Patterns of population subdivision and gene flow in the ant *Nothomyrmecia macrops* reflected in microsatellite and mitochondrial DNA markers

M. SANETRA* and R. H. CROZIER
Department of Zoology and Tropical Biology, James Cook University, Townsville, Qld 4811, Australia

Abstract

The Australian endemic ant *Nothomyrmecia macrops* is renowned for having retained a large proportion of ‘primitive’ morphological and behavioural characters. Another less studied peculiarity of this species is the production of short-winged (brachypterous) female sexuals, which presumably are poor dispersers. The males, in contrast, bear a full set of normally developed wings and thus may disperse widely. We investigated patterns of genetic differentiation within and among three distantly separated populations in South Australia using nine polymorphic microsatellite loci and four regions of mitochondrial DNA (*COI, COII, Cytb, IrRNA*). We sampled eight subpopulations, one in the Lake Gilles CP, two near Penong and five around Poochera where distances ranged from 360 km to sites separated by 2–10 km. Only little differentiation was found at the local scale (within the assumed dispersal distance of males) using nuclear markers, whereas the three distant locations were moderately differentiated (*F*$_{ST}$ = 0.06). Mitochondrial DNA genetic structure was much more pronounced on all scales (*θ*$_{ST}$ = 0.98), with regular differences in both haplotype composition and frequency even occurring among closely located sites. This lack of congruence between nuclear and mitochondrial markers strongly suggests limited female dispersal and male-biased gene flow among populations. As to the conservation status of the species there is no evidence for severe population reductions in the recent past, which would have left populations genetically depauperate.

Keywords: female philopatry, hierarchical population structure, male-biased gene flow, microsatellites, mitochondrial DNA, *Nothomyrmecia macrops*, primitive ants

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Introduction

Social insect species can display striking differences in wing morphology of the sexual forms, in ants ranging from fully winged males and females to complete wing loss in one or the other sex (Hölldobler & Wilson 1990; Heinze & Tsuji 1995). However, reduction of wings is much more common in female than in male sexuals where it is known only from a few genera (Heinze *et al.* 1993; Heinze & Tsuji 1995; Yamauchi *et al.* 2001). These differences in dispersal ability suggest unequal contributions of the sexes in mediating gene flow and generating population genetic structure. As an important consequence, sex-specific dispersal rates as well as ploidy levels significantly affect some of the statistical estimators widely used by population geneticists (Berg *et al.* 1998). Behavioural differences may also lead to divergent patterns of male and female dispersal, particularly in social organisms (Melnick & Hoelzer 1992). For instance, polygynous ant queens, though usually bearing a full set of wings, often re-enter their mother colony to reproduce while the males can disperse widely (Hölldobler & Wilson 1990; Bourke & Franks 1995).

The formation of long-lived, perennial colonies is a common feature of social insect populations, so that they have a great potential for developing genetic structure at different hierarchical levels. It is therefore interesting to investigate the effects of social organization on the dispersal and spatial structuring of populations (Chapuisat *et al.* 1997; Beye *et al.* 1998; Pamilo 1998). Limited dispersal of founding
females from their natal colony or the establishment of new colonies by budding (a colony splits into two or more daughter colonies) can result in increased relatedness among neighbours, known as population viscosity (Hamilton 1964; Pamilo 1998). In ants, increased viscosity has regularly been found in conjunction with polygyny (e.g. Pamilo 1983; Crozier et al. 1984; Chapuisat et al. 1997; Beye et al. 1998; Géraud et al. 2000; Tsutsui & Case 2001) and very recently in the ponerine ant _Diacamma cyaneiventre_, a monogynous species that lacks a winged queen caste and reproduces by Budding (Doums et al. 2002). However, the genetic effects of restricted female dispersal in independently founding species remain largely unstudied.

The Australian endemic ant _Nothomyrmecia macrops_ Clark, 1934 exhibits many morphological and social traits that are considered ancestral in the Formicidae (Taylor 1978; Hölldobler & Taylor 1984; Hölldobler & Wilson 1990; Jaisson et al. 1992) but a few derived features have also been identified. These are the vestigial worker ocelli, the ventral stridulatory organ and, most importantly, the short wings of the female sexuals (Taylor 1978). Female brachyptery occurs in a few other ant genera as well, being particularly diverse in the most closely related genus _Myrmecia_ (Clark 1951). Although mating behaviour and dispersal are not well known in _Nothomyrmecia_, it seems likely that mating takes place close to the mother nest. Recent genetic work has shown that queens can mate multiple times (one to three) and that males and their mates are unrelated (Sanetra & Crozier 2001). After mating the brachypterous queens may flutter from vegetation, however, the occurrence of long-range dispersal is unlikely. In contrast, males are normally winged and presumably good flyers (Taylor 1978). Interestingly, newly mated queens of this monogynous species display alternative reproductive strategies in that they can either found colonies independently or achieve readoption into their orphaned natal colony as a form of queen replacement (Sanetra & Crozier 2002).

_N. macrops_ was first recorded in the southeastern part of Western Australia but it has not been seen in this area since (Shattuck 1999). The currently known distribution is confined to the Eyre Peninsula in South Australia where the species occurs locally in the mallee eucalypt regions between Penong and the Lake Gilles Conservation Park (Watts et al. 1998; Fig. 1). One might expect that _N. macrops_ is more widely distributed than currently recognized because of the cryptic lifestyle of these ants. Workers are strictly nocturnal and arboreal foragers that are only found on the ground for a short time span as they leave and return to their nests. Nests contain fewer than 100 workers, and are built in soil with a very inconspicuous entrance (Taylor 1978; Shattuck 1999). Despite the likelihood of a more extended distribution, individual habitat patches are often small and isolated from one another, and a large proportion of seemingly suitable mallee woodland does not appear to be inhabited by the species. However, the density of _Nothomyrmecia_ colonies within habitat patches can be very high (this study), apparently indicating a situation with long-lived and saturated nest sites (Sanetra & Crozier 2002). This peculiar combination of general rarity and local abundance raises intriguing questions about genetic diversity and speciation in these cryptic ants.
differentiation, variability and migration. Considerable variation at one allozyme locus suggested that effective population sizes of Nothomyrmecia populations may be larger than expected from available field data (Ward & Taylor 1981), but more extensive population genetic data are needed.

Species with philopatric females and dispersing males are expected to show different patterns of genetic structure between the nuclear and the mitochondrial genome (Avise 1994; Ross & Shoemaker 1997). Thus, sex-biased gene flow can be revealed by a comparison of population genetic measures from nuclear and mitochondrial markers, such as $F_{ST}$ and $\phi_{ST}$, translated into gene flow levels (Ennos 1994; Berg et al. 1998). Mitochondrial DNA (mtDNA) is a good marker for studying matrilineal movements (Crozier 1990; Avise 1994), and the occurrence of significant structure is considered indicative of low female dispersal (Ross & Shoemaker 1997; Tay et al. 1997). In contrast, extensive male-mediated gene flow can be sufficient to reinforce nuclear genetic homogeneity across sites notwithstanding limited female dispersal (Crozier et al. 1984; Ross & Shoemaker 1997; Tay et al. 1997). The few studies of social insects employing both sets of markers in concert have documented the occurrence of male-biased gene flow in polygynous populations and its absence in monogynous populations of the same or closely related species (Ross & Shoemaker 1997; Ross et al. 1999; Seppä et al. 2002). Male-biased dispersal in the monogynous ant D. cyaniventris so far stands as an exception (Doums et al. 2002).

In this study we used both polymorphic microsatellite loci and mtDNA to elucidate patterns of genetic subdivision of N. macrops on different geographical scales, including most of the populations that are known at present. Among the major goals was to show how sex-specific dispersal rates can affect the genetic structuring of populations. It can be predicted that limited female dispersal leads to significant population differentiation but this is that foraging areas do not overlap widely as all nests are expected from available field data (Ward & Taylor 1981), and individuals that were retrieved by digging up nests entirely. The methods used to track down workers and queens on the eucalypt tree trunks and locating the nests are described by Sanetra & Crozier (2001). Foragers were sampled independently in that they were taken from different trees usually separated by at least 5 m to avoid sampling individuals from the same colony. The basis for this is that foraging areas do not overlap widely as all nests recorded were within a distance of 2 m from the tree upon which their workers were found foraging. If trees were more proximate (< 10 cases) individual microsatellite genotypes were compared to ascertain membership to different colonies.

Sampling localities for Myrmecia species in Australia were as follows: M. gulosa (Fabricius): near Waterfall, New South Wales. M. pyriformis Smith: La Trobe University Campus, Bundoora, Melbourne, Victoria. M. froggatti Forel: Atherton Tablelands, between Mt. Garnet and Innot Hot Springs, Queensland.

Microsatellite genotyping

DNA was extracted from single heads or larvae of N. macrops in 500 μL of 5% Chelex solution using a standard procedure (e.g. Crozier et al. 1999). Nine microsatellite loci designed for N. macrops, Nmac11, Nmac13, Nmac18, Nmac28, Nmac39, Nmac43, Nmac45, Nmac47 and Nmac53, were chosen to study the genetic structure of populations. Between 21 and 36 individuals were genotyped (mean 30.6, total 245) from each of the eight sites (LG: 19 colony samples, 15 foraging workers, 2 foraging queens = 36 independent samples; same order as before, Po-1: 4-25-4 = 33; Po-2: 0-33-2 = 35; Po-3: 1-24-9 = 34; Po-4: 2-20-11 = 33; Po-5: 2-19-0 = 21; Pe-1: 2-16-13 = 31; Pe-2: 2-12-7 = 21).
From the colony samples a single individual was randomly selected per colony because colony members are highly related (e.g. Ross & Shoemaker 1997). Primer sequences, amplification conditions and radioactive detection procedures for these loci are reported elsewhere (Sanetra & Crozier 2000). As a second method, primers were labelled with the fluorescent dyes HEX (Nmac43, 101–153 bp; Nmac11, 186–210 bp; Nmac53, 306–340 bp), TET (Nmac45, 131–177 bp; Nmac39, 202–222 bp; Nmac47, 289–339 bp) and 6-FAM (Nmac13, 108–132 bp; Nmac28, 150–235 bp; Nmac18, 280–306 bp) for three sets of size-compatible loci. Aliquots of 10 µL polymerase chain reactions (PCR) from all loci were combined in a final volume of 20 µL containing 2, 0.5, 1.5, 1.0, 1.0, 1.5, 1, 2 and 2 µL of Nmac11, Nmac13, Nmac18, Nmac28, Nmac39, Nmac43, Nmac45, Nmac47 and Nmac53 PCR products, respectively. Two microlitres of this cocktail mixed with a size standard were then electrophoresed in a single lane and visualized on an ABI Prism 377 DNA Sequencer (Applied Biosystems). Alleles were scored using GENSCAN Version 2.0.1 (Perkin–Elmer/ABI) software. The accuracy of 1 bp distinctions is known to be difficult using the latter method (e.g. Haberl & Tautz 1999).

At three loci where several alleles differed by only 1 bp (Nmac18, Nmac39, Nmac43), ambiguities were resolved by running known marker individuals from radioactive assays in adjacent lanes.

**mtDNA sequencing**

We sequenced a total of 2231 bp from three protein coding genes, cytochrome c oxidase I and II (COI, COII), cytochrome b (Cytb) and a segment of the large ribosomal subunit RNA (lrRNA) in *N. macrops* (185 individuals, see Table 1) and three outgroup species of the closely related genus Myrmecia, namely *M. gulosa*, *M. pyriformis* and *M. froggatti*. Primer sets for these regions were largely based on universal primers used in other studies of insects and included: cytochrome c oxidase I: C1-J-1751 (Simon et al. 1994) and CI145′-CCAAAAATTTTCTTTTCTTTTCTT-3′ (E Hasegawa, pers. commun.) framing a 496-bp fragment; cytochrome c oxidase II: for *Nothomyrmecia COII-Noth-Fw* (5′-ACATGATCTATAATTTTACAGG-3′) and COII-Noth-Rv (5′-TAGCCCACAAATTTCAGAACTT-3′) framing a 552-bp fragment (designed for this study); for *Myrmecia* L3034 (5′-TATATGGCAGATTAGTGC-3′) and H3665 (5′-CCCCAAATTCTGACAGTTG-3′) (AT Beckenbach, pers. commun.); cytochrome b: CB-J-10933 and CB-N-11367 (Simon et al. 1994) framing a 433-bp fragment, L10621 (5′-TATTGAGAAAYTGGCTC-3′) and H11370 (5′-AACTCCTCCTAGTTTATTNGG-3′) framing a 748-bp fragment (AT Beckenbach, pers. commun.); lrRNA: 874-16SIR and 875-16SmF (Cameron et al. 1992) framing an = 550-bp fragment.

Each PCR mixture contained 100 ng of DNA, a reaction buffer of 50 mM KCl, 2.5 mM MgCl₂, 10 mM Tris–HCl (pH 8.8), 250 µM dNTPs, 0.5 µM of each of the forward and reverse primers, and 1 U Taq DNA polymerase (Promega) in a volume of 50 µL. Amplification for all sequences involved an initial 94 °C step for 2 min, followed by 35 cycles of 94 °C for 30 s, annealing for 30 s, extension for 1–3 min, and a final extension of 5 min at 72 °C, using a Perkin–Elmer 9700 thermocycler. The annealing temperature and extension conditions varied according to primer pair and species. *Nothomyrmecia*: 45 °C, 3 min at 60 °C for the COI and COII regions, 43 °C, 1–3 min at 60 °C for the Cytb regions and 45 °C, 1 min at 68 °C for the lrRNA region. *Myrmecia*: 40 °C, 3 min at 60 °C for the COII region (L3034–H3665 primers), and 40 °C, 1–3 min at 60 °C for the Cytb regions but *M. froggatti* did not amplify well with the L10621–H11370 primers (the remaining regions as for *Nothomyrmecia*). The amplified products were cleaned using the PEG purification method and sequenced using the Big Dye Terminator cycle sequencing ready reaction kit (Perkin–Elmer) with an ABI Prism 377 DNA auto-sequencer. Raw sequences were edited, aligned and open reading frames for protein coding genes identified using SEQUENCHER Version 3.1 (Gene Codes Corp.). Those individuals that matched known haplotypes were sequenced in one direction, whereas

| Haplotype | A | B | C | D | E | F | G | H | I | J | K | L | M | n | h  |
|----------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LG       | 0.60 | 0.23 | 0.08 | 0.06 | 0.03 |   |   |   |   |   |   |   | 35 | 0.59 |
| Po-1     | 0.04 |   |   |   |   |   |   | 0.96 |   |   |   |   | 24 | 0.08 |
| Po-2     | 0.03 | 0.43 |   |   |   | 0.54 |   |   |   |   |   |   | 35 | 0.54 |
| Po-3     | 1.00 |   |   |   |   |   |   |   |   | 22 |   |   |   |   | 0.00 |
| Po-4     | 1.00 |   |   |   |   |   |   |   |   | 26 |   |   |   |   | 0.00 |
| Po-5     | 0.55 | 0.05 | 0.40 |   |   |   |   |   |   | 22 |   | 0.56 |
| Pe-1     | 1.00 |   |   |   |   |   |   |   |   | 11 |   |   | 10 |   | 0.00 |
| Pe-2     | 1.00 | 0.02 | 0.02 | 0.01 | 0.01 | 0.27 | 0.15 | 0.01 | 0.05 | 0.10 | 0.12 | 0.06 | 0.05 | 185 | 0.86 |

new haplotypes were always sequenced in both directions to ensure the accuracy of nucleotide designations.

Resulting consensus sequences were compared with the published sequence of the honeybee to confirm the identity of fragments of COI (Pos. 2075–2545 in Apis mellifera), COII (Pos. 3652–4176 including six gaps) and Cytb (Pos. 11 125–11 859). LrRNA sequences were different in length yielding 502 bp in Nothomyrmecia, 512 bp in M. pyriformis, 513 bp in M. gulosa and 497 bp in M. froggatti. The alignment with the corresponding 516 bp segment of the honeybee sequence (Pos. 13 412–13 927) required the insertion of numerous gaps and was 525 bp long. GenBank Accession nos for (Pos. 13 412–13 927) required the insertion of numerous gaps and was 525 bp long. GenBank Accession nos for Nothomyrmecia, M. pyriformis, M. gulosa and M. froggatti (in the order COI, COII, Cytb and LrRNA) are AY191783–191786, AY191787–191790, AY191791–191794 and AY191795–191798, respectively.

Data analysis
The final data set for microsatellite analysis comprised 213 independent samples of foragers and single randomly chosen individuals from each of 32 colonies (total 245, see sampling section). The unbiased expected heterozygosity at each locus in every population was estimated as $H_E = 2n(1 - \Sigma p_i^2)/(2n - 1)$, where $p_i$ is the frequency of each of the alleles at a locus and $n$ is the number of individuals analysed (Nei 1987). Expected numbers of heterozygotes and homozygotes in each population were obtained by multiplying $H_E$ and $(1 - H_E)$ by the sample size and then compared among populations using a G-test of independence (Sokal & Rohlf 1995). Effective population size was estimated from the equation $n_e = 4N\mu + 1$ where $\mu$ is the mutation rate and $n_e$ is the effective number of alleles given as $n_e = 1/\Sigma p_i^2$ (Hartl & Clark 1997).

The program genepop (Raymond & Rousset 1995), via its web implementation (Version 3.1c), was used to test if the study populations conformed to Hardy–Weinberg expectations by examining the genotype frequency distributions of samples from the same site. Genotypic linkage disequilibrium among all pairs of loci within each site was investigated using Fisher’s exact test as implemented in genepop. $P$-Values were estimated using the Markov chain method with the parameters 1000 dememorization steps, 1000 batches and 1000 iterations per batch for all tests. Bonferroni corrections were applied when multiple tests were performed (Sokal & Rohlf 1995) and probability values for all loci combined or all populations combined were obtained using Fisher’s method of combined probability (Sokal & Rohlf 1995).

Pairwise $F_{ST}$ estimates between sites were computed according to Weir & Cockerham (1984), and $R$-statistics according to Rousset (1996) using genepop. Allelic differentiation was calculated using a probability test. A Mantel test was used to estimate the association between genetic differentiation and geographical distance between all pairs of sampling sites (range 2–360 km) and for a subset of the range (2–10 km). The relationship between transformed values of $F_{ST}$ and log-transformed metric distances according to Rousset (1997) was also considered.

We employed an analysis of molecular variance (AMOVA, Excoffier et al. 1992) to assess the distribution of microsatellite and mtDNA variation. A nested three-level AMOVA was performed by partitioning the total sum of squares into components representing variation among populations, among sites within populations and among individuals within sites using the ARLEQUIN Version 2.001 software (Schneider et al. 2001). To study haplotype differentiation this method also yields analogues to $F$-statistics designated as $\phi$-statistics, for which we used the number of pairwise differences between haplotypes as Euclidean distance measure or assumed all haplotypes were evolutionarily equally related (in terms of sequence divergence). The probabilities that the molecular variances and fixation indices at different levels were significantly positive (indicating differentiation) were determined by permutation analysis using 1000 randomly permuted data sets. Haplotype diversity was measured as $h = n(1 - \Sigma p_i^2)/(n - 1)$ where $n$ is the number of gene copies in the sample and $p_i^2$ the sample frequency of the $i$-th haplotype (Nei 1987).

A minimum spanning haplotype network was generated using statistical parsimony as implemented in the program tcs Version 1.13 (Clement et al. 2000). We also estimated phylogenetic trees from the mtDNA sequences with maximum parsimony and maximum likelihood using PAUP* Version 4.0b10 (Swofford 2002). Tree-bisection-reconnection (TBR) branch swapping and MulTrees option were employed for both the likelihood and parsimony analyses. The maximum likelihood tree was generated with a heuristic search using the HKY85 model (Hasegawa et al. 1985) with estimated transition/transversion ratios and the shape parameter of the gamma function was also estimated from the data (number of rate categories 4). The program TREESTRUCT Version 5.0 (Schmidt et al. 2000) was used to compare the nucleotide composition of each sequence to the frequency distribution assumed in the maximum likelihood model using 5% chi-square tests. One thousand bootstrap replicates were generated for each the parsimony and the likelihood trees. We used the Kishino-Hasegawa test to attain statistical significance between alternative tree topologies (Kishino & Hasegawa 1989).

Relative male and female gene flow in the range of 2–10 km was estimated according to Berg et al. (1998). We first calculated $N_m n_f$ from the mtDNA data using the equation $F_{ST} = 1/(2N_m n_f + 1)$ assuming equal sex ratios $N_f = N_m = 1/2$. $N$ is the effective population size and $m$ the migration rate with subscripts $m$ and $f$ denoting the respective male and female portions. We then estimated male gene flow under the male-haploid DAM model (dispersal after
mating) using Wright’s classical formula $F_{ST} = 1/(1 + 4N_m)$ where $N = 9N_fN_m/(2N_f + 4N_m)$ for haplodiploids (Hartl & Clark 1997) and $m = (3m_f + m_m)/3$ with $m_f$ and $m_m$ being the migration rate of females and males, respectively. Although the mating behaviour of *N. macrops* is largely unknown, it seems most likely that females exhibit a stationary sexual calling behaviour and disperse after mating. However, absolute numbers of immigrating males and females should be interpreted with caution as many authors have pointed out the drawbacks of such estimates (e.g. Bossart & Prowell 1998; Whitlock & McCauley 1999).

**Results**

**Microsatellite variation**

The nine microsatellite loci displayed high levels of variability in the three populations (Table S1, online supplementary material) with mean expected heterozygosities ($H_E$) in Lake Gilles, Poochera and Penong of 0.85, 0.80 and 0.81 (average 0.81), respectively. The expected ratios of heterozygotes: homozygotes did not differ significantly among populations for all loci combined ($G_e = 3.03, P = 0.89$). However, significant differences in variability were detected at two loci, *Nmac18* ($G_e = 18.15, P = 0.006$) and *Nmac53* ($G_e = 23.11, P = 0.0008$). The Poochera population, though by far the largest sample, had lower heterozygosities values ($H_E = 0.66$) at these two loci compared with the other two populations ($H_E = 0.84–0.89$). In each population we found an average of 13.3 alleles across all loci (Lake Gilles 12.0, Poochera 16.6, Penong 11.2). Total number of alleles per locus ranged from 13 to 28 considering all populations studied. There were no indels inferred among these haplotypes and the number of haplotypes per population ranged between two and six (Fig. 3, Table 2).

Additional haplotypes in the three outgroup species of the genus *Myrmecia* required the insertion of numerous gaps in the 18S rRNA alignment (see Materials and methods). Resulting sequences for *Cytb* were shorter in *Myrmecia* (726, 693, 491 bp for *gulosa*, *pyriformis*, *froggatti*, respectively) due to poor performance of the L10621–H11370 primer pair. A comparison with an already published *Cytb* sequence of *M. gulosa* from Helensburgh, NSW (Crozier et al. 1995) revealed only one transition in the overlapping region of 429 bp (framed by the CB1–CB2 primers). The minimum sequence divergences between *Nothomyrmecia* and *Myrmecia* were large, 23.2% for *M. gulosa*, 20.1% for *M. pyriformis* and 19.5% for *M. froggatti*.

**Population subdivision**

The overall estimates of $F_{ST} = 0.06$ and $R_{ST} = 0.07$, which measure all effects of population substructure combined, indicated moderate genetic differentiation at the microsatellite loci. In stark contrast, the corresponding value of $\phi_{ST} = 0.98$ for mtDNA was more than an order of magnitude larger and close to unity when estimates of interhaplotype sequence divergence were used ($\phi_{ST} = 0.69$ without haploypic evolutionary relationships considered). Allele frequencies at the microsatellite loci (Table S1) differed significantly as judged by exact probability tests for allelic differentiation yielding a highly significant test result for all populations combined. Pairwise comparisons among sites within Poochera (Table 3) produced the lowest $F_{ST}$-values but the null hypothesis of allelic homogeneity was still strongly rejected on the basis of combining
probabilities for all loci \((P < 0.0008)\), indicating significant structuring even at the finest scale assayed in this study. Likewise, permutation tests showed that all pairwise comparisons of \(\theta_{ST}\) as shown in Table 4 were significant except for two adjacent patches, Po-3 and Po-4, in which the same haplotype was shared in all individuals (see Fig. 4).

We examined the microsatellite data for evidence of isolation-by-distance using all sites within and among populations. The relationship between transformed values of \(F_{ST}/(1 - F_{ST})\) and the logarithm of the geographical

| Variable sites observed in COI, COII, Cytb and lrRNA sequences of N. macrops. The thirteen haplotypes are shown as italic letters A to M. The numbers at the top designate the locations of polymorphic sites within the resolved sequence fragment. Codon positions are indicated below and nonsynonymous changes are marked with an asterisk |
|---|---|---|---|
| Consensus | COI | COII | Cytb |
| Lake Gilles | T | A | G | T | G | T | C | C | T | A | C | T | G | T | G | T |
| B | A | C | A | T | A | A | T | G | T | G | T | G | T | G | T | G |
| C | A | C | A | T | A | A | T | G | T | G | T | G | T | G | T | G |
| D | A | C | A | T | A | A | T | G | T | G | T | G | T | G | T | G |
| E | A | C | A | T | A | A | T | G | T | G | T | G | T | G | T | G |
| Poochera | F | A | C | A | G | G | A | C | T | G | A | C | T | G | A | C |
| G | A | C | A | G | T | G | A | C | T | G | A | C | T | G | A | C |
| H | A | C | A | G | T | G | A | C | T | G | A | C | T | G | A | C |
| I | A | C | A | G | T | G | A | C | T | G | A | C | T | G | A | C |
| J | A | C | A | G | T | G | A | C | T | G | A | C | T | G | A | C |
| K | A | C | A | G | G | A | C | T | G | A | C | T | G | A | C | T |
| Penong | L | C | G | C | A | C | T | A | C | G | G | A | C | G | G |
| M | C | G | C | A | C | T | A | G | C | G | G | A | C | G | G |
| Codon Pos. | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 1* | 3 | 3 | 3 | 3 | 3 |

**Fig. 2** Multilocus microsatellite estimates of pairwise differentiation \(F_{ST}/(1 - F_{ST})\) are plotted against the logarithm of map distances (in km) including all sampling sites of *Nothomyrmecia macrops* within and among populations.

**Fig. 3** Parsimony network for *Nothomyrmecia macrops* showing the relationship among mitochondrial haplotypes. Thick lines delineate the three major clusters of haplotypes, with number of nucleotide differences described in base pairs (bp). The dotted line indicates an alternative connection. Thin lines represent single base changes and thus connect haplotypes that are more closely related. Open squares indicate interior nodes in the network that were not represented in the sample (inferred intermediate haplotypes).
distances is shown in Fig. 2. Three main clusters can be discerned from left to right: (i) very small values representing the among-site variation within local populations ($F_{ST} = 0.01–0.04$, $R_{ST} = −0.01–0.07$; Table 3) in the range of 2–10 km; (ii) medium to large values among two of the study populations ($F_{ST} = 0.04–0.05$, $R_{ST} = 0.05–0.10$, distances 155–165 km; $F_{ST} = 0.06–0.10$, $R_{ST} = 0.07–0.13$, distances 185–205 km, for Lake Gilles-Poochera and Poochera-Penong, respectively); and (iii) the largest geographical distances of 350–360 km from Lake Gilles to Penong (ln distance $≈ 6$ in Fig. 2), which, however, were not reflected by their comparatively small fixation indices ($F_{ST} = 0.04–0.05$, $R_{ST} = 0.05–0.07$). A matrix comparison between all pairs of sites resulted in a significant association between genetic differentiation and geographical distance ($r = 0.71$, $P = 0.002$ and $r = 0.37$, $P = 0.018$ for the transformed values), which held true on the small scale (2–10 km) using a subset of the data including the five sites around Poochera (see Fig. 4 for a map of these locations) only for the untransformed data ($r = 0.76$, $P = 0.03$ and $r = 0.12$, $P = 0.29$ for the transformed values).

Hierarchical analyses of molecular variance (Table 5) revealed that the greatest amount of total mtDNA variation was accounted for by differences among populations (90.4%, $\phi_{SP} = 0.90$), indicating very strong macrogeographical structure at the population level. The among- and within-site variance components were much smaller (2–7%), yet haplotypic differentiation among sites was also quite large ($\phi_{SP} = 0.75$). In contrast to results from analyses of mtDNA, most of the microsatellite variation occurred among individuals within sites (93.7%), whereas a much lesser but significant amount was found among populations (4.4%) and among sites within populations (1.9%). Fixation indices at different levels underline the general trend that there is substantially more variation among populations (as measured by $F_{PT}$ and $\phi_{PT}$) as there is among sites within populations (as measured by $F_{SP}$ and $\phi_{SP}$) (Table 5).

Spatial differentiation in both haplotype composition and frequency was found among sites separated by 2–10 km (Table 1), suggesting a high degree of female philopatry over relatively short geographical distances. On this
microgeographical scale, however, haplotypes were all closely related and never separated by more than four base substitutions. When looking at the haplotype distribution among patches around the township of Poochera (Fig. 4) it appears that haplotypes were shared to varying extents but usually there were marked differences even between sites located close to each other. For instance, not a single haplotype was shared between Po-3 and Po-5 (\(\phi_{ST} = 0.80\)), separated by only 10 km. Likewise, the two sites near Penong having about the same distance between them, each appeared fixed for a specific haplotype (Table 1). However, no haplotypic differences were observed between the sites closest to town (Fig. 4), behind the Poochera Roadhouse (Po-3) and the area around the Cemetery (Po-4). Po-2 and Po-5 were the most diverse sites each containing three haplotypes (Table 1) but only moderately differentiated (\(\phi_{ST} = 0.29\); sharing 40–50% of haplotype G). Sites Po-1 and Po-2, which are only 2 km apart and not

### Table 5 Three level hierarchical analyses of nuclear (microsatellite) and mtDNA diversity in populations of *Notomyrmecia macrops* sampled from eight sites in southern Australia

<table>
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<tr>
<th></th>
<th>Microsatellites</th>
<th>MtDNA (Euclidean distance)</th>
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<tbody>
<tr>
<td></td>
<td>F-statistics</td>
<td>(\phi)-statistics</td>
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<tr>
<td>Among populations</td>
<td></td>
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<tr>
<td>Among sites</td>
<td>(F_{PT} = 0.04)</td>
<td>(\phi_{PT} = 0.90)</td>
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<tr>
<td>Within populations</td>
<td>(F_{SP} = 0.02)</td>
<td>(\phi_{SP} = 0.75)</td>
</tr>
<tr>
<td>All sites</td>
<td>(F_{ST} = 0.06)</td>
<td>(\phi_{ST} = 0.98)</td>
</tr>
<tr>
<td>Within sites</td>
<td>93.74</td>
<td>2.43</td>
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</table>

The total diversity is partitioned among the three levels of the hierarchy being compared. \(F_{PT}\) and \(\phi_{PT}\) are the fixation indices of the three populations (Lake Gilles, Poochera, Penong) relative to the total combined population, \(F_{SP}\) and \(\phi_{SP}\) are the indices of the sites relative to the population aggregates. The proportion of diversity among all sites (in parentheses) equals the sum of the variances among populations and among sites within populations, which corresponds to the global estimate of \(F_{ST}\) and \(\phi_{ST}\). Significance was tested by nonparametric permutation tests. P-Values refer to the probabilities of obtaining a larger component of variance by chance under the null hypothesis that the variance component is zero (estimated from 1000 permutations).
separated by agricultural land but interconnected by suitable habitat, exhibited unexpectedly large differences in their haplotype composition.

Genealogical relationships

A minimum spanning parsimony network in Fig. 3 clearly demonstrates that haplotypes were population specific and geographically absolutely localized, with five haplotypes in the Lake Gilles region, six in Poochera and two in Penong. The three haplotype clusters differed by 15–21 base substitutions, which indicates a relatively deep evolutionary separation among geographical regions.

The parsimony analyses resulted in five most-parsimonious trees (425 steps, consistency index = 0.98, retention index = 0.92). The strict consensus tree indicated three distinct groups corresponding with their geographical distribution. Bootstrap analysis provided good support for these major divisions, 100, 99 and 88% for Penong, Poochera and Lake Gilles, respectively. Moreover, the Lake Gilles and Poochera haplotypes were grouped together in 61% of bootstrap replicates. The likelihood analysis also recovered a tree containing three distinct clades identical to those of the parsimony analyses, with the exception that the Penong and Poochera haplotypes were associated with each other in the most likely tree (−ln likelihood = 4353.44; Fig. S1, online material. Bootstrap support for this latter grouping was low (45%) but maximum likelihood branch length was significant. However, all analyses produced tree topologies that were not significantly different in terms of their likelihood scores using the Kishino-Hasegawa test.

Sequence divergence was similar among the three internal groups. Haplotypes from Penong differed from the Poochera haplotypes by a mean of 1.06 ± 0.014% (±SE) and from the Lake Gilles haplotypes by a mean of 0.99 ± 0.017%. The Poochera and Lake Gilles haplotypes differed from each other by a mean sequence divergence of 0.84 ± 0.009%. Myrmecia outgroup species were clearly set apart with an average sequence divergence of 20.9% to the Nothomyrmecia haplotype cluster. M. gulosus and M. pyriformis differed significantly in their base composition so that only M. froggatti was retained as outgroup for subsequent analyses.

Gene flow estimates

Assessing gene flow among sites within populations from microsatellite $F_{ST}$ yielded an estimate of total gene flow of $N_{m} = 12.5$, including the five subpopulations around Poochera and the two near Penong (2–10 km range). Relative contributions of males and females to total gene flow were obtained from the difference between estimates of microsatellite and mitochondrial differentiation, being $F_{ST} = 0.02$ and $\phi_{ST} = 0.75$ (Table 5). The number of immigrating females per generation was estimated for $\phi_{ST}$ using pairwise differences and $\phi_{ST}$ equidistant to be $N_{m} = 0.17$ and 0.23, respectively. In contrast, the approximate numbers of migrating males, $N_{m} = 24$ for both estimates, was much larger. From this it follows that male gene flow is 100–140 times higher than female gene flow.

Discussion

Population structure

Our measures for genetic subdivision among the three studied populations of the ‘primitive’ ant Nothomyrmecia macrops in South Australia showed significant structure at both microsatellite and mtDNA markers. Although the mtDNA data showed very strong genetic differentiation in all population comparisons, results from microsatellite analysis yielded relatively weak to moderate levels of population subdivision. Contrasting population genetic structure displayed by the two genomes has been reported previously in other animal groups, such as mammals (Nyakaana & Arctander 1999), birds (Pietrney et al. 2000; Scribner et al. 2001) and fishes (Bernatchez & Osinov 1995), but also in social insects (Doums et al. 2002; Seppä et al. 2002). Part of the discordance can be explained by the differences in the modes of inheritance in the two marker systems, as the effective population size of mtDNA is only one quarter that of nuclear DNA. However, several studies have found a discordance larger than expected, which then has been explained in terms of sex-biased gene flow among those populations (e.g. Nyakaana & Arctander 1999; Doums et al. 2002).

Genetic differentiation of N. macrops at nuclear markers ($F_{ST} = 0.06$) over a distance of 360 km was fairly low relative to most other studies that have examined large-scale population structure in ants using microsatellites (Ross & Shoemaker 1997; Giraud et al. 2000; Goropashnaya et al. 2001; Tsutsui & Case 2001; Doums et al. 2002). For instance, the fixation index between populations of native fire ants in Argentina was 0.04 with a distance of ≈ 160 km between them (Ross et al. 1997). In two ponerine ants, which like N. macrops are primitive species with restricted dispersal abilities, genetic differentiation among localities was found to be exceptionally high (for Gnamptogenys striatula $F_{ST} = 0.17$, range 50 km, Giraud et al. 2000; for Diacamma cyanecentrum $F_{ST} = 0.26$, range 188 km, Doums et al. 2002). In contrast, populations of Lasius niger, a very common species that carries out conspicuous mating flights, were not genetically differentiated over distances of up to 1000 km (vander Have et al. 1988). In N. macrops, differentiation was also shown to increase substantially from the spatial level of sites within populations ($F_{sp} = 0.02$) to that of populations ($F_{pt} = 0.04$; Table 5). This makes clear that regional differences are stronger than variation among sites.

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Microsatellite data and their transformation into statistical estimators for population genetic structure have recently become a matter of debate (Jarne & Lagoda 1996; Gaggiotti et al., 1999; Hedrick 1999; Balloux et al. 2000; Balloux & Lugon-Moulin 2002). Specifically, it has been stated that microsatellites are likely to underestimate genetic divergence between populations because of their high degree of polymorphism (Hedrick 1999; Balloux et al. 2000). This is particularly true for population structure estimates, such as $F$- and $R$-statistics, when populations are isolated and gene flow is restricted (Balloux et al. 2000). We used both $F$- and $R$-statistics as their relative performance depends on many factors that cannot generally be disentangled (Balloux & Lugon-Moulin 2002). Based on simulation studies of $F_{ST}$ and $R_{ST}$ performances, Gaggiotti et al. (1999) demonstrated that $F_{ST}$ yields the better estimate under realistic conditions when the number of loci is small ($< 10$) or the sample size is small ($< 50$). More reliable results using $F_{ST}$ are also expected in studies of rare and endangered species with small population sizes due to the high variance associated with $R_{ST}$ (Balloux & Lugon-Moulin 2002). Given the extent of population isolation and fragmentation caused by extensive human activities in the mallee regions of South Australia during the last 100 years or so, current gene flow among populations of *N. macrops* is probably very low. In addition, our microsatellite markers were extremely variable in all subpopulations, so that the maximum possible $F_{ST}$ is greatly reduced (Hedrick 1999). We therefore conclude that our $F_{ST}$ values in the large-scale analysis are likely to considerably underestimate the true amount of genetic differentiation.

The extent of genetic differentiation at the microsatellite loci was related to geographical distance, showing a significant isolation-by-distance effect. Available data on other ant species are heterogeneous in that regard encompassing both associations between genetic and geographical distance as well as the lack of such correlations (Seppä & Pamilo 1995; Chapuisat et al. 1997; Chapuisat & Crozier 2001; Goropashnaya et al. 2001; Tsutsui & Case 2001). However, the two geographically most distant populations of *N. macrops*, Lake Gilles and Penong, did not display fixation indices of the expected size. This may be due to the probable long-term separation of these populations in general (see below). Because microsatellite evolution is subject to size homoplasy where many newly produced alleles are the same size but not identical-by-descent (Jarne & Lagoda 1996), saturation of genetic information may become a serious issue for groups separated by a large number of generations. Disregarding homoplasy then can lead to underestimating the actual divergence among populations (Garza & Freimer 1996; Viard et al. 1998; Taylor et al. 1999). For instance, homoplasy frequently occurs at interrupted repeats between subspecies of the honeybee (Estoup et al. 1995) but its frequency at pure repeat loci is supposedly larger and has yet to be determined. Hence, the possible impact of size homoplasy in highly divergent populations deserves further attention, although some recent work suggests that its detection does not invariably produce higher estimates of population structure (Angers et al. 2000).

*N. macrops* displayed an unusually high genetic mtDNA differentiation, not only at the spatial level of population, but also at a microgeographical scale among sites ($\theta_{ST} \approx 0.75$). Because the species is patchily distributed in local pockets of mallee woodland discrete populations separated by clearings or other barriers can occur in relatively small areas. In a few polygynous ants comparable fixation indices, although usually not exceeding 0.5, have been reported at similar geographical scales (Ross & Shoemaker 1997; Tay et al. 1997, Liatard & Keller 2001). As yet the monogynous *N. macrops* with its high local mtDNA differentiation appears to be paralleled only by *Diacamma cyaneiventris*, the colonies of which are headed by a single mated worker or gamergate (Doums et al. 2002). Interestingly, the current list of low gene flow species includes the entire range of gyne polymorphism in ants from fully developed wings over brachyptery to complete flightlessness in the form of gamergates. Thus, there is growing evidence that the physical inability to fly is unlikely to be the primary cause of limited female dispersal in ants (Liatard & Keller 2001) and that selection on female behaviour may be more important. However, it is worth noting that some ants with wings might not be able to fly, e.g. as proposed for microgynes of *Polyrachis* (Heinze & Hölldobler 1993).

**Phylogeography**

The observation of complete phylogeographical partitioning of the mitochondrial haplotypes depicted in Fig. 3 suggests that gene flow among the three populations has been zero over some period of evolutionary time, so that sufficient time has elapsed to attain complete lineage sorting (Avise 2000). Thus, we are looking at geographical variation of allopatric populations rather than at present-day patterns of gene flow. The current interpopulation relationships are probably the result of a subsequent loss of haplotypes since the divergence from an ancestral population. Estimates of mean sequence divergence among populations of *N. macrops* are $\approx 0.96\%$, which indicates a relatively deep evolutionary split almost reaching the level of subspecies in some other social Hymenoptera. Within the three major mtDNA lineages of honeybees mean sequence divergence is $\approx 0.3\%$, whereas sequences in different groups differ by 2.0% or more (Smith 1991). In more ancient groups, however, such as the Australian endemic ant genus *Myrmecia*, differences between species appear to be generally larger than those among well-reconized species of honeybee (Crozier et al. 1995).
Reconstructions of the geological history of southern Australia are scarce but throughout the Cretaceous and early tertiary period of epicontinental seas provide a landscape of water-filled basins hostile to terrestrial insects, such as the Murray and Lake Eyre basin, which were periodically interconnected by dry land (Cranston & Naumann 1991). It thus occurs that the origins of some of Australia’s arid zone biota may have to be sought far back in time (e.g. Barker & Greenslade 1982). Because N. macrops has particularly limited dispersal powers, a similar scenario provides a reasonable explanation for the present-day patterns of population genetic differentiation in that historical separation was mainly by changes in sea level and old river systems, especially given the geological stability of Australia in general.

Our molecular results support the extremely isolated taxonomic position of the monotypic genus Nothomyrmecia in the ant phylogeny (Taylor 1978; Hölldobler & Wilson 1990). There is now good evidence that the large and diverse genus Myrmecia constitutes the sister group of Nothomyrmecia (Ward & Brady 2002; Taylor et al. 2002), yet the minimum sequence divergence to species of the gulosas and aberrans group was as high as 20%. The recent discovery of new material of the fossil ant genus Prionomyrmex from Baltic amber has stimulated some taxonomic controversy over the maintenance of the subfamily Nothomyrmecinae and also the genus name Nothomyrmecia itself (Baroni Urbani 2000; Ward & Brady 2002). Using the methods given by Baroni Urbani (2000) the proposed synonymy of Nothomyrmecia with Prionomyrmex Mayr, 1868 does not appear justifiable because the well-developed postpetiole and the propodeal tubercles are clear distinctions between the two and have not been dealt with appropriately (P Ward, pers. commun.).

Gene flow patterns

Fine-scale population structure revealed patterns of contemporary gene flow among interbreeding subpopulations in the range of 2–10 km. Here, a comparison of genetic structure using mtDNA and nuclear markers is most insightful to assess the extent of sex-biased dispersal (Ennos 1994; McCauley 1995; Berg et al. 1998). At microsatellite markers, gene flow does not appear to be strongly reduced up to distances of 10 km. From this we conclude that the males are reasonably good dispersers, so that they can fly in from more distant patches and have mating success. Male-biased dispersal is in line with previous suggestions that Nothomyrmecia males should have good flying abilities according to their wing and thorax morphology (Taylor 1978; Hölldobler & Taylor 1984). The picture was completely different when we used mtDNA, indicating an extremely low extent of female dispersal. Results are thus consistent with the supposition of the exclusive occurrence of the short-winged female form. Aptery or brachyptery is not uncommon in the closely related genus Myrmecia in which wing morphology is subject to ample polymorphism (Clark 1951; Haskins & Haskins 1955). Although its general adaptiveness is unproven, it should be advantageous in small scattered populations held in enclaves by narrow ecological requirements (Taylor 1978). Wing reduction has also been interpreted as an adaptation to arid habitats (Tinaut & Heinze 1992) and drought stress (Wheeler 1917).

In other ants in which similar aspects of population structure have been studied, strongly male-biased gene flow has mostly been observed in polygynous species or forms. In Solenopsis invicta (Ross & Shoemaker 1997; Ross et al. 1999) and a number of Formica species (Seppä et al. 2002) restricted female gene flow is a simple consequence of the fact that polygynous queens are often readopted into their natal colony after mating. Doums et al. (2002) recently showed sex-biased gene flow in D. cyaniventre (for details on its biology see Introduction) in which the extent of male bias was 20–30 times across localities a few kilometres apart. In that regard, N. macrops can be seen as an interesting case in which a monogynous species has reduced the dispersal ability of queens and yet the independent type of colony founding prevails (Taylor 1978; Sanetra & Crozier 2002).

Genetic diversity and conservation

Like many species in Australia, N. macrops is likely to have experienced severe habitat fragmentation due largely to utilization of the mallee regions for agriculture. Populations often exist on small patches of remnant roadside mallee, which constitute a significant proportion of the species remaining habitat according to a recent survey (Watts et al. 1998). Only two of the currently known sites are located in protected areas, being the Lake Gilles Conservation Park and the Chadinga Conservation Reserve. Nevertheless, the status of N. macrops as an endangered species has remained uncertain because of its extremely cryptic lifestyle. Genetic data are thus very helpful to shed new light on the conservation status of the species.

Our results show that appreciable levels of genetic variation exist in all three main populations. The average expected heterozygosity of 81% at the microsatellite loci is well within the levels of variation reported for other very common ant genera, such as Myrmica (Evans 1993), Atta (Fjerdingstad et al. 1998) and Camponotus (Crozier et al. 1999). For example, N. macrops has higher microsatellite diversity than was found in small and geographically isolated populations of Formica cinerea in Europe (Goropashnaya et al. 2001). Overall haplotype diversity was very similar to that observed in the ponerine ant D. cyaniventris except...
that in this species a considerable fraction of localities showed no variation at all (Doums et al. 2002). Therefore, the extent of genetic diversity in natural populations of *N. macrops* does not show any signs of reduction of genetic variation. Loss of genetic variation may lead to inbreeding depression, inducing a decline in population size that may culminate in extinction (O’Brien 1994; Frankham & Ralls 1998).

Calculations based on the effective number of alleles suggested effective population sizes in individual habitat patches > 1000, which indicates that the species still occurs in reasonable numbers. Earlier estimates from Poochera, although based on one allozyme locus only, were in the order of 10^4 (Ward & Taylor 1981). These results correspond to recent findings that where the species occurs it can be quite abundant (Watts et al. 1998; this study). Moreover, no serious decline in abundance was noticed in the very confined subpopulation behind the Poochera Roadhouse over more than 20 years since the original discovery by Taylor (1978). The occurrence of *N. macrops* in small enclaves does not appear to be a particular threat to the species’ survival.

We agree with Watts et al. (1998) that there is no reason to classify *N. macrops* endangered provided that no major clearing of mallee vegetation takes place. Further research using more populations as well as the discovery of new sites is nevertheless needed before its final conservation status can be assessed. In the meantime, we recommend a management strategy aimed at conserving a broad range of the existing genetic variation, as many of the sites sampled possess unique genetic characteristics. Preventing the loss of local populations would thus preserve significant molecular biodiversity (Crozier 1997). This can mostly be achieved by saving the long stretches of roadside mallee along the Eyre Highway no. 1 and some adjacent areas. In conclusion, the future of this conspicuous member of the Australian ant fauna is promising given reasonable control of anthropogenic effects on its remaining habitat.

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**Supplementary Material**

Supplementary material is available from http://www.blackwellpublishing.com/products/journals/suppmat/MEC/MEC1900/MEC1900sm.htm

**References**


The research presented is part of several studies in the laboratory of Ross Crozier examining the phylogeny, social evolution and population genetics of Australian ants. Matthias Sanetra is an evolutionary biologist who integrates molecular genetic tools and life history patterns to address questions about social organization, species diversification and the evolution of mutualism and parasitism. Ross Crozier’s research interests are very broad but mainly are in evolutionary genetics of social groups including the evolutionary theory of social behaviour, the molecular ecology of social insect populations, and molecular phylogenetics.