the morphological appearance of *M.* cf. *wasmanni*, and this was confirmed by the results for the 6 individuals selected for morphometrics; hence we consider it very unlikely that the two workers suggested to be *M. minor* by microsatellites and mtDNA had *M. minor* morphology and, using a plausibility argument, consider the presence of *M. minor* in the molecular results to be the result of hybridisation followed by backcrossing.

M50: 1B→4B (*M. minor*, *M.* cf. *wasmanni, M. capitatus*)→7B, but: in no colony gene flow between *M. capitatus* and any other species inferred, and we assume that there was a single individual of pure *M. capitatus* in the colony-sample which was destroyed by DNA extraction, and therefore opt for 7A (*M. capitatus*)→3A; →5C→6A (*M. minor*); →3A

M52: 1B→4A (*M. minor*, *M.* cf. *wasmanni*)→5C→6A (*M.* cf. *wasmanni*); →3C

M57: 1B→4A (*M. minor*, *M.* cf. *wasmanni*)→5C→6B (*M. minor*, *M.* cf. *wasmanni*); →3D (two)

M58: 1B→4A (*M. minor*, *M.* cf. *wasmanni*)→5C→6A (*M.* cf. *wasmanni*); →3C

M60: 1B→4A (*M. minor*, *M.* cf. *wasmanni*)→5C→6A (*M. minor*)→3C; this inference is based on the assumption that presence of both pure species in a colony would have resulted in their identification via the morphometric data for that colony, given that all workers remaining after molecular analysis were examined when selecting workers for morphometric analysis; here, however, all ca. 50 workers of the sample homogenously had the morphological appearance of *M.* cf. *wasmanni*, and this was confirmed by the results for the 6 individuals selected for morphometrics; hence we consider it very unlikely that the worker suggested to be *M. minor* by microsatellites and mtDNA had *M. minor* morphology and, using a plausibility argument, consider the presence of *M. minor* in the molecular results to be the result of hybridisation followed by backcrossing.

M61: 1B→4A (*M. minor*, *M.* cf. *wasmanni*)→5C→6A (*M. minor*); →3D (two)

M62: 1B→4A (*M. minor*, *M.* cf. *wasmanni*)→5C→6A (*M. minor*); →3D (two)

M63: 1B→4B (*M. minor*, *M.* cf. *wasmanni, M. capitatus*)→7A (*M. capitatus*)→3A; →5C→6A (*M. minor*); →3C

MC1: 1A→2A (*M. capitatus*)→3A

MC2: 1A→2A (*M. capitatus*)→3A

MC3: 1A→2A (*M. capitatus*)→3A

MC11: key not applicable because no data on microsatellites and mtDNA; minimum inference: consistent with only *M. capitatus* present in the colony, no indication of more than one pair of parents

MC12: key not applicable because no data on microsatellites and mtDNA; minimum inference: consistent with only *M. capitatus* present in the

colony, no indication of more than one pair of parents

MC13: key not applicable because no data on microsatellites and mtDNA; minimum inference: consistent with only *M. capitatus* present in the colony, no indication of more than one pair of parents

Mes: key not applicable because no data on microsatellites; minimum inference: presence of *M. capitatus* and hybrids between *M. minor* and *M.* cf. *wasmanni*, no indication of more than one pair of parents, neither for *M. capitatus* nor the hybrids of *M. minor* and *M.* cf. *wasmanni*

MM1: 1A→2A (*M. minor*)→3A

MM3: 1A→2A (*M. minor*)→3A

MW4: 1A→2A (*M. minor*)→3A

References

Allaby, M. (1999). *A dictionary of zoology*. Oxford: Oxford University Press.

André, E. (1883). Les fourmis. In E. André (Ed.), *Species des hyménoptères d'Europe et d'Algérie. Tome deuxieme* (pp. 345–404). Beaune: Edmond André.

Arnold, M. L. (2006). *Evolution through genetic exchange*. Oxford: Oxford University Press.

Arthofer, W., Schlick-Steiner, B. C., Steiner, F. M., Konrad, H., Espadaler, X., & Stauffer, C. (2005). Microsatellite loci for the study of habitat fragmentation in the harvester ant *Messor structor*. *Conservation Genetics, 6*, 859–861.

Baer, B. (2011). The copulation biology of ants (Hymenoptera: Formicidae). *Myrmecological News, 14*, 55–68.

Baroni Urbani, C. (1971). Catalogo delle specie di Formicidae d'Italia (Studi sulla mirmecofauna d'Italia X). *Memorie della Società Entomologica Italiana, 50*, 5–287.

Barton, N. H. (2001). The role of hybridization in evolution. *Molecular Ecology, 10*, 551–568.

Beckenbach, A. T. (2009). Numts and mitochondrial pseudogenes. *Myrmecological News, 12*, 217–218.

Becquet, C., Patterson, N., Stone, A. C., Przeworski, M., & Reich, D. (2007). Genetic structure of chimpanzee populations. *Public Library of Science Genetics, 3*, e66.

Beibl, J., D'Ettorre, P., & Heinze, J. (2007). Cuticular profiles and mating preference in a slave-making ant. *Insectes Sociaux, 54*, 174–182.

Bensasson, D., Zhang, D.-X., Hartl, D. L., & Hewitt, G. M. (2001). Mitochondrial pseudogenes: evolution's misplaced witnesses. *Trends in Ecology & Evolution, 16*, 314–321.

Buschinger, A. (2009). Social parasitism among ants: a review (Hymenoptera: Formicidae). *Myrmecological News, 12*, 219–235.

Callen, D. F., Thompson, A. D., Shen, Y., Phillips, H. A., Richards, R. I., Mulley, J. C., et al. (1993). Incidence and origin of "null" alleles in the (AC)n microsatellite markers. *American Journal of Human Genetics, 52*, 922–927.

Carlin, N. F., & Hölldobler, B. (1983). Nestmate and kin recognition in interspecific mixed colonies of ants. *Science, 222*, 1027–1029.

Ciborowski, K. L., Consuegra, S., de Leaniz, C. G., Beaumont, M. A., Wang, J., & Jordan, W. C. (2007). Rare and fleeting: an example of interspecific recombination in animal mitochondrial DNA. *Biology Letters, 3*, 554–557.

Costedoat, C., Pech, N., Salducci, M.-D., Chappaz, R., & Gilles, A. (2005). Evolution of mosaic hybrid zone between invasive and endemic species of Cyprinidae through space and time. *Biological Journal of the Linnean Society, 85*, 135–155.

Coyne, J. A., & Orr, H. A. (2004). *Speciation*. Sunderland: Sinauer.

Crozier, R. H., & Pamilo, P. (1996). *Evolution of social insect colonies. Sex allocation and kin selection*. Oxford: Oxford University Press.

Czechowski, W. (2001). Mixed colony of *Formica pratensis* Retz. + *Formica cinerea* Mayr + *Formica sanguinea* Latr. (Hymenoptera: Formicidae) and its presumed origin. *Annales Zoologici (Warszawa), 51*, 205–209.

Dasmahapatra, K. K., Silva-Vasquez, A., Chung, J. W., & Mallet, J. (2007). Genetic analysis of a wild-caught hybrid between non-sister *Heliconius* butterfly species. *Biology Letters, 3*, 660–663.

Ehrich, D. (2006). AFLPdat: a collection of R functions for convenient handling of AFLP data. *Molecular Ecology Notes, 6*, 603–604.

Emery, C. (1908). Beiträge zur Monographie der Formiciden des paläarktischen Faunengebietes. (Hym.) Teil III. *Deutsche Entomologische Zeitschrift, 1908*, 437–465.

Errard, C. (1984). Evolution, en fonction de l'âge, des relations sociales dans les colonies mixtes hétérospécifiques chez les fourmis des genres *Camponotus* et *Pseudomyrmex*. *Insectes Sociaux, 31*, 185–198.

Feldhaar, H., Foitzik, S., & Heinze, J. (2008). Lifelong commitment to the wrong partner: hybridization in ants. *Philosophical Transactions of the Royal Society B: Biological Sciences, 363*, 2891–2899.

Felsenstein, J. (1985). Confidence-limits on phylogenies—an approach using the bootstrap. *Evolution, 39*, 783–791.

Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhook, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology, 3*, 294–299.

Funk, D. J., Nosil, P., & Etges, W. J. (2006). Ecological divergence exhibits consistently positive associations with reproductive isolation across disparate taxa. *Proceedings of the National Academy of Sciences of the United States of America, 103*, 3209–3213.

Gaubert, P., Taylor, P. J., Fernandes, C. A., Bruford, M. W., & Veron, G. (2005). Patterns of cryptic hybridization revealed using an integrative approach: a case study on genets (Carnivora, Viverridae, *Genetta* spp.) from the southern African subregion. *Biological Journal of the Linnean Society, 86*, 11–33.

Gavrilets, S., & Losos, J. B. (2009). Adaptive radiation: contrasting theory with data. *Science, 323*, 732–737.

Gompert, Z., Fordyce, J. A., Forister, M. L., Shapiro, A. M., & Nice, C. C. (2006). Homoploid hybrid speciation in an extreme habitat. *Science, 314*, 1923–1925.

Goodman, S. J., Barton, N. H., Swanson, G., Abernethy, K., & Pemberton, J. M. (1999). Introgression through rare hybridization: a genetic study of a hybrid zone between red and sika deer (genus *Cervus*) in Argyll, Scotland. *Genetics, 152*, 355–371.

Grant, P. R., & Grant, B. R. (1994). Phenotypic and genetic effects of hybridization in Darwin's finches. *Evolution, 48*, 297–316.

Grasso, D. A., Mori, A., Bottini, B., & Le Moli, F. (2002). Colony founding in the harvesting ant *Messor minor* (Hymenoptera, Formicidae). *Insect Social Life, 4*, 17–22.

Grasso, D. A., Mori, A., Giovannotti, M., & Le Moli, F. (2004). Interspecific interference behaviours by workers of the harvesting ant *Messor capitatus* (Hymenoptera, Formicidae). *Ethology Ecology & Evolution, 16*, 197–207.

Grasso, D. A., Mori, A., & Le Moli, F. (1999). Recruitment and trail communication in two species of *Messor* ants (Hymenoptera, Formicidae). *Italian Journal of Zoology, 66*, 373–378.

Grasso, D. A., Mori, A., & Le Moli, F. (2004b). Competizione intra- ed interspecifica tra colonie incipienti di formiche mietitrici del genere *Messor* (Hymenoptera, Formicidae). *XIX Congresso Nazionale Italiano di Entomologia,* pp. 301–304.

Harkness, R. W., & Isham, V. (1988). Relations between nests of *Messor wasmanni* in Greece. *Insectes Sociaux, 35*, 1–18.

Helms Cahan, S., & Keller, L. (2003). Complex hybrid origin of genetic caste determination in harvester ants. *Nature, 424*, 306–309.

Höfener, C., Seifert, B., & Krüger, T. (1996). A genetic model for disruptive selection on colony social organisation, reproduction, and ecotype distribution in wood ants inhabiting different woodland habitats. *Insectes Sociaux, 43*, 359–373.

Hölldobler, B., & Markl, H. (1990). Notes on interspecific, mixed colonies in the harvester ant genus *Pogonomyrmex*. *Psyche, 96*, 237–238.

Hopper, K. R. (1999). Risk-spreading and bet-hedging in insect population biology. *Annual Review of Entomology, 44*, 535–560.

ICZN = International Commission on Zoological Nomenclature. (1999). *International Code of Zoological Nomenclature* (4th ed.). London: International Trust for Zoological Nomenclature.

Isingrini, M., Lenoir, A., & Jaisson, P. (1985). Preimaginal learning as a basis of colony-brood recognition in the ant *Cataglyphis cursor*. *Proceedings of the National Academy of Sciences of the United States of America, 82*, 8545–8547.

Koide, Y., Ikenaga, M., Sawamura, N., Nishimoto, D., Matsubara, K., Onishi, K., et al. (2008). The evolution of sex-independent transmission ratio distortion involving multiple allelic interactions at a single locus in rice. *Genetics, 180*, 409–420.

Krausse, A. H. (1910). Über Stridulationstöne bei Ameisen. *Zoologischer Anzeiger, 35*, 523–526.

Krausse, A. H. (1911). Über *Messor structor* Ltr. und einige andere Ameisen auf Sardinien. *Bullettino della Società Entomologica Italiana, 41*, 14–18.

Kulmuni, J., Seifert, B., & Pamilo, P. (2010). Segregation distortion causes large-scale differences between male and female genomes in hybrid ants. *Proceedings of the National Academy of Sciences of the United States of America, 107*, 7371–7376.

Kumar, S., Tamura, K., Jacobsen, I. B., & Nei, M. (2001). MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics, 17*, 1244–1245.

Latreille, P. A. (1798). *Essai sur l'histoire des fourmis de la France*. Brive: F. Bourdeaux.

MacArthur, R. H., & Wilson, E. O. (1967). *The theory of island biogeograpy*. Princeton: Princeton University Press.

Mallet, J. (2005). Hybridization as an invasion of the genome. *Trends in Ecology & Evolution, 20*, 229–237.

Mallet, J. (2007). Hybrid speciation. *Nature, 446*, 279–283.

Maruyama, M., Steiner, F. M., Stauffer, C., Akino, T., Crozier, R. H., & Schlick-Steiner, B. C. (2008). A DNA and morphology based phylogenetic framework of the ant genus *Lasius* with hypotheses for the evolution of social parasitism and fungiculture. *BioMed Central Evolutionary Biology, 8*, 237.

McDevitt, A. D., Edwards, C. J., O'Toole, P., O'Sullivan, P., O'Reilly, C., & Carden, R. F. (2009). Genetic structure of, and hybridisation between, red (*Cervus elaphus*) and sika (*Cervus nippon*) deer in Ireland. *Mammalian Biology, 74*, 263–273.

McMeniman, C. J., & Barker, S. C. (2005). Transmission ratio distortion in the human body louse, *Pediculus humanus* (Insecta: Phthiraptera). *Heredity, 96*, 63–68.

Miller, C. R., Joyce, P., & Waits, L. P. (2002). Assessing allelic dropout and genotype reliability using maximum likelihood. *Genetics, 160*, 357–366.

Mori, A., & Le Moli, F. (1998). Mating behaviour and colony founding of the slave-making ant *Formica sanguinea*. *Journal of Insect Behavior, 11*, 235–245.

Nordborg, M., Hu, T. T., Ishino, Y., Jhaveri, J., Toomajian, C., Zheng, H., et al. (2005). The pattern of polymorphism in *Arabidopsis thaliana*. *Public Library of Science Biology, 3*, e196.

Pattengale, N. D., Alipour, M., Bininda-Emonds, O. R. P., Moret, B. M. E., & Stamatakis, A. (2010). How many bootstrap replicates are necessary? *Journal of Computational Biology, 17*, 337–354.

Pritchard, J. K., Stephens, M., & Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics, 155*, 945–959.

Pritchard, J. K., Wen, X., & Falush, D. (2010). *Documentation for structure software: version 2.3*. http://pritch.bsd.uchicago.edu/structure_software/release_versions/v2.3.3/structure_doc.pdf. Accessed 29 September 2010.

Pusch, K., Heinze, J., & Foitzik, S. (2006). The influence of hybridization on colony structure in the ant species *Temnothorax nylanderi* and *T. crassispinus*. *Insectes Sociaux, 53*, 439–445.

Pusch, K., Meindl, C., & Heinze, J. (2006). Heterospecific colony fusion in two *Temnothorax* (Hymenoptera: Formicidae) sibling ants. *Myrmecologische Nachrichten, 9*, 43–46.

Pusch, K., Seifert, B., Foitzik, S., & Heinze, J. (2006). Distribution and genetic divergence of two parapatric sibling ant species in Central Europe. *Biological Journal of the Linnean Society, 88*, 223–234.

Randi, E. (2008). Detecting hybridization between wild species and their domesticated relatives. *Molecular Ecology, 17*, 285–293.

Rissing, S. W., & Pollock, G. B. (1991). An experimental analysis of pleometrotic advantage in the desert seed-harvester ant *Messor pergandei* (Hymenoptera; Formicidae). *Insectes Sociaux, 38*, 205–211.

Ross, K. G., & Trager, J. C. (1990). Systematics and population genetics of fire ants (*Solenopsis saevissima* complex) from Argentina. *Evolution, 44*, 2113–2134.

Santschi, F. (1927). Revision des *Messor* du groupe *instabilis* Sm. (Hymenopt.). *Boletín de la Real Sociedad española de Historia natural (Madrid), 27*, 225–250.

Schlick-Steiner, B. C., Steiner, F. M., Konrad, H., Markó, B., Csősz, S., Heller, G., et al. (2006). More than one species of *Messor* harvester ants (Hymenoptera: Formicidae) in Central Europe. *European Journal of Entomology, 103*, 469–476.

Schlick-Steiner, B. C., Steiner, F. M., Konrad, H., Seifert, B., Christian, E., Moder, K., et al. (2008). Specificity and transmission mosaic of ant nest-wall fungi. *Proceedings of the National Academy of Sciences of the United States of America, 105*, 941–944.

Schlick-Steiner, B. C., Steiner, F. M., Seifert, B., Stauffer, C., Christian, E., & Crozier, R. H. (2010). Integrative taxonomy: a multi-source approach to exploring biodiversity. *Annual Review of Entomology, 55*, 421–438.

Schmidt, B. C., & Sperling, F. A. H. (2008). Widespread decoupling of mtDNA variation and species integrity in *Grammia* tiger moths (Lepidoptera: Noctuidae). *Systematic Entomology, 33*, 613–634.

Schwarz, D., Matta, B. M., Shakir-Botteri, N. L., & McPheron, B. A. (2005). Host shift to an invasive plant triggers rapid animal hybrid speciation. *Nature, 436*, 546–549.

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

Seifert, B. (2000). Rapid range expansion in *Lasius neglectus* (Hymenoptera, Formicidae)—an Asian invader swamps Europe. *Deutsche Entomologische Zeitschrift, 47*, 173–179.

Seifert, B. (2006). Social cleptogamy in the ant subgenus *Chthonolasius* Ruzsky, 1912—survival as a minority. *Abhandlungen und Berichte des Naturkundemuseums Görlitz, 77*, 251–276.

Seifert, B. (2008). Removal of allometric variance improves species separation in multi-character discriminant functions when species

are strongly allometric and exposes diagnostic characters. *Myrmecological News, 11*, 91–105.

Seifert, B. (2009). Cryptic species in ants (Hymenoptera: Formicidae) revisited: we need a change in the alpha-taxonomic approach. *Myrmecological News, 12*, 149–166.

Seifert, B., & Goropashnaya, A. V. (2004). Ideal phenotypes and mismatching haplotypes—errors of mtDNA treeing in ants (Hymenoptera: Formicidae) detected by standardized morphometry. *Organisms Diversity & Evolution, 4*, 295–305.

Seifert, B., Kulmuni, J., & Pamilo, P. (2010). Independent hybrid populations of *Formica polyctena X rufa* wood ants (Hymenoptera: Formicidae) abound under conditions of forest fragmentation. *Evolutionary Ecology, 24*, 1219–1237.

Senn, H. V., & Pemberton, J. M. (2009). Variable extent of hybridization between invasive sika (*Cervus nippon*) and native red deer (*C. elaphus*) in a small geographical area. *Molecular Ecology, 18*, 862–876.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H., & Flook, P. (1994). Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America, 87*, 651–701.

Solida, L., Grasso, D. A., Celant, A., Fanfani, A., Mori, A., & Le Moli, F. (2007). Foraging activity in two species of *Messor* harvester ants: preliminary data on size-matching and diet breadth. *Redia, 90*, 71–73.

Solida, L., Scalisi, M., Fanfani, A., Mori, A., & Grasso, D. A. (2010). Interspecific space partitioning during the foraging activity of two syntopic species of *Messor* harvester ants. *Journal of Biological Research, 13*, 3–12.

Soltis, P. S., & Soltis, D. E. (2003). Applying the bootstrap in phylogeny reconstruction. *Statistical Science, 18*, 256–267.

Stamatakis, A., Hoover, P., & Rougemont, J. (2008). A rapid bootstrap algorithm for the RAxML web servers. *Systematic Biology, 57*, 758–771.

Steiner, F. M., Crozier, R. H., & Schlick-Steiner, B. C. (2009) ("2010"). Colony structure. In L. Lach, C. Parr, & K. Abbott (Eds.), *Ant ecology* (pp. 177–193). Oxford: Oxford University Press.

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

Steiner, F. M., Seifert, B., Moder, K., & Schlick-Steiner, B. C. (2010). A multisource solution for a complex problem in biodiversity research: Description of the cryptic ant species *Tetramorium alpestre* sp.n. (Hymenoptera: Formicidae). *Zoologischer Anzeiger, 249*, 223–254.

Swofford, D. L. (1998). *PAUP*: Phylogenetic Analysis Using Parsimony (*and other methods). Version 4.0b3*. Sunderland: Sinauer.

Tamura, K., & Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution, 10*, 512–526.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., & Higgins, D. G. (1997). The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research, 25*, 4876–4882.

Turelli, M., Barton, N. H., & Coyne, J. A. (2001). Theory and speciation. *Trends in Ecology & Evolution, 16*, 330–343.

Umphrey, G. J. (2006). Sperm parasitism in ants: Selection for interspecific mating and hybridization. *Ecology, 87*, 2148–2159.

Van der Have, T. M., Pedersen, J. S., & Boomsma, J. J. (2011). Mating, hybridisation and introgression in *Lasius* ants (Hymenoptera: Formicidae). *Myrmecological News, 15*, 109–115.