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Genetics, behaviour and chemical recognition of the invading ant *Pheidole megacephala*

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Abstract

Introduced species often become ecologically dominant, displacing native species and posing a serious threat to ecosystem function and global biodiversity. Ants are among the most widespread and damaging alien species; introductions are often accompanied by population-level behavioural and genetic changes contributing to their success. We investigated the genetic structure, chemical profile and nestmate recognition in introduced populations of the invasive big-headed ant, *Pheidole megacephala*, in Australia. Behavioural analyses show that workers are not aggressive towards conspecifics from different nests, even at large geographical scales (up to 3000 km) and between populations encompassing a wide range of environmental conditions. By contrast, interactions with workers of other species invariably result in agonistic behaviours. Genetic analyses reveal that populations have low genetic diversity. No genetic differentiation occurs among nests of the same population; differentiation between populations, though significant, remains weak. Chemical analyses indicate that cuticular lipids are similar between colonies of a population, and that differentiation between populations is low. Altogether, these results indicate that the big-headed ant *P. megacephala* forms a large unicolonial population across northern/eastern Australia.

Keywords: biological invasions, cuticular hydrocarbons, genetic structure, invasive ant, *Pheidole megacephala*, unicoloniality

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Introduction

The rate of biological invasions by exotic plant and animal species (whether through accidental or deliberate introduction) has risen dramatically over the last century, with increased global trade and human mobility (e.g. Mack et al. 2000; Pascal et al. 2005). Owing to their potential for profound ecological and socio-economical disruptions, nonindigenous species are now recognized as a major threat of the 21st century (Vitousek et al. 1997; Wilcove et al. 1998; Mack et al. 2000; Sala et al. 2000; Mooney & Cleland 2001; Lockwood et al. 2006). The main causes of the success of invasive species stem from the combination of historical, genetic and ecological factors of both the invaders and the invaded ecosystem (Lodge 1993; Williamson 1996; Sakai et al. 2001; Facon et al. 2006; Suarez & Tsutsui 2008).

Ants are among the most abundant and influential invertebrate groups in terrestrial ecosystems. More than 12,450 ant species have been described to date (http://antbase.org/- September 25 2008); they occur in almost every environment on Earth (except Antarctica, Greenland and some inhospitable islands) and play a diversity of roles in terrestrial ecosystems. However, many species of ants have become established outside their native ranges (Williams 1994; McGlynn 1999), and a subset of them are among the world’s worst invasive species (Lowe et al. 2001). In their introduced habitats, they reduce the diversity and abundance of native ants through continuous warfare, they affect other taxa through direct or indirect competition, and may alter the composition of entire ecosystems and landscapes (Passera 1994; Moller 1996; Holway et al. 2002a; Sanders et al. 2003). Invasive ants share some characteristics that facilitate their dispersal from their native ranges, their introduction into new habitats through local movements and by long distance jumps, their long-term establishment and their spread in a new context. These include a close association with humans, general nesting requirements, omnivory, polygyny (occurrence of multiple reproductive queens per colony), population genetic viscosity due to colony reproduction by...
budding, and reduced intraspecific aggression between nests of the same unicolonial population (Passera 1994; Moller 1996; Holway et al. 2002a).

The ecological domination of invasive ant species is often attributed, in part, to their propensity to be unicolonial, i.e. to form expansive, polygynous populations within which individuals mix freely among physically separate nests (Holway et al. 2002a). By reducing costs associated with territoriality and aggression between nests, unicoloniality allows high worker densities and contributes to interspecific dominance and habitat saturation by the competitive exclusion of other ant species (Holway et al. 2002a). Laboratory rearing shows that nonaggressive colonies experience a reduced mortality and an increased foraging activity, resulting in higher rates of resource retrieval, greater brood production and larger worker populations (Holway et al. 1998). Consistent with these observations, among the five ants listed on the 100 world’s worst alien species (Lowe et al. 2001), all except one show a social organization based on unicoloniality. Furthermore, the presence of several queens accelerates colony growth (Vargo & Fletcher 1989) and increases the chance that transported propagules contain reproductive females able to initiate new colonies (Hee et al. 2000; Aron 2001). It was first suggested that unicoloniality is a derived trait that evolved after the introduction into new environments, due to a reduced diversity at recognition alleles through a genetic bottleneck (Tsutsui et al. 2000) or from a selection process against less common alleles ['genetic cleansing'; Giraud et al. (2002)]. However, expansive populations including polygynous supercolonies that compete for resources and potentially interbreed were documented in the native range of the Argentine ant (Suarez et al. 1999; Tsutsui et al. 2000). Therefore, Pedersen et al. (2006) suggested that unicoloniality is not a derived form of social organization that evolved after the introduction. This interpretation remains however, disputed by Suarez et al. (2008) who pointed out the strong competition between supercolonies from the same population and important differences in the genetic architecture between native and introduced populations of the species. Clearly, differences in the interpretation of the data stem from different definitions of unicoloniality. Suarez et al. (2008) refer to the traditional definition of unicoloniality, i.e. to situations where ‘entire populations consist of nests without clear colony boundaries, whose members intermix’ (Bourke & Franks 1995), a population level phenomenon. On the other hand, for Pedersen et al. (2006), unicolonial populations consist of one or several supercolonies, a colony level phenomenon. Here, we borrowed the population level definition of unicoloniality.

In social insects, discrimination of nestmates from non-nestmates relies on the use of nonvolatile olfactory cues, mainly cuticular lipids, that constitute a ‘colony odour’ (Vander Meer et al. 1998; Howard & Blomquist 2005). Cuticular profiles identify each individual’s colony of origin and are used to form a recognition template against which to compare labels of other individuals. These recognition cues may derive from the environment (e.g. food or nesting material), they may have a genetic basis, or they may originate from both exogenous (environmental) and endogenous (genetic) sources (Vander Meer et al. 1998; Howard & Blomquist 2005). In unicolonial invasive species, the lack of aggressiveness between nests could stem from reduced genetic diversity at genes that encode components of their cuticular profiles due to events of range expansion and population bottlenecks directly affecting recognition capacity.

In this study, we investigated the association between genetic architecture, chemical signature and nestmate recognition in the big-headed ant Pheidole megacephala. This species reportedly is native from southern Africa (Wilson & Taylor 1967); it has spread to most tropical and subtropical zones of the world, including several Pacific Islands (Wetterer 2007). The most serious consequence of P. megacephala invading natural habitats is the elimination of native ants and its potential cascading effects on other trophic levels. Two recent surveys in semi-natural rainforest of northern Australia illustrated the impressive dynamics of the infestation. Data collected in 1996 and 2005 showed that the infested area had doubled in nine years and that P. megacephala abundance increased more than 20-fold while abundance of other macro-invertebrates decreased (Hoffmann et al. 1999; Hoffmann & Parr 2008). The species interferes with agricultural production by tending scale insects and plant lice, and by facilitating the introduction of new potentially invasive plant species by seed transportation (Hoffmann et al. 1999; Wetterer 2007).

Despite its ecological and economic importance, P. megacephala has received surprisingly little attention. In this paper, we present the first genetic data on the population structure of this species in ecologically distinct sites (wildlife and human-disturbed habitats) from Australia, using microsatellite markers. We also examined for the first time the pattern of variation of worker cuticular profiles in relation to population genetic structure and geographical location. Finally, we measure intra- and intersite tolerance on the basis of aggression tests between workers of nests at all sites, and we correlate levels of intraspecific aggression between nest pairs to the similarity of their cuticular lipid profiles to determine the potential role of these compounds as labels for nestmate recognition.

Materials and methods

Study species

The ant Pheidole megacephala was introduced into Australia about a century ago. It was first reported in north Queensland;
since then, its range expanded and now includes subtropical southern Queensland, warm-temperate coastal New South Wales, the mediterranean Perth region, the Darwin region of the top end of the Northern Territory and, over the last decade, different areas of the Tiwi Islands where it has negatively affected the ecological integrity of the native island biota (Hoffmann et al. 1999; Andersen et al. 2004) (Fig. 1).

Field collection and sampling
Fieldwork was conducted in spring 2005, in two regions about 3000 km apart, Darwin (Northern Territory) and Brisbane (Queensland) (Fig. 1). Colony material was collected at four sites belonging to distinct ecological environments: (i) in a wildlife rainforest habitat at Howard Springs Nature Reserve ('HS'; 12°27'28" S–131°3'6" E; N = 18 nests); and in 3 human-disturbed habitats; (ii) in an urban park in Whittle Park ('WP'; 12°23'48" S–130°55'3" E; N = 16); (iii) along an urban road in Surfers Paradise ('SP'; 27°59'30" S–153°25'48" E; N = 11); and (iv) in gardens of a suburb in Chuwar ('CH'; 27°33'46" S–152°47'18" E; N = 16). The distances between the two sites sampled in Darwin and Brisbane were 20 and 80 km, respectively. Samples of workers and queens were collected from each nest and kept alive for behavioural assays. *Pheidole megacephala* have dimorphic worker castes (majors and minors), but the great majority of the workers exploring the soil surface are minors. Therefore, we used only minor workers for the behavioural assays, chemical and genetic analyses. A subsample of 50 workers from each nest was immediately used for chemical extraction. From these, eight workers per nest were chosen randomly and stored in ethanol for subsequent genetic analyses. All queens were chemically and genetically analysed.

**Behavioural assays**
Intraspecific aggression between pairs of workers was quantified immediately after collection using standardized
fighting trials (Holway et al. 1998). Two individuals were placed together in a neutral arena (diameter = 2 cm, height = 1 cm) coated with Fluon™. Interactions were scored over five min according to a scale ranging from 1 to 4, with 1 = short antennation (< two s); 2 = prolonged antennation; 3 = aggression, i.e. lunging and attempts of biting; and 4 = fight, i.e. prolonged biting and pulling. Levels 1 and 2 are referred to as nonaggressive behaviours, whereas levels 3 and 4 are referred to as aggressive behaviours. Five trials were conducted for each nest pair and the highest score was averaged across trials. Pairs of behaviours. Five trials were conducted for each nest pair.

Levels 1 and 2 are referred to as nonaggressive behaviours, whereas levels 3 and 4 are referred to as aggressive behaviours. Five trials were conducted for each nest pair and the highest score was averaged across trials. Pairs of behaviours. Five trials were conducted for each nest pair and the highest score was averaged across trials. Pairs of 

**Extraction and isolation of cuticular lipids**

Samples of about 50 workers from each nest were washed in 1 mL of hexane for five min just after collection. Chemical profiles were obtained on a Thermo Polaris Q™ electron impact ion trap mass spectrometer interfaced to a Thermo Trace GC Ultra™ gas chromatograph equipped with a DB-5MS fused silica capillary column (30 m × 0.25 mm, diameter × 0.25 μm film thickness) (Thermo Finnigan). Qualitative and quantitative data were acquired by running the Thermo Xcalibur™ data system (Thermo Finnigan). Following splitless injection of 1 μL of the sample, oven temperature was maintained at 80 °C for two min, increased to 270 °C at 20 °C/min, and then to 310 °C at 3 °C/min, using helium as carrier gas (1.2 mL/min). The injection port and transfer line were set at 240 °C and 310 °C, respectively. We used the relative amount (i.e. area) of the peaks corresponding to cuticular lipids as a quantitative measure of each profile. Compounds with a relative peak area of less than 1% in all profiles were excluded from the data. Cuticular lipids were identified by analysis of their mass spectra produced by both electron impact and chemical ionization with methane.

**Molecular procedures and genetic analyses**

**DNA extraction.** Genetic analyses were performed on a sample of 144 workers, 16 queens and their spermatheca from Howard Springs Nature Reserve; 128 workers, 24 queens and their spermatheca from Whittle Park; 88 workers, 7 queens and their spermatheca from Surfers Paradise; and 128 workers, 36 queens and their spermatheca from Chuwar. Individual ants were ground in digestion solution (100 mM NaCl, 50 mM Tris, 1 mM EDTA, 0.5% SDS, and 200 μg/mL proteinase K). To isolate sperm DNA, queens were dissected and the spermatheca removed and placed in a drop of distilled water. The envelope was ruptured with forceps and seminal fluid was recovered using a micropipette, then resuspended in 20 μL of digestion solution. Individual ants and seminal fluid were incubated for two hours at 55 °C in 100 μL of digestion solution; DNA was purified through phenol/chloroform extractions and ethanol precipitation following standard protocols (Sambrook & Russell 2001). The DNA pellet was resuspended in 100 μL of sterile H₂O.

**Genomic DNA genotyping.** Genotypes of ants were determined at eight statistically independent microsatellite loci: Pmeg-06, Pmeg-07, Pmeg-09, Pmeg-10, Pmeg-11, Pmeg-12, Pmeg-14 and Pmeg-15 (Fournier et al. 2008). Polymerase chain reaction (PCR) amplifications were carried out in a 10 μL volume, containing 2 μL of genomic DNA (about 4–8 ng of DNA), 5 μL of Qiagen HotStarTaq Master Mix (providing a final concentration of 0.5 unit HotStarTaq DNA polymerase, 1 × PCR buffer with 1.5 mM MgCl₂, and 200 μM of each dNTP) and 0.3 μM of each primer’s pair. PCR were performed with a MJ Research PTC-200 thermocycler. After an initial denaturing step of 15 min at 95 °C, the PCR consisted of 35 cycles of 30 s at 94 °C, 90 s at the annealing temperature (see detailed protocol in Fournier et al. 2008), and 60 s at 72 °C, followed by a final extension step of 30 min at 60 °C. Microsatellite loci were analysed using an ABI 3100 automated sequencer (Applied Biosystems); the lengths of PCR products were determined using Genemapper Software (Applied Biosystems) and used to construct a multilocus genotype for each individual.

**Statistical analyses**

**Genetic markers.** Basic descriptive statistics, including allelic richness (an estimate of allelic diversity that compensates for unequal sample size), expected (Hₑ) and observed (Hₒ) heterozygosities were calculated using Fstat 2.9.3 (Goudet 2001) and averaged across loci. Departure from the Hardy–Weinberg equilibrium for each locus across and within populations was tested using genepop 3.4 (Raymond & Rousset 1995). We compared the allelic information between native [South Africa; data from Fournier et al. (2008)] and introduced (Australian) regions. In addition, a hierarchical analysis of molecular variance (AMOVA) was performed using genalex 6 software (Peakall & Smouse 2006) to assess the component of genetic diversity attributable to: (i) variance between Australian and South African regions; (ii) variance between sites within regions; and (iii) variance among individuals within sites. We computed estimates of population divergence using both variance in allelic frequency (F-statistics; Weir & Cockerham 1984) and allelic size (R-statistics; Michalakis & Excoffier 1996).

Genetic differentiation was analysed over pairs of nests in each site and over pairs of sites in Australia by testing for...
analyses were conducted across three levels represented based on 999 permutations using genetic structure was tested by a Mantel test (Mantel 1967) (Lewis & Zaykin 1999). The effect of spatial separation on in a new environment – show reduction of both allele

\[ \sum p_i^2 \]

where \( p_i \) is the population frequency of allele \( i \) at locus \( j \).

**CUTICULAR PROFILE.** The diversity of cuticular compounds over nests, i.e. the constancy of the proportions of peaks in the cuticular profiles, was estimated using Nei’s index, \( I = 1 - \sum p_i^2 \) and \( p_i \) is the relative proportion of peak \( i \) over all chemical peaks identified as cuticular lipids. Non-parametric Kruskal–Wallis tests were used for multiple independent comparisons among sites; a \textit{posteriori} nonparametric pairwise comparisons of within-population cuticular compounds variation among sites were performed applying Dunn’s procedure (Siegel & Castellan 1988).

The similarity of cuticular profiles between workers from different nests in a site (the within-site cuticular profiles variation) was estimated by nest-by-nest Euclidean distances based on the relative amounts of chemical compounds. Euclidean distances vary between 0 and 1; 0 means that the chemical profiles are identical, whereas 1 indicates that no compound is shared by the two profiles. Non-parametric Kruskal–Wallis tests were used for multiple independent comparisons among sites; Dunn’s multiple comparisons tests were performed to define specific differences of within-population cuticular compounds variation among sites.

To discriminate between different patterns of chemical profile amongst sites, we performed a canonical discriminant function analysis on relative amounts (percentages) of the chemical peaks identified. Each peak had an equal statistical weight and cuticular profile was reduced to a few essential and independent components. To estimate the chemical variation attributable to the differences among sites and among nests within sampling sites, we applied the analysis of molecular variance (AMOVA) approach to a matrix of nest-by-nest Euclidean distances based on the relative amounts of chemical compounds. The significance of each variance component was tested by a random permutation test (999 permutations) using Genalex 6.

Correlations between divergence in chemical profiles (i.e. mean pairwise Euclidean distance of relative amounts of chemical compounds between nests, each sampled in a different site) and geographical distance among sites were tested by performing a Mantel test (Mantel 1967) based on 999 random permutations, using Genalex 6.

Association between variation of the cuticular profile and genetic and spatial data. To test for a possible association between variations of the cuticular profile and genetic or spatial
data, we performed Mantel correlation tests using distance matrices from interpopulation chemical (as pairwise Euclidean distances), geographical and genetic (as pairwise $F_{ST}$) distances.

Deviations of the variables from normality were tested using the Shapiro-Wilk test: normally distributed data sets were consequently analysed with parametric tests; when logarithmic or angular transformations did not suffice we used nonparametric statistics (Sokal & Rohlf 1995). Unless otherwise specified, means are presented ± SE and all statistical tests are two-tailed; they were carried out with the computer program spss Inc., 1989–2005).

**Results**

Field observations showed that no other ant species were found when *Pheidole megacephala* was present, and that the density of nests and ant workers were invariably high.

**Patterns of aggression between nests**

In all 552 of the 5-min aggression tests ($n = 187, 145, 85$ and $135$ confrontations for the sites WP, HS, SP and CH, respectively), workers showed no sign of aggressiveness, the maximum level of intraspecific aggression being equal to level 2 (Table 1). Similarly, encounters between workers from different sites were always peaceful ($n = 45$ confrontations; maximum scored = 2) (Table 1). As a consequence, no relationship was found between the level of intraspecific aggression and the distance between nests pairs, within or between sites (Spearman’s coefficient of rank correlations, all $P > 0.163$).

In contrast, interactions between *P. megacephala* workers and two local competitors (*Linepithema humile* and *Iridomyrmex purpureus*; $n = 10$ confrontations with each species) always resulted in very high levels of aggression. Both opponents initiated aggression; individuals started fights with prolonged biting and pulling (level four) resulting in the death of one or other of the two antagonists.

**Patterns of variation of the genetic markers**

Genetic diversity in Australia, measured as allelic richness and observed and expected heterozygosity, was relatively low compared to that reported for South Africa (Fournier et al. 2008). While the number of alleles ranged from 4 to 10 in South African populations, we found two to four alleles in our Australian sample. Similarly, levels of observed heterozygosity per locus in Africa varied between 0.350 and 0.900, whereas it ranged from 0.146 to 0.477 in Australia (Table 2). The hierarchical gene diversity analysis revealed that 59% and 86% of the total genetic variance was the result of within sites differences, when it was estimated from the variance in allelic frequency and size, respectively. In addition, a significant proportion of the allelic variance was attributed to differences among regions (34% and 12% for $F_{RT}$ and $R_{RT}$, respectively), and, at a lesser extent, among populations (7% and 2% for $F_{SR}$ and $R_{SR}$, respectively) (Table 3). Given a loss of 3.75 alleles on average per locus, we calculated the minimum number of queens that must have been introduced in Australia to account for the observed diversity in the study sample. We performed 200 simulations and assumed conservatively that no alleles were lost after the introduction of the first population in Australia. Our simulation estimated that at least two unrelated queens (i.e. six haploid genomes) must have been introduced in Australia.

Across the eight loci examined, no statistical difference in the genetic variability occurred between the four sites (Table 1). The genotypic proportions observed did not deviate from those expected under the Hardy–Weinberg

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Table 1: Statistics (mean ± SE) on behavioural assays (Ag: level of aggressiveness), on genetic analyses (Ar: allelic richness; $H_E$: observed heterozygosity; $H_{ST}$: expected heterozygosity; $F_{ST}$: fixation index), and chemical analyses ($I$: Nei index; $E$: Euclidean distance). Comparisons of statistics between sites were performed using nonparametric Kruskal–Wallis tests followed by posthoc Dunn procedures (different letters inside the parentheses indicate significant differences). Comparisons within- vs. between-sites were assessed using Mann–Whitney tests. ns: $P > 0.05$; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

<table>
<thead>
<tr>
<th></th>
<th>Ag</th>
<th>Ar</th>
<th>$H_O$</th>
<th>$H_E$</th>
<th>$F_{ST}$</th>
<th>I</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whittle Park (WP)</td>
<td>1.03 ± 0.013(a)</td>
<td>1.334 ± 0.018(a)</td>
<td>0.290 ± 0.019(a)</td>
<td>0.296 ± 0.020(a)</td>
<td>0.064 ± 0.026(b)</td>
<td>0.948 ± 0.002(b)</td>
<td>0.060 ± 0.003(a)</td>
</tr>
<tr>
<td>Howard Springs (HS)</td>
<td>1.103 ± 0.025(a)</td>
<td>1.386 ± 0.012(a)</td>
<td>0.361 ± 0.013(a)</td>
<td>0.352 ± 0.011(a)</td>
<td>-0.003 ± 0.012(a)</td>
<td>0.935 ± 0.003(a)</td>
<td>0.059 ± 0.003(a)</td>
</tr>
<tr>
<td>Surfers Paradise (SP)</td>
<td>1.141 ± 0.038(a)</td>
<td>1.355 ± 0.014(a)</td>
<td>0.342 ± 0.018(a)</td>
<td>0.325 ± 0.010(a)</td>
<td>0.017 ± 0.022(a)</td>
<td>0.930 ± 0.005(a)</td>
<td>0.122 ± 0.014(b)</td>
</tr>
<tr>
<td>Chuwar (CH)</td>
<td>1.104 ± 0.026(a)</td>
<td>1.300 ± 0.018(a)</td>
<td>0.217 ± 0.019(a)</td>
<td>0.226 ± 0.013(a)</td>
<td>0.011 ± 0.017(a)</td>
<td>0.935 ± 0.007(a,b)</td>
<td>0.135 ± 0.015(b)</td>
</tr>
<tr>
<td>Chi-squared values</td>
<td>10.315*</td>
<td>0.801 ns</td>
<td>4.412 ns</td>
<td>3.642 ns</td>
<td>49.366***</td>
<td>11.626**</td>
<td>35.068***</td>
</tr>
<tr>
<td>Within-sites</td>
<td>1.086 ± 0.012</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.020 ± 0.004</td>
<td>—</td>
<td>0.085 ± 0.004</td>
</tr>
<tr>
<td>Between-sites</td>
<td>1.067 ± 0.038</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.151 ± 0.003</td>
<td>—</td>
<td>0.128 ± 0.004</td>
</tr>
<tr>
<td>Mann–Whitney U$^2$</td>
<td>12 626 ns</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>90 324***</td>
<td>—</td>
<td>5451***</td>
</tr>
</tbody>
</table>
equilibrium for most single-locus comparisons within each site, except a significant heterozygote deficiency observed at *Pmeg-12* for CH (*P* = 0.038). *F*-statistics indicate a positive inbreeding coefficient in three out of the four sites studied (WP, HS, CH; Table 4); the combined analysis of the four sites gives an overall estimates of *F*<sub>IT</sub> = 0.199. Consistent with this result, the average relatedness ± standard errors between the queens and their mates was 0.364 ± 0.053 (95%CI: 0.259–0.469). Within each site no genetic differentiation occurred between nests, indicating that they belonged to a single, huge colony (Table 1).

A total of one, two, and seven private alleles (i.e. occurring at only one site) were, respectively, found in CH, WP, and HS; no private allele was observed in SP. Over all four Australian populations sampled, colony pairs shared on average 27.1% of their alleles (range: 0–77.3%; median: 26.1%). Accordingly, the tree-level hierarchical analysis revealed a

**Table 2** Comparison of genetic diversity (mean ± SE) between Australian and South African populations, measured as number of alleles (*Na*), allelic richness (*Ar*) and observed and expected (*H*<sub>O</sub>, *H*<sub>E</sub>) heterozygosity. Data for the South African population are taken from Fournier et al. (2008). Comparisons were assessed using Mann–Whitney tests. ns: *P* > 0.05; *: *P* < 0.05; **: *P* < 0.01; ***: *P* < 0.001

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Variance components</th>
<th>Per cent variation</th>
<th>Fixation indices</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among regions</td>
<td>1.128</td>
<td>34.26%</td>
<td><em>F</em>&lt;sub&gt;RT&lt;/sub&gt; = 0.343*</td>
</tr>
<tr>
<td>Among populations</td>
<td>0.212</td>
<td>6.43%</td>
<td><em>F</em>&lt;sub&gt;SR&lt;/sub&gt; = 0.098*</td>
</tr>
<tr>
<td>Within populations</td>
<td>1.953</td>
<td>59.31%</td>
<td><em>F</em>&lt;sub&gt;ST&lt;/sub&gt; = 0.407*</td>
</tr>
<tr>
<td>Among regions</td>
<td>2651.418</td>
<td>12.42%</td>
<td><em>R</em>&lt;sub&gt;RT&lt;/sub&gt; = 0.124*</td>
</tr>
<tr>
<td>Among populations</td>
<td>458.769</td>
<td>2.15%</td>
<td><em>R</em>&lt;sub&gt;SR&lt;/sub&gt; = 0.025*</td>
</tr>
<tr>
<td>Within populations</td>
<td>18 237.703</td>
<td>85.43%</td>
<td><em>R</em>&lt;sub&gt;ST&lt;/sub&gt; = 0.146*</td>
</tr>
</tbody>
</table>

**Table 3** Hierarchical analysis of molecular variance (AMOVA) of microsatellite loci among *Pheidole megacephala* populations. The components of genetic variance were estimated (i) among Australian and South African regions; (ii) among populations within regions; and (iii) among individuals within populations. ns: *P* > 0.05; *: *P* < 0.05; **: *P* < 0.01; ***: *P* < 0.001

<table>
<thead>
<tr>
<th>Site</th>
<th><em>Na</em> ± SE</th>
<th><em>Ar</em> ± SE</th>
<th><em>H</em>&lt;sub&gt;O&lt;/sub&gt; ± SE</th>
<th><em>H</em>&lt;sub&gt;E&lt;/sub&gt; ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australia (n = 488)</td>
<td>5.125 ± 0.742</td>
<td>2.545 ± 0.226</td>
<td>0.314 ± 0.036</td>
<td>0.392 ± 0.046</td>
</tr>
<tr>
<td>Min. 3</td>
<td>1.978</td>
<td>0.146</td>
<td>0.166</td>
<td></td>
</tr>
<tr>
<td>Max. 9</td>
<td>3.673</td>
<td>0.477</td>
<td>0.507</td>
<td></td>
</tr>
<tr>
<td>South Africa (n = 20)</td>
<td>7.125 ± 0.833</td>
<td>6.129 ± 0.631</td>
<td>0.626 ± 0.071</td>
<td>0.737 ± 0.031</td>
</tr>
<tr>
<td>Min. 4</td>
<td>3.549</td>
<td>0.350</td>
<td>0.583</td>
<td></td>
</tr>
<tr>
<td>Max.10</td>
<td>8.148</td>
<td>0.900</td>
<td>0.826</td>
<td></td>
</tr>
<tr>
<td>Mann–Whitney U</td>
<td>16 ns</td>
<td>1***</td>
<td>6***</td>
<td>0***</td>
</tr>
</tbody>
</table>

**Table 4** Deficit in heterozygotes and relatedness between nestmates in the four sites sampled (†relative to both South African and Australian populations, ‡relative to the Australian populations only, §relative to the collection site)

<table>
<thead>
<tr>
<th>Site</th>
<th><em>F</em>&lt;sub&gt;IS&lt;/sub&gt;</th>
<th><em>F</em>&lt;sub&gt;IT&lt;/sub&gt;</th>
<th><em>r</em>&lt;sup&gt;†&lt;/sup&gt;</th>
<th><em>r</em>&lt;sup&gt;‡&lt;/sup&gt;</th>
<th><em>r</em>&lt;sup&gt;§&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whittle Park (WP)</td>
<td>0.092 ± 0.084</td>
<td>0.151 ± 0.097</td>
<td>0.402 ± 0.051</td>
<td>0.371 ± 0.052</td>
<td>0.013 ± 0.040</td>
</tr>
<tr>
<td>Howard Springs (HS)</td>
<td>0.052 ± 0.022</td>
<td>0.049 ± 0.032</td>
<td>0.124 ± 0.029</td>
<td>0.092 ± 0.029</td>
<td>−0.005 ± 0.022</td>
</tr>
<tr>
<td>Surfers Paradise (SP)</td>
<td>0.042 ± 0.054</td>
<td>0.058 ± 0.056</td>
<td>0.448 ± 0.025</td>
<td>0.096 ± 0.025</td>
<td>0.032 ± 0.042</td>
</tr>
<tr>
<td>Chuwar (CH)</td>
<td>0.157 ± 0.084</td>
<td>0.167 ± 0.09</td>
<td>0.142 ± 0.033</td>
<td>0.102 ± 0.034</td>
<td>0.020 ± 0.028</td>
</tr>
</tbody>
</table>

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A moderate but significant genetic differentiation among sites ($F_{\text{sites-Australia}} = 0.140; 95\% \text{ CI} = 0.047–0.212$). This result was confirmed by significant genotypic differentiation between pairs of sites for all pairwise comparisons (all $P < 0.001$), and by a positive relatedness among nestmate workers (mean ± SE = 0.174 ± 0.086; one tailed $t$-test, $t = 6.681; P < 0.001$) when all nests of the four collection sites were used as a reference (Table 4). Correlation analysis showed that the geographical and genetic distances between the sites were not significantly associated (Mantel test, $r_{\text{geo,gen}}; P = 0.836$), as expected under a scenario of jump dispersal.

Gene flow, assessed using the private alleles estimates of $Nm$, was consistent with the $F_{ST}$ estimates and genotypic differentiation observed between pairs of sites. Howard Springs Natural Park (HS) showed consistently low gene flow with other regions with $Nm$ varying between 0.98 and 7.96, whereas Whittle Park (WP), Surfers Paradise (SP) and Chuwar (CH) showed higher gene flow ranging from 9.76 to 28.85.

Despite the low genetic diversity in terms of allelic richness and levels of heterozygosity, no bottleneck signature (i.e. significant heterozygosity excess than expected from the observed allele number) was detected in the four sites under study when the set of all polymorphic loci were analysed (one-tailed Wilcoxon test for $H$ excess, infinite allele mutation, all $P > 0.191$; two-phased mutation model, all $P > 0.422$). Moreover, none of the allele distributions showed a departure from the standard L-shape in the mode-shift test.

The average genetic relatedness among worker nestmates did not differ from zero when nests of the same site were taken as the reference population (one tailed $t$-test, all $P > 0.232$; Table 4). As a result, the estimated number of queens contributing to reproduction (estimated from worker relatedness) was extremely high. Genotyping of seminal fluid contained in spermatheca of 83 queens indicated that 72 (87%) were singly mated and 11 (13%) were double-mated. The probability of nondetection due to two males bearing the same alleles at all loci was reasonably low and varied from 0.02 to 0.06.

**Patterns of variation of the cuticular profile**

Cuticular lipids yielded 47 peaks ranging in size from $C_{19}$ to $C_{40}$ (Fig. 2). Electronic and chemical ionizations revealed the presence of lipids including linear alkanes, alkenes and methyl-branched alkanes. Proportion of compounds in the cuticular profiles ($I$) differed significantly between the four sites (Kruskal–Wallis test, $P = 0.009$; Table 1), with a significantly higher diversity in WP than in HS and SP (Dunn’s procedures, $P < 0.05$ for both comparisons). Within each site, cuticular profiles were very similar among nests (mean Euclidean distance ± SE = 0.085 ± 0.004; range: 0.059–0.135; Table 1). Within-site cuticular profiles variation differed significantly between the four sites under study (Kruskal–Wallis test, $P < 0.001$; Table 1). A posteriori pairwise comparisons showed that variation was identical for HS and WP (the two sites collected in the

![Fig. 2 Cuticular profile of *Pheidole megacephala* workers. Cuticular lipids yield 47 peaks; they are indicated by an arrow (two cuticular lipids are outside the retention time shown here). The 23 lipids likely involved in nestmate recognition are pointed by plain arrows.](image)
Northern Territory) and for SP and CH (the two sites collected in Queensland) (Dunn’s procedures, all \( P > 0.05 \)), but lower in HS and WP compared to SP and CH (all \( P < 0.01 \)) (Table 1).

In agreement with these results, a canonical discriminant function (CDF) analysis performed on relative amounts of the 47 chemical peaks identified discriminate the four sites significantly (Fig. 3). Among these, the relative proportion of 24 compounds varied significantly between the four study populations (Fig. 2). The two first principal components account for 98.6% of the overall variance among groups; the canonical correlation values associated to these two CDFs are, respectively, \( C_{c1} = 0.999 \) and \( C_{c2} = 0.996 \). 100% of original grouped cases were correctly classified; overall, 22.2% of cross-validated samples were assigned correctly to their site by the two CDFs (WP, 44.4%; HS, 0%; SP, 16.7%; CH, 16.7%). The results of AMOVA show that 30% of the total variation was attributable to differences between sites and 70% was accounted for by the differences among nests within sampling sites (variance components were significant: random permutation test, \( P = 0.010 \)). The peaceful behaviour observed during encounters between ants from the four different sites (see above) indicates that the 24 heterogeneous chemicals can be eliminated as likely nestmate recognition cues.

Within the four sites, there was no significant correlation between divergence in chemical compounds (i.e. Euclidean distances between pairs of nests) and geographical distance among nests (Mantel tests, all \( P > 0.138 \)).

Association between variation of the cuticular profile and genetic and spatial data

Correlation tests showed that the chemical distances between populations were not significantly correlated with geographical (Mantel test, \( r_{\text{geo,chem}}; P = 0.180 \)) or genetic distances (Mantel test, \( r_{\text{gen,chem}}; P = 0.120 \)).

Discussion

Our results show that the big-headed ant *Pheidole megacephala* forms large unicolonial populations with very high numbers of reproductive queens in northern/eastern Australia. Workers are not aggressive towards conspecifics from different nests. This lack of aggression occurs even at large geographical scales (up to 3000 km) and between populations encompassing a wide range of environmental conditions. By contrast, aggression is invariably extremely high between heterospecific workers, generally leading to the death of one protagonist. In agreement with these results, within the four study populations we found no genetic differentiation between nests and a high similarity in the cuticular profiles. Importantly, the peaceful behaviour of workers also occurs despite genetic and chemical differentiations between populations. Our data reveal that the weak genetic differentiation between populations primarily stems from the presence of private alleles in three of the four study sites. The total genetic diversity is low and hierarchical gene diversity analysis indicated that most genetic variance was attributed to within sites differences. Chemical analyses also show that the four populations differ in the relative proportion of the cuticular compounds identified. Among these, we found several double-bonded and methylated hydrocarbons, two classes of compounds reportedly involved in nestmate recognition (Vander Meer et al. 1998; Howard & Blomquist 2005). Altogether, these results suggest that all four populations studied belong to the same supercolony and arise from independent introductions from the same population established in the Australian continent.

Unicoloniality has been reported in other invasive ants such as *Linepithema humile* (Newell & Barber 1913; Markin 1970), *Anoplolepis gracilipes* (Greenslade 1972; Haines & Haines 1978), *Wasmannia auropunctata* (Clark et al. 1982), polygyne forms of *Solenopsis invicta* (Porter et al. 1988; Vander Meer et al. 1990) or *Lasius neglectus* (Boomsma et al. 1990). However, the extent of the networks of nests varies according to species and populations. A single 450-km-long supercolony of the clonal ant *W. auropunctata* inhabits New Caledonia (Fournier et al. 2005a; Foucaud et al. 2006); two supercolonies of the Argentine ant *L. humile* coexist in Europe, the main one ranging from Italy to the Spanish Atlantic coast (Giraud et al. 2002); and a mosaic of colonies of the yellow crazy ant *A. gracilipes*, ranging from small isolated nests to
supercolonies, splits up different regions in Borneo (Drescher et al. 2007). Variations in the distribution of supercolonies, as well as the relations between them, reflect the invasion history of the species. For instance, the New Caledonian supercolony of the little fire ant most likely arose from a single introduction of only one male and one female genotype, of which daughter populations spread through serial introduction events (Foucaud et al. 2006). In contrast, the coexistence of two mutually aggressive supercolonies of the Argentine ant in Europe is consistent with two independent introduction events (Jaquiéry et al. 2005). The main supercolony of the Argentine ant in Europe could have resulted from a single founding event involving as few as 6–13 unrelated queens (Giraud et al. 2002). In P. megacephala, we estimated that at least two mated queens, i.e. six haploid genomes, must have been introduced in Australia to account for the observed diversity. The number of introduced individuals influences directly the genetic diversity of the newly established population which, in turn, affects the diversity of the recognition cues encoded in the ants’ cuticular profiles (Beye et al. 1998; Stuart & Herbers 2000; Tsutsui et al. 2000). Our data did not pinpoint an association between the genetic and chemical distances. The large variability in environmental conditions between the study sites may scramble the genetic part of the cuticular hydrocarbon profiles (see also Ugelvig et al. 2008). Under these conditions, the significant but low genetic differentiation between populations could not be sufficient to induce aggressive interactions between non-nestmate workers. Consistent with this result, workers are nonaggressive despite a weak average pairwise allele-shared distance value of 27%. This contrasts with those reported by Tsutsui et al. (2000) in L. humile, where workers from nests sharing less that 60% alleles display moderate to high intraspecific aggression, whereas those sharing more than 75% alleles were nonaggressive. However, a significantly different pattern was reported in this species: colony pairs were almost aggressive despite high allele sharing (70%) and peaceful despite relatively low allele sharing (50%) (Buczkowski et al. 2004). Altogether, these results confirm no clear relationship between intraspecific aggression and genetic similarity when using neutral microsatellite markers (Buczkowski et al. 2004). Finally, because workers are peaceful irrespective of the environmental conditions and genetic relationships, our study gives no evidence of an intraspecific recognition mechanism in P. megacephala. However, one may not exclude that intraspecific recognition occurs in some populations, but it is not expressed in the populations under study.

Reduction in allelic diversity and pronounced bottleneck are genetic consequences accompanying introduction of invasive species in new environments (Wares et al. 2005; Dlugosch & Parker 2008). Our within- and between-population genetic analyses are only partly consistent with these expectations: we found a relatively low genetic diversity, but no heterozygosity excess. The lack of heterozygosity excess indicates no sign of founder effect. However, one may not exclude that populations recovered from such an effect, what may happen when the bottleneck is brief or recent, when the invading propagule is large and/or if population growth is rapid (Wares et al. 2005; Dlugosch & Parker 2008). Pheidole megacephala fulfils at least this latter condition. Many queens head colonies of the species and reproduction takes place all the year round. In addition, queens are highly fertile and can successfully revive an experimental nest even if accompanied by only a few workers (Chang 1985). An alternative explanation is that the nondetection of a bottleneck signature stems from an artefact of the analysis, since all populations have few polymorphic loci, reducing the detection power of the heterozygosity excess method applied in this study (Piry et al. 1999). In addition, spatial population structure may bias inferences of demographic and evolutionary processes (Leblois et al. 2006). Yet, our study shows that the reduction in genetic diversity in Australian populations of P. megacephala is well marked. Comparison with native African colonies indeed reveals two-times higher allelic richness in Africa than Australia (i.e. a proportional loss of allelic diversity of 0.526 (Fournier et al. 2008). Such a reduction in genetic diversity in introduced populations, as compared to native populations, has been reported in other invasive ant species. For instance, Solenopsis invicta, Linepithema humile and Wasmannia auropunctata reportedly experience a proportional loss of allelic diversity of 0.183, 0.492, and 0.718, respectively (Ross et al. 1993; Tsutsui et al. 2000; Fournier et al. 2005b).

Reduced genetic diversity may have two detrimental consequences (Allendorf & Lundquist 2003). First, it usually leads to inbreeding depression and limits population growth. In Hymenoptera, a direct cost of inbreeding is the production of homozygous diploid males, which are generally sterile. Such males arise because of the complementary sex-determining system (csd) of Hymenoptera, whereby heterozygous individuals at the sex locus develop into females, whereas hemizygous and homozygous individuals develop into males (Crozier 1971). However, the male-haploid sex determining system allows rapid purge of deleterious alleles and, hence, forsake below-average genotypes (Crozier 1970; Schmid-Hempel et al. 2007; but see Zayed & Packer 2005). Second, low genetic diversity limits evolutionary opportunities of populations to evolve. But a minimum level of genetic diversity could be maintained thanks to multiple introductions and the concomitant interbreeding between individuals from introduced populations. This process creates new exotic variants and contributes to the evolutionary success of invading species (Suarez & Tsutsui 2008). It has been recently documented as providing most of the evolutionary potential of an invasive...
population of the snail *Melanoides tuberculata* (Facon et al. 2008). Our results indicate a significant level of inbreeding in the populations studied. A positive inbreeding coefficient may result either from a Wahlund effect (i.e. a subpopulation structure) or from true inbreeding due to matings between close relatives, as pointed out by the positive relatedness between the queens and their mates. The remarkable success of *P. megacephala* in its introduced range indicates that the species is not endangered by the potential effects of low genetic diversity and associated inbreeding.

Two main hypotheses, not mutually exclusive, have been proposed to account for the evolution of unicoloniality. First, unicoloniality may have resulted from a reduction of genetic diversity and associated inbreeding. Polygyny tends to counter-select the set-up of kin discrimination mechanism and to favour acceptance of unrelated individuals and workers exchanges (Holzer et al. 2006). Our results indeed show that colonies of *P. megacephala* are headed by a high number of queens. Second, mechanisms leading to a cessation of aggressiveness could be based on a reduction of self-nonself recognition. These mechanisms may include: (i) the sharing of a collective gestalt odour (acquired when individuals mix together in winter nests; Elias et al. 2005) instead of a colony-specific odour; or (ii) the loss of genetic diversity at recognition loci following genetic bottlenecks (Holway et al. 1998; Tsutsui et al. 2000; but see Pedersen et al. 2006) or genetic cleansing (Giraud et al. 2002). The occurrence of differences in chemical profiles between populations does not plead in favour of the collective gestalt odour hypothesis. Rather, our data show a limited genetic diversity in Australian populations, consistent with a founder effect. The lack of aggressive behaviour in the big-headed ant, despite a low but significant chemical differentiation between the study sites, could also result from the threshold of self-nonself recognition being not reached or from the absence of discrimination behaviour (failing to respond to chemical differences). On the other hand, Steiner et al. (2007) recently proposed that the first stage of the formation of a supercolony may be the abandonment of aggression between members belonging to different colonies, despite the maintenance of self-nonself discrimination. Consistent with their hypothesis, the authors showed that workers of the noninvasive, monogynous ant *Lasius australicus* behave peacefully towards conspecifics from other colonies, despite high within-colony relatedness and self-nonself discrimination. Agonistic behaviour towards conspecifics in populations of *P. megacephala* could be counter-selected to favour the conquest of new territories and the search of food, and in turn, the saturation of the environment. Whether *P. megacephala* has lost its aggressiveness and forms supercolonies in its native range awaits further genetic, chemical and behavioural analyses.

The role of human activities is central in invasion processes, as invasions generally occur through human-trade, as well as from and to anthropogenically disturbed areas. Like other invasive ants (Tsutsui & Case 2001; Ugelvig et al. 2008), jump dispersal via human mediated transport is very likely in *P. megacephala* as no correlation between geographical and genetic distances among sites was detected. On the other hand, human modifications to the environment may favour the introduction of an alien species by modifying biotic and abiotic environmental conditions (Davis & Pelsor 2001; Keane & Crawley 2002; Holway & Suarez 2006) (*Solenopsis invicta*, Tschinkel 1988; *Anoplolepis gracilipes*, O’Dowd et al. 1999; *Linepithema humile*, Tsutsui & Case 2001; Holway et al. 2002b; Carptiero et al. 2004; *Wasmannia auropunctata*, Foucaud et al. unpublished data). The present work emphasizes the close relationship between the human presence and the invasion of *P. megacephala* in northern/eastern Australia. High gene flow arises between the three human-modified environments (Whittle Park, Surfers Paradise and Chuwar), whereas it is reduced with the wildlife habitat (Howard Springs Nature Reserve).

In conclusion, this study shows that encounters between two *P. megacephala* workers never yield aggression, irrespective of the chemical, genetic or geographical distances between the actors. This set of characters may have been pivotal in the ecological success of the species. If the lack of intraspecific aggressiveness is a recurrent factor present to most ant invasions, the underlying mechanisms leading to this ‘entente cordiale’ are nevertheless multifarious. Beside the lack of intraspecific aggressiveness, our study suggests that other factors are involved in the ecological success of this species: it can live in the vicinity of humans, it is very aggressive towards other species and does not seem to suffer from a reduction in genetic diversity. These life-history traits make *P. megacephala* a redoubtable species, able to invade human-modified environments and, once introduced, well able to acclimatize to varying conditions in the new range.

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