Genetic Structure, Nestmate Recognition and Behaviour of Two Cryptic Species of the Invasive Big-Headed Ant *Pheidole megacephala*

Denis Fournier1*, Maurice Tindo2, Martin Kenne2, Paul Serge Mbenoun Masse3, Vanessa Van Bossche1, Eliane De Coninck4, Serge Aron1

1 Evolutionary Biology and Ecology, Université Libre de Bruxelles, Brussels, Belgium, 2 Département de Biologie des Organismes Animaux, Faculté des Sciences, Université de Douala, Douala, Cameroun, 3 Laboratoire de Zoologie, Faculté des Sciences, Université de Yaoundé I, Yaoundé, Cameroun, 4 Royal Museum for Central Africa, Tervuren, Belgium

Abstract

**Background:** Biological invasions are recognized as a major cause of biodiversity decline and have considerable impact on the economy and human health. The African big-headed ant *Pheidole megacephala* is considered one of the world’s most harmful invasive species.

**Methodology/Principal Findings:** To better understand its ecological and demographic features, we combined behavioural (aggression tests), chemical (quantitative and qualitative analyses of cuticular lipids) and genetic (mitochondrial divergence and polymorphism of DNA microsatellite markers) data obtained for eight populations in Cameroon. Molecular data revealed two cryptic species of *P. megacephala*, one inhabiting urban areas and the other rainforests. Urban populations belong to the same phylogeographic group than those introduced in Australia and in other parts of the world. Behavioural analyses show that the eight populations sampled make up four mutually aggressive supercolonies. The maximum distance between nests from the same supercolony was 49 km and the closest distance between two nests belonging to two different supercolonies was 46 m. The genetic data and chemical analyses confirmed the behavioural tests as all of the nests were correctly assigned to their supercolony. Genetic diversity appears significantly greater in Africa than in introduced populations in Australia; by contrast, urban and Australian populations are characterized by a higher chemical diversity than rainforest ones.

**Conclusions/Significance:** Overall, our study shows that populations of *P. megacephala* in Cameroon adopt a unicolonial social structure, like invasive populations in Australia. However, the size of the supercolonies appears several orders of magnitude smaller in Africa. This implies competition between African supercolonies and explains why they persist over evolutionary time scales.

Introduction

The recent increase in human activity and commerce has, intentionally or unintentionally, greatly contributed to the dispersal of exotic species and their introduction into new habitats. In most cases, introduced species do not survive and, among those introduced species that do, many do not cause problems of any sort. However, some species’ overwhelming invasive success upsets the balance in the invaded ecosystems and constitutes an important threat to biodiversity [1]. These biological invaders have a considerable impact on the economy and on public health [2].

Biological invaders represent a paradox for evolutionary biologists [3]. Introduced populations usually suffer a dramatic reduction in genetic diversity due to bottleneck effects and genetic drift. They may experience inbreeding depression leading to more recessive, deleterious traits. Often, they have slow population growth because low densities lead to a failure to mate (i.e. the Allee effect [4]). Yet, in their introduced range biological invaders (which, by definition, are present in biogeographical regions where they did not evolve) frequently triumph in the competition for ecological resources over native populations that are shaped by natural selection to fit their environment and local conditions. A rich and expanding body of literature has been produced to help to understand why some species become successful invaders and to decipher the processes involved in biological invasions [5,6,7,8,9]. It ensures that demographic, ecological and evolutionary factors, associated both with the invader and with the environment, jointly influence the success of an invasion [10,11,12]. Migratory ability, freedom from natural enemies, biotic or abiotic changes in a given area, the phylogeographical history of the invader, phenotypical adaptive traits, and hybridization events occurring in the new...
environment can shape the interaction between the invader and the invaded environment.

Ants are among the most ecologically successful groups of organisms [13]. About 150 ant species (of the 12,642 species described to date, http://www.antbase.org/ — December 2011) have successfully established populations outside their native range [14]; many have caused major economic losses as well as changes in both species composition and the functioning of ecosystems [15]. Some life-history traits may have promoted their success as invaders; the world’s five most invasive ant species are all omnivorous, they have adopted an opportunistic nesting behaviour, they are found living in human-disturbed environments, their nests have a large number of reproductive queens (polygyny), and they show exacerbated aggressiveness towards other ant species but a reduced intraspecific aggressiveness at the population level [16,17,18,19]. In ants and other social insects, individuals discriminate colony members (nestmates) from foreign ones (non-nestmates) through multicomponent chemical signatures present on the surface of the cuticle [20,21,22]. The lack of intraspecific aggressiveness results in the formation of supercolonies where nests have no clear boundaries, and workers, brood and queens move freely between the nests. Supercolonies are made up of such a large number of nest units that individuals from distant nests are unlikely to come into direct contact with one another [23]. Populations may then be composed of the assemblage of supercolonies co-existing in close proximity or, at the endpoint of a continuum, they are uniclonial and consist of a single, huge supercolony.

The evolutionary forces leading to unicoloniality may be diverse [24,25,26], but their ecological consequences are similar: unicoloniality eliminates the costs of intra-specific competition and combines the worker forces of different nests to conquer new territories. Moreover, polygyny increases the fertility of the nests. The combination of unicoloniality and polygyny provides a decisive advantage to invasive ants because it results in extremely high population densities and allows them to efficiently monopolise environmental resources [27]. Recent findings have, however, challenged the role of unicoloniality in the invasive success of introduced populations by pointing out the absence of clear colonial boundaries leading to the formation of supercolonies and/or unicolonial populations in the native range of two invasive ants, the Argentine ant Linepithema humile [23,24] and the little fire ant Wasmannia auropunctata [28]. Thus, unicoloniality is not necessarily a derived trait that evolves after introduction, and other demographic, ecological or evolutionary factors could explain the ecological dominance of invasive populations.

The African big-headed ant Pheidole megacephala forms a complex including at least ten subspecies, all described in Sub-Saharan Africa [29,30]. According to their geographic distribution, Wheeler [29] hypothesized an Ethiopian or Malagasy origin of the species; but this remains debated [31]. This extremely widespread and destructive species is listed as one of the 100 worst invasive organisms by the International Union for the Conservation of Nature (IUCN) [32]. P. megacephala is dominant in the many areas it has invaded [31]. In Australia, it has been shown that threatens native biodiversity and natural ecosystems, infests houses, and negatively impacts horticultural and agricultural production [33]. The species’ ecological success can be linked to its unicolonial social structure. Workers are not aggressive towards conspecifics from different nests, even over large geographical scales (up to 3000 km) and between populations encompassing a wide range of environmental conditions [34]. The lack of aggressiveness is associated with the absence of genetic differentiation between nests, and reduced chemical differentiation between populations [34]. While the consequences of its invasive success and the life history traits of the African big-headed ant P. megacephala have been widely documented in the Pacific [31,34,35], studies on the species in Africa are scarce [36,37] and no data are available concerning its population genetics and social structure.

We adopted a pluralistic approach to investigate populations of P. megacephala in Cameroon, west Central Africa (Figure 1). First, we used mtDNA sequence variation to assess phylogenetic relationships between individuals from Cameroon and those previously sampled in invaded areas. Second, by using DNA microsatellite loci, we studied the genetic diversity and genetic differentiation of Cameroonian populations 0.8–230 km apart. Third, our genetic data were supplemented by worker aggression tests between individuals from different nests and populations to determine colony boundaries. Fourth, we conducted gas chromatography and mass spectrometry (GC-MS) analyses to make qualitative and quantitative comparisons of the cuticular profiles within and between populations. Finally, we tested for a possible correlation between behavioural, spatial, chemical and genetic data. In addition, we gauged the contribution of the different levels of population structure to patterns of chemical and genetic variation. Our results are compared with those previously reported for P. megacephala in South-Africa and Australia to investigate possible evolutionary changes.

Results

Phylogenetic relationships

The phylogenetic tree reconstructed on the basis of mtDNA sequences (Figure 2) reveals that Cameroonian populations of P. megacephala form two phylogenetic groups (P. megacephala var. 1 and P. megacephala var. 2) that evolved independently from a very long time. These two phylogenetic groups exactly match with the two ecological zones studied, P. megacephala var. 1 corresponding to the specimens collected in urban areas and P. megacephala var. 2 to those sampled in rainforest. The position of P. sexspinosa and P. xerophila on the phylogenetic tree, two sister species of P. megacephala [38], indicates that the two phylogenetic groups identified correspond to two cryptic species of the African big-headed ant. Moreover, our genetic analyses show that mtDNA sequences of P. megacephala var. 1 (i.e. urban samples) and of Australian specimens belong to the same phylogenetic group. They are also very close to those isolated from samples collected in Mauritius [39]; DF, personal data), South Africa [40] and Madagascar [41] (sequences and locations were retrieved by crossing databases available at http://www.antweb.org/ and http://www.boldsystems.org/). Furthermore, the Basic Local Alignment Search Tool (BLAST) shows high homologies between sequences obtained from P. megacephala var. 1 and sequences published for samples collected in Seychelles (E-values very close to zero), and BLAST for P. megacephala var. 2 gave the highest scores with samples collected in Gabon and Comoros (http://www.antweb.org/ and http://www.boldsystems.org/).

Morphological studies

The size of individuals estimated by the maximum head width was different between the two cryptic species. Minor were lower in P. megacephala var. 1 (urban) than in P. megacephala var. 2 (rainforest) populations (mean ± se = 0.617 ± 0.009 and 0.672 ± 0.011, respectively; Mann-Whitney test, p<0.001). Morphological observations at electron microscope also show that minor of P. megacephala var. 1 have shorter spines on the propodeum than minor of P. megacephala var. 2 (see Supplementary materials, Figures S1 and S2). In addition, hairs on the petiole of P. megacephala var. 1 end in a point, whereas they form a brush in P. megacephala var. 2 (see Supplementary materials, Figure S3).
Behavioural assays

No aggressive behaviour was observed in control experiments involving two workers from the same nest (1.067 ± 0.064, min-max 1–2, n = 15 tests). Behavioural assays testing the responses of two major or two minor from different nests yielded consistent results (n = 39 tests, Pearson’s correlation, r_p = 0.905, p < 0.001). By contrast, encounters between individuals from different populations resulted in aggressive responses in 60.10% of the 386 trials (P. megacephala var. 1: mean worker aggressiveness ± se = 2.947 ± 0.070; P. megacephala var. 2: 1.933 ± 0.188) (Figure 3A). Agonistic interactions were also observed, though to a lesser extent, between workers originating from different nests in five out of the eight populations sampled. In populations of P. megacephala var. 1, 46.12% of the responses were aggressive in YU (n = 722 trials; mean worker aggressiveness ± se = 2.526 ± 0.109) and 3.38% in YS (n = 148; 1.432 ± 0.068); in populations of P. megacephala var. 2, 9.09% of aggressive interactions was observed in AK (n = 11; 1.333 ± 0.220), 12.94% in CA (n = 201; 1.687 ± 0.089) and 7.14% in CB (n = 56; 1.468 ± 0.116) (Figure 3A). However, no aggressiveness occurred within populations YM (n = 30 trials; 1.133 ± 0.067) and YN (n = 5; 6) of P. megacephala var. 1 or CC (n = 7; 1.292 ± 0.042) of P. megacephala var. 2 (Figure 3A).

Our behavioural data suggested the existence of supercolonies in each of the two cryptic species of P. megacephala. Two supercolonies, SC-1 and SC-2, cohabit in population YU where workers displayed aggressive behaviours in roughly one test out of two (Figures 1 and 3A). Nests from populations YM and YN could be assigned to supercolony SC-1, and those from population YS to supercolony SC-2. Supercolony SC-3 corresponds to the nests of population CA and supercolony SC-4 groups the nests of populations AK, CB and CC (Figure 1). Consistent with this nest distribution, in both cryptic species workers from the same supercolony tolerated each other (mean worker aggressiveness ± se = 1.660 ± 0.048), whereas workers from different supercolonies were aggressive towards each other (3.489 ± 0.092; Mann-Whitney test, p < 0.001) (Figure 3B). In the latter situation, both opponents acted aggressively and fought until the death of one of the antagonists.

Genetic diversity, relatedness and population structure

One hundred and eight alleles over eight microsatellites loci were found across all of the eight populations sampled. Thirty-three alleles were detected exclusively in P. megacephala var. 1 populations (i.e. urban areas) and 52 in P. megacephala var. 2...
Measurements of genetic diversity computed for *P. megacephala* var. 2 (number of alleles per locus \( A = 5.563 \pm 0.568 \), allelic richness based on the smallest sample size \( N = 22 \) \( A_{[22]} = 4.233 \pm 0.306 \) and genetic diversity \( H = 0.609 \pm 0.033 \) were higher than for *P. megacephala* var. 1 \( A = 4.333 \pm 0.393 \), \( A_{[22]} = 3.330 \pm 0.236 \) and \( H = 0.548 \pm 0.036 \). The inbreeding coefficient computed for *P. megacephala* var. 2 \( f = 0.097 \pm 0.053 \) was lower than for *P. megacephala* var. 1 \( f = 0.120 \pm 0.067 \) (Table 1). The allelic richness was significantly different between the two groups \( p = 0.011 \); however, they did not differ by their number of alleles, their genetic diversity or their inbreeding coefficient \( p = 0.065, 0.063 \) and 0.627, respectively.

The mean genetic relatedness between workers from the same nest was significantly greater than zero for all of the populations studied (mean \( \pm se = 0.680 \pm 0.029 \) and 0.699 \( \pm 0.022 \) for *P. megacephala* var. 1 and *P. megacephala* var. 2, respectively; Table 1). Similarly, workers within supercolonies were closely related (Table 2). In contrast, relatedness between pairs of supercolonies was low and not different from zero (mean \( \pm se = 0.068 \pm 0.044 \), 95% confidence interval \([-0.036;0.172] \) for *P. megacephala* var. 1 and 0.181 \( \pm 0.097 \) \([-0.047;0.409] \) for *P. megacephala* var. 2). Accordingly, genetic differentiation estimated by the \( F_{st} \) index confirmed significant levels of population structure between supercolonies of two phylogenetic groups \( F_{st} = 0.183 \) and 0.030 for *P. megacephala* var. 1 and *P. megacephala* var. 2, respectively) and low gene flows between supercolonies belonging to different phylogenetic groups \( F_{st} \) varied from 0.216 \( (Nis = 0.91) \) to 0.319 \( (Nis = 0.53) \). In line with these results, Bayesian cluster analyses of the microsatellite data set detected genetic substructures even without including the sampling locations of the individuals. The resulting posterior probabilities were highly concordant between the replicated runs, with the highest average value indicating the most likely number of population clusters, \( K \). Our whole data yielded a best estimate of \( K = 2 \) (Figure 4A), which was also confirmed by a peak in the \( \Delta K \) statistic. Clusters C1 and C2 consisted of 35 and 29 nests, and corresponded exactly to the two cryptic species (Figure 4B). The probability for each worker to be correctly assigned to the phylogenetic group from which it was sampled was high. In addition, the Bayesian analyses applied to each cryptic species clearly indicated the presence of genetic clusters corresponding to the four supercolonies, as revealed through the behavioural assays (Figure 5).

Within supercolonies, differentiation between nests collected from Yaoundé and its periphery (i.e. *P. megacephala* var. 1) was statistically lower than differentiation between nests collected from the Campo Forest Reserve (i.e. *P. megacephala* var. 2) \( F_{st} = 0.178 \pm 0.006 \) and 0.228 \( \pm 0.006 \), respectively; Mann-Whitney test, \( p < 0.001 \). The maximum distance between nests in the same supercolony varied from 1 to 49 km; on the other hand, the minimum distance between nests belonging to two different supercolonies was 46 m (Figure 1).

Estimates of genetic diversity, inbreeding and relatedness coefficients within supercolonies are given in Table 2. Across the two cryptic species, supercolonies showed a positive and statistically significant \( f \) value, indicating non-random mating. Inbreeding coefficients ranged from 0.207 to 0.431, and corresponded to approximately 50 to 75% full-sib mating given the sperm stored in queen’s spermatheca) \( (0.694 \pm 0.038 \), 95% CI: 0.612–0.777 \). The mean genetic relatedness between cohabiting queens was high and equal to 0.634 \( \pm 0.075 \) (95% CI: 0.467–0.806). Estimates of the effective number of queens per supercolony – corrected for the effect of inbreeding - revealed a low degree of polygyny and corresponds to groups with 1–4 singly-mated queens. This is largely below the number of queens observed in the field (up to 71 queens were collected from a single nest of the population YS).

**Diversity and variation in cuticular hydrocarbon profiles (CHC)**

Cuticular lipids yielded 47 peaks ranging in size from C19 to C40. Electronic and chemical ionizations did not show qualitative
nor quantitative differences of CHC profiles isolated from urban populations (P. megacephala var. 1) with those previously described for Australian populations [34] (Nei’s index of diversity, P. megacephala var. 1: mean ± se = 0.933 ± 0.009; Australia: 0.938 ± 0.002; Mann-Whitney U-test, $p = 0.260$). Conversely, diversity of profiles isolated from individuals collected in rainforest (P. megacephala var. 2) was significantly lower than that observed in Australian populations ($I = 0.765 ± 0.015$; Mann-Whitney test, $p < 0.001$). Ionisation mass spectrometry revealed the presence of lipids including linear alkanes, methyl-branched alkanes and alkenes [34].

Discriminant analyses on 12 principal components (cross-validated) allowed 71.7% of the Cameroonian nests to be accurately classified in their phylogenetic group (YU 95.5%; YN 50.0%; YS 75.0%; CA 85.7%; CB 37.5%; CC 0%; AK 0%;) (Figure 6A). The first and second canonical discriminant functions (CDFs) accounted for 97.9% and 0.8% of the total variance, respectively. Canonical correlation values (range: 0.379 to 0.997) indicated that the canonical varieties might explain the differences between nests from the two cryptic species. Likewise, the cuticular hydrocarbon profiles of nests clustered according to behavioural and genetic supercolonies (Figure 6B). The first two principal components accounted for 99.7% of the overall variance between groups; the canonical correlation values associated with these two CDFs were, respectively, $C_{c1} = 0.996$ and $C_{c2} = 0.626$. Overall, 70.0% of the cross-validated samples were correctly assigned to their supercolonies by the two CDFs (SC-1 60.0%; SC-2 72.7%; SC-3 85.7%; SC-4 57.1%).

Within each cryptic species, the diversity of the chemical profiles measured by the Nei index $I$ did not differ between nests, nor between supercolonies (Kruskal-Wallis tests followed by Dunn’s procedures, all $p > 0.05$; Table 1). In contrast, profiles of minor collected from the rainforest were significantly less diverse than the profiles of urban populations ($I = 0.765 ± 0.015$ and 0.933 ± 0.009, respectively; Mann-Whitney test, $p < 0.001$). The similarity in chemical profiles estimated by the Euclidean distances $E$ was different between the two ecological zones. The chemical differentiation between nests was lower in P. megacephala var. 1 (urban) than in P. megacephala var. 2 (rainforest) populations ($E = 0.151 ± 0.006$ and 0.444 ± 0.006, respectively; Mann-Whitney test, $p < 0.001$; Table 1).

**Associations between behavioural, genetic, chemical and spatial data**

In populations of P. megacephala var. 1, correlation analyses showed that aggressiveness was closely related to genetic (Mantel
test, \( r = 0.487, p<0.001 \)) and spatial distances \( (r = 0.502, p<0.001) \). In contrast, chemical distances had no influence on aggressiveness \( (r = -0.054, p = 0.597) \). In \( P. megacephala \) var. 2, aggressiveness was not associated with any of the three distances estimated (all \( p>0.527 \)). Genetic differentiation, CHC profile dissimilarity and spatial distances were all positively correlated (all \( r>0.276, p<0.001 \)), except in \( P. megacephala \) var. 2 where chemical and spatial distances were not associated \( (r = 0.036, p = 0.430) \). For both cryptic species, the diversity of cuticular compounds measured by the Nei index \( I \) was associated with neither the allelic richness (Spearman’s rank correlations, \( p>0.569 \)), nor with the genetic diversity \( (p>0.366) \).

**Contributions of various levels of population structure to patterns of chemical and genetic variation**

In the analyses of molecular variance (AMOVA), the total genetic differentiation among populations was 0.482 (\( F_{CT} \)) of which 0.352 (\( F_{ST} \)) was due to the among-cryptic species component and 0.200 (\( F_{SC} \)) was due to the among-population within-cryptic species component (Table 3). AMOVA analyses of chemical compound differentiation provided similar results, with differentiation among populations being 0.412 of which 0.367 was due to the among-species component and 0.071 to the among-population within-species component (Table 3). The distributions of genetic and chemical variations were comparable whether we used populations or supercolonies as reconstructed by the behavioural assays (Table 4).

**Discussion**

Our results show that two cryptic species of *Pheidole megacephala* cohabit in Cameroon, and that these species differ in their ecological niche: *P. megacephala* var. 1 occupies urban areas and *P. megacephala* var. 2 is found in rainforests. These two reproductively isolated groups may have cohabited for a long time in Cameroon. Alternatively, urban populations may result from a recent introduction from other populations originating from Africa, or not. The presence of private alleles supports a reduced gene flow between species. Despite this reproductive isolation, our data show that they share similar reproductive and dispersal strategies. Within each phylogenetic group, nests are organized into mutually aggressive supercolonies, and supercolonies are genetically differen-

---

**Table 1. Number of nests sampled, location and descriptive population statistics (mean ± se and [95% confidence interval]) for genetic and chemical analyses of eight Cameroonian populations of the invasive ant *P. megacephala***

| Genetic data | Chemical data |
|--------------|--------------|
| **P. megacephala var. 1** (urban populations) | **P. megacephala var. 2** (rainforest populations) |
| YM (3 nests) | AK (4 nests) |
| 3.57±0.429 | 4.00±0.802 |
| 3.296±0.439 | 3.512±0.557 |
| 0.533±0.059 | 0.597±0.042 |
| 0.075±0.090 | -0.103±0.161 |
| 0.746±0.102 | 0.711±0.052 |
| 0.134±0.061 | 0.202±0.045 |
| 0.836±0.036 | 0.456±0.026 |
| 0.075±0.801 | 0.026±0.022 |
| | 0.773±0.027 |
| | 0.472±0.022 |
| | 0.330±0.398 |

---

**Table 1 (continued)**

| Genetic data | Chemical data |
|--------------|--------------|
| **P. megacephala var. 1** (urban populations) | **P. megacephala var. 2** (rainforest populations) |
| N3.86053 E11.50730 | N2.78759 E10.27990 |
| 4.250±0.675 | 4.00±0.802 |
| 3.679±0.498 | 3.512±0.557 |
| 0.602±0.073 | 0.597±0.042 |
| -0.031±0.079 | -0.103±0.161 |
| 0.397±0.104 | 0.711±0.052 |
| 0.088 | 0.202±0.045 |
| 0.944±0.004 | 0.456±0.026 |

---

**Table 1 (continued)**

| Genetic data | Chemical data |
|--------------|--------------|
| **P. megacephala var. 1** (urban populations) | **P. megacephala var. 2** (rainforest populations) |
| N3.89885 E11.49714 | N2.57509 E9.89992 |
| 3.143±0.634 | 6.125±0.895 |
| 2.368±0.343 | 3.851±0.463 |
| 0.416±0.094 | 0.622±0.042 |
| 0.025±0.207 | 0.392±0.096 |
| 0.792±0.038 | 0.656±0.035 |
| 0.144±0.020 | 0.366±0.003 |
| 0.944±0.004 | 0.927±0.013 |
| 0.111±0.007 | 0.168±0.010 |

---

**Table 1 (continued)**

| Genetic data | Chemical data |
|--------------|--------------|
| **P. megacephala var. 1** (urban populations) | **P. megacephala var. 2** (rainforest populations) |
| N3.89885 E11.49714 | N2.57509 E9.89992 |
| 3.143±0.634 | 6.125±0.895 |
| 2.368±0.343 | 3.851±0.463 |
| 0.416±0.094 | 0.622±0.042 |
| 0.025±0.207 | 0.392±0.096 |
| 0.792±0.038 | 0.656±0.035 |
| 0.144±0.020 | 0.366±0.003 |
| 0.944±0.004 | 0.927±0.013 |
| 0.111±0.007 | 0.168±0.010 |

**Note:** Mean ± se population genetic statistics over eight loci. A: number of alleles; Ar: allelic richness; H: gene diversity; f: inbreeding coefficient; r: relatedness; Fst: Wright’s measure of population subdivision; I: Nei index; E: Euclidean distance. Statistical comparisons between ecological zones were conducted using non-parametric, Mann-Whitney tests (different letters indicate significant differences).

doi:10.1371/journal.pone.0031480.t001
entiated. Genetic differentiation between supercolonies may result from at least two factors. First, new queens are recruited among daughters, as revealed by the high relatedness among cohabiting queens \((r = 0.637 \pm 0.075)\). Second, mating takes place in the nests between related individuals \((r = 0.694 \pm 0.038)\) between a queen and her mate), and results in a relatively high level of inbreeding. This reproductive pattern and the significant correlation between the genetic and geographical distances indicate that the main mode of colony reproduction occurs *via* dependent foundation, whereby a queen and a few workers leave the natal nests to start a new colony nearby. These breeding and dispersal strategies contribute maintaining genetic homogeneity in each supercolony, and simultaneously generate high levels of inbreeding. In Hymenoptera, a direct cost of inbreeding is the production of diploid males. However, in spite of the high inbreeding coefficient, no such diploid males were found in our sample \((X = 38\), unpublished data\). As suggested for other Hymenoptera [34,43], the male-haploid sex determining system may help purging deleterious alleles [44,45].

The two cryptic species show life history traits that are characteristic of introduced populations, both in Yaoundé where preliminary field observations indicate that *P. megacephala* is dominant in the human-disturbed zone and could negatively affect native ant fauna [personal observations, Mbounou et al. unpublished data] and [36], and in less disturbed habitats of the Campo Forest Reserve. Nests are numerous and form supercolonies extending over 1 to 49 km (Figure 1), with high numbers of reproductive queens. In Yaoundé and its periphery, the supercolonies are very close to each other, with nests belonging to different supercolonies being separated by only 46 m. Interestingly, field observations showed that the contact zone is propitious to the presence of two other ant species, namely *Odontomachus troglodytes* and *Myrmecia opaciventris*. In contrast to Australian populations, *P. megacephala* in Cameroon does not form a huge, single uniclonial population spreading over thousands of kilometres, but a mosaic of mutually aggressive supercolonies.

### Table 2. Statistics (mean ± se and [95% confidence interval]) for genetic and chemical analyses of the four supercolonies defined from behavioural assays.

|                       | Genetic data | Chemical data |
|-----------------------|--------------|---------------|
|                       | A            | Ar            | H            | f             | f             | Fst            | I              | E              |
| **P. megacephala var. 1** (urban populations) |              |               |              |               |               |                |                |                |
| SC-1 (13 nests)       | 11.250 ± 1.971 | 11.086 ± 1.931 | 0.769 ± 0.034 | 0.363 ± 0.065 | 0.709 ± 0.054 | 0.273 ± 0.014 | 0.946 ± 0.001 | 0.100 ± 0.007 |
|                       | [0.210–0.517] | [0.599–0.819]  | [0.246–0.300]  | [0.087–0.113]  |                |                |                |                |
| SC-2 (22 nests)       | 8.250 ± 1.485 | 7.880 ± 1.455 | 0.596 ± 0.071 | 0.207 ± 0.078 | 0.663 ± 0.036 | 0.146 ± 0.005 | 0.926 ± 0.013 | 0.172 ± 0.010 |
|                       | [0.022–0.392] | [0.587–0.739]  | [0.135–0.157]  | [0.153–0.192]  |                |                |                |                |
| **P. megacephala var. 2** (rainforest populations) |              |               |              |               |               |                |                |                |
| SC-3 (15 nests)       | 6.000 ± 0.866 | 5.720 ± 0.791 | 0.599 ± 0.054 | 0.431 ± 0.104 | 0.721 ± 0.026 | 0.216 ± 0.006 | 0.705 ± 0.019 | 0.364 ± 0.017 |
|                       | [0.185–0.678] | [0.665–0.776]  | [0.203–0.227]  | [0.330–0.398]  |                |                |                |                |
| SC-4 (14 nests)       | 5.625 ± 0.754 | 5.499 ± 0.733 | 0.606 ± 0.047 | 0.254 ± 0.099 | 0.676 ± 0.036 | 0.242 ± 0.012 | 0.801 ± 0.018 | 0.455 ± 0.008 |
|                       | [0.019–0.489] | [0.598–0.754]  | [0.219–0.265]  | [0.439–0.471]  |                |                |                |                |

SC-1: nests YM-13, YM-18, YM-19, YN-22, YN-23, YU-01, YU-02, YU-12, YU-24, YU-40, YU-41, YU-42, YU-43. SC-2: nests YS-31, YS-32, YS-33, YS-34, YS-35, YS-36, YS-37, YS-38, YU-03, YU-04, YU-05, YU-06, YU-07, YU-08, YU-09, YU-10, YU-11, YU-25, YU-26, YU-27, YU-39, YU-44. SC-3: nests CA-52, CA-53, CA-54, CA-55, CA-56, CA-57, CA-58, CA-59, CA-60, CA-61, CA-62, CA-63, CA-64, CA-66, CA-67. SC-4: nests XK-66, XK-68, XK-91, AK-92, CB-70, CB-71, CB-72, CB-74, CB-75, CB-76, CB-77, CB-79, CC-80, CC-83.

**A**: number of alleles; **Ar**: allelic richness; **H**: gene diversity; **f**: inbreeding coefficient; **Fst**: Wright’s measure of population subdivision; **I**: Nei index; **E**: Euclidean distance.
doi:10.1371/journal.pone.0031480.t002

Such a difference between populations has been documented for two other invasive ants between invaded and native ranges. Supercolonies of the Argentine ant *Linepithema humile* have been found spanning 6000 kilometres across Europe [25] and extending for 1000 km in California [24], whereas populations are organized into smaller supercolonies in its native range [23,46,47]. Likewise, a single 450-km-long supercolony of the little fire ant *Wasmannia auropunctata* has been reported in New Caledonia [48], while supercolonies encountered in the native range are several orders of magnitude smaller [28,49].

Our data also reveal that populations collected in urban areas in Cameroon belong to the same phylogenetic group than those from Australia and South Africa. However, genetic diversity and allelic richness appear significantly different among Cameroon (P. megacephala var. 1 populations; present study), South Africa [40] and Australia [34] (one-way ANOVAs followed by Tukey’s post hoc tests, \(F=14.151, p<0.001\); Figure 7). Australian populations exhibit a remarkably low genetic diversity compared to South African and Cameroonian populations; in contrast, no difference occurs between the African populations (Figure 7). An analysis of molecular variance (AMOVA) shows that of the total molecular variance, 34% \((F_{RT}=0.340)\) is attributed to divergence among countries, 9% \((F_{ST}=0.135)\) to populational differences within region, and 57% \((F_{ST}=0.429)\) to individual differences within populations (Table 5). Such genetic changes have been studied in detail in the Argentine ant *L. humile* [24,25], the fire ant *Solenopsis invicta* [50] and the little fire ant *W. auropunctata* [51], and have been shown to result from bottlenecks at introduction. Reduced genetic diversity may have negative effects by limiting population growth [52,53] and by diminishing the ability to adapt to a new environment [54]. However, successive introductions with interbreeding between introduced populations [55,56,57], and the purge of deleterious alleles linked to the drastic reduction in genetic diversity [58,59,60] may greatly limit the negative effects of low genetic diversity, as exemplified by the invasive success of introduced populations. According to a stepping-stone model,
once populations are established in urban areas, they probably serve as sources for secondary introductions elsewhere, and as one goes along introductions, nests form networks. Thus, supercolonies can be considered as a step along a continuum of social organization where multicoloniality and unicoloniality represent the two extremes. This process was documented for the Argentine ant Linepithema humile [61,62]: its extent from Rosario (Argentina) to California (US) via Buenos Aires and the south-eastern United States was accompanied by an increase in the size of the colonies. Phylogenetic relationships and genetic differentiation between supercolonies, as well as their allelic composition raise the possibility that urban P. megacephala colonies could be the source of introduced populations.

Cuticular lipids present on the surface of the cuticle of ants mediate nestmate recognition between individuals [20,21,22]. Ants react according to either a gradual or a threshold effect [63]. In the first situation, they exhibit scaled aggressiveness from rejection to acceptance, the intensity of the response depending on the qualitative and/or quantitative variations between the cuticular profiles of the protagonists [21,22,63]; in the second situation, ants are accepted or rejected. Our data do not show a correlation between aggressiveness and the differentiation of the cuticular hydrocarbon profile; rather, they suggest that the behaviour of P. megacephala workers depends on a threshold beyond which the response is aggressive. This threshold could be expressed through variations in the relative abundance of chemical compounds in the cuticular profile, or through the existence of chemical compounds specific to the supercolony. In the yellow crazy ant Anoplolepis gracilipes, aggressiveness between supercolonies increases with the dissimilarity in cuticular hydrocarbon profiles, and the proportion of qualitatively different cuticular hydrocarbon profiles is positively correlated with the proportion of alleles that differed between supercolony pairs [64]. However, the composition of the cuticular profiles is generally determined by the interaction between heritable and environmental cues derived from food or nesting materials. Our data show that cuticular profiles in P. megacephala do not differ between supercolonies belonging to the same environment, despite a significant genetic differentiation between supercolonies. In addition, our results indicate that there is no association between genetic and chemical diversity. This suggests that the presence of private alleles is not expressed in the cuticular compounds specific to the supercolony. Therefore, it seems likely that the environment plays a significant role in the cuticular cues involved in nestmate recognition in this species. However, we cannot excluded that nestmate discrimination in P. megacephala relies upon highly sensitive responses to few

---

**Figure 4. Bayesian cluster analysis.** A: $\Delta K$ (a measurement of the rate of change in the structure likelihood function) values as a function of $K$, the number of putative supercolonies. In this case, $K=2$. B: Graphical representation of the data set for the most likely $K=2$, where each colour corresponds to a suggested cluster and each individual is represented by a vertical bar. The populations are indicated in the X-axis (AK: Akok; CA, CB and CC: Campo Forest Reserve; YM, YN, YS and YU: Yaoundé and its periphery). The Y-axis represents the probability for which an individual will be assigned to each cluster.

doi:10.1371/journal.pone.0031480.g004
specific compounds masked in the overall odour profile and not detected in our analyses [65], and/or that only some classes of chemicals or individual compounds involved in nestmate recognition are correlated with genetic and behavioural patterns [66], or that this species uses chemicals other than cuticular hydrocarbons for nestmate recognition [67]. Further investigations consisting in treating individuals with known compounds, or to transfer cuticular lipid extracts between ants from different supercolonies [68] should be conducted to clarify the support of nestmate recognition in P. megacephala. Furthermore, our data reveal a greater chemical diversity in cuticular profiles for ants collected from urban areas than for those collected from the Campo Forest Reserve habitats. Our sampling was conducted during the dry season, which is less favourable to population and colony growth. At this period of the year, it seems likely that urban areas offer a greater diversity in the choice of habitats and food sources than forest areas. Consistent with this, we observed that ants in Yaoundé were opportunistic generalists and used a variety of nesting sites and food sources, while in the forest they were usually confined to plant roots (e.g. Pennisetum purpureum Schumann-cher [Poaceae] and Costus afer Ker-Gawler [Costaceae]) where they tended aphids. Chemical differentiation between urban and rainforest populations may therefore be induced by environmental conditions, but it may also stem from phylogenetic differences. Whether these chemical divergences lead to or result from reproductive isolation could remain crucial for understanding the evolutionary history of the two cryptic species.

Our study shows that the African big-headed ant P. megacephala forms unicolonial populations in Cameroon, both in natural habitats and urban areas. It has been suggested that unicoloniality is a key factor responsible for the ecological dominance of ants. Many invasive ant species (e.g. Linepithema humile [69], Anoplolepis gracilipes [70], Wasmannia auropunctata [71], Solenopsis invicta [72], Lasius niloticus [73], Paratrechina longicornis [74], P. megacephala [34] and Tapinoma sessilis [75]) are indeed unicolonial. However, recent findings challenge the role of unicoloniality in the invasive success of introduced populations and have highlighted the evolutionary contradictions posed by this social organization in terms of kin selection. First, introduced populations of a given species do not necessarily have a different social or genetic structure than native ones [23,28,47], and native populations may also attain high densities [76,77]. Second, unicolonial populations are characterized by the presence of numerous egg-laying queens, so that workers display reproductive altruism towards random members instead of relatives [78,79]. Interestingly, our data show that this is not the case in the present study, where relatedness between workers within supercolonies is high (r = 0.692 ± 0.014) due notably to a high kinship among breeders. Our study emphasizes a fundamental distinction between African and introduced populations in Australia in terms of nest structure. In Australia, populations are organized into a single supercolony spreading over 3000 km [34]. In Cameroon, on the other hand, P. megacephala forms a mosaic of mutually unrelated supercolonies within which individuals are related. In this context, competition...
(e.g., conquering new territories or food sources) between supercolonies favours reproductive altruism within supercolonies [23,80,81], and hinders the propagation of selfish detrimental traits by selectively eliminating less competitive supercolonies to the advantage of more competitive ones [78,82].

One commonly proposed explanation for invasive success is the enemy release hypothesis (ERH), which states that the lack of natural predators and competitors in an exotic environment favours the successful establishment and spread of species outside of their native range [83]. Our study shows that *P. megacephala* var. 1 share behavioural and evolutionary traits that are characteristic of introduced *P. megacephala* populations in Australia. Moreover, field observations in Cameroon indicate that this species largely dominates other native ants in urban habitats. The ERH may act in synergy with other factors facilitating the introduction and naturalization of *P. megacephala*. First, propagules composed of a queen and a few workers may experience high survivorship and rapid growth. A study conducted in Australia showed that the invasion by *P. megacephala* is consistent with the introduction of only six haploid genomes [34]. Second, *P. megacephala* combines a high aggressiveness towards other ant species with highly efficient predatory capacities. In invaded ranges, this species captures many different types of prey and is more efficient than native ants in preying on termites [37]. This stems from the effective exploitation of the landmarks deposited by termites and competing ants, and the efficient short- and long-range recruitment of conspecifics. In the ant *Wasmannia auropunctata*, one of the most invasive and destructive pests in the world [84], the combination of several life history traits seems particularly relevant in explaining its ecological dominance. (i) Its clonal reproductive system, whereby males and females are clonally produced [85], maintains favourable combinations of genes over time and thus offers an adaptive advantage over populations that reproduce sexually. (ii) Its unicolonial population structure provides numerical and competitive advantages over multicolonial ants [48]. (iii) Its proximity to humans allows it access to profoundly modified habitats that may, over time, increase its ability to become invasive [84,86]. In fact, the main factor associated with the ecological dominance of *W. auropunctata* is the human activities [49]; its reproductive system and social organisation strengthen the chances of successful invasion, but are neither necessary nor enough to explain the success of the invasion. Likewise, the success of other invasive ant species such as *Solenopsis invicta* [87], *Anoplolepis gracilipes* [88], *Linepithema humile* [89], *Paratrechina longicornis* [90] and *Lasius neglectus* [91] also seems correlated to anthropogenic disturbances. Biotic homogenization and habitat degradation within urbanized regions

| Table 3. Results of the hierarchical analyses of molecular variance (AMOVAs) evaluating the amount of genetic and chemical variance between and within groups. |
| Source | df | MS | Percentage of variation (%) | F value | p |
|---|---|---|---|---|---|
| Genetic variance |  |  |  |  |  |
| Between cryptic species (*F_{BS}*) | 1 | 2.849 | 35% | 0.352 | 0.001 |
| Between populations (*F_{PS}*) | 6 | 0.282 | 13% | 0.200 | 0.001 |
| Within populations (*F_{ST}*) | 56 | 0.110 | 52% | 0.482 | 0.001 |
| Total | 63 | 100% |  |  |  |
| Chemical variance |  |  |  |  |  |
| Between cryptic species (*F_{BS}*) | 1 | 3.759 | 37% | 0.367 | 0.001 |
| Between populations (*F_{PS}*) | 5 | 0.256 | 5% | 0.071 | 0.021 |
| Within populations (*F_{ST}*) | 54 | 0.152 | 59% | 0.412 | 0.001 |
| Total | 60 | 100% |  |  |  |

Nests were assigned to their population.
doi:10.1371/journal.pone.0031480.t003

| Table 4. Results of the hierarchical analyses of molecular variance (AMOVAs) evaluating the amount of genetic and chemical variance between and within supercolonies. |
| Source | df | MS | Percentage of variation (%) | F value | p |
|---|---|---|---|---|---|
| Genetic variance |  |  |  |  |  |
| Between cryptic species (*F_{BS}*) | 1 | 2.849 | 30% | 0.296 | 0.001 |
| Between populations (*F_{PS}*) | 6 | 0.805 | 21% | 0.304 | 0.001 |
| Within populations (*F_{ST}*) | 56 | 0.104 | 49% | 0.510 | 0.001 |
| Total | 63 | 100% |  |  |  |
| Chemical variance |  |  |  |  |  |
| Between cryptic species (*F_{BS}*) | 1 | 3.759 | 36% | 0.356 | 0.001 |
| Between populations (*F_{PS}*) | 2 | 0.382 | 5% | 0.082 | 0.006 |
| Within populations (*F_{ST}*) | 57 | 0.153 | 59% | 0.409 | 0.001 |
| Total | 60 | 100% |  |  |  |

doi:10.1371/journal.pone.0031480.t004
can certainly promote the evolution and the expression of invasive characteristics [92].

In conclusion, our study shows that Cameroon hosts two cryptic, reproductively isolated species of the big-headed ant *P. megacephala*. These species are restricted to certain ecological zones: *P. megacephala* var. 1 is found in urban areas and *P. megacephala* var. 2 in rainforests. Urban populations are phylogenetically close to populations in Australia, Mauritius, South Africa and Madagascar. Rainforest populations have high homologies with populations in Gabon, Madagascar and Comoros. Populations of both cryptic species are organised into a mosaic of supercolonies and adopt a unicolonial structure. Further analyses should decipher the cryptic species complex of *P. megacephala* and to identify the sub-species (if not all) particularly suited to adapt to and invade new environments. The ecological and evolutionary strategies adopted in native areas should be considered as a first phase in the expansion of and invasion by this species. More attention should also be placed on how anthropogenic activities affect the biotic and abiotic conditions of the environment, and to what extent invasional meltdown processes [93] could facilitate invasion. The next step in understanding the worldwide expansion of the ant *P. megacephala* will be to investigate the routes and means of introduction to prevent new invasions.

**Materials and Methods**

**Field collection and sampling**

Sixty-four nests (i.e. an aggregation of workers, brood, and/or queens in a single place) were sampled from eight populations in Cameroon in February 2009 (Figure 1 and Table 1; The Ministry of Scientific Research and Innovation provided the research permit # 019/MINRESI/B00/C00/C10/C13). Nests were collected from two ecological zones, a rainforest (29 nests sampled from along forest tracks in the Campo Forest Reserve) and a human-disturbed area (35 nests located in Yaoundé and its periphery). The Campo rainforest belongs to the Biafran Atlantic low altitude forest district; it is characterized by a rich and diverse flora dominated by the plant family *Caesalpiniaceae* [94,95]. The nests were located mainly below the roots of plants such as *Pennisetum purpureum* Schumacher (Poaceae) and *Costus afer* Ker-Gawler (Costaceae), or, more rarely, in dead branches or termite mounds. Different species of the genus *Pheidole* and species belonging to other genera (*e.g.* *Camponotus*, *Tetramorium*, *Pachycondyla*, *Myrmicaria*, *Cataulacus*, *Dorylus*, *Odontomachus*, *Oecophylla*) were sympatric with *P. megacephala*. In contrast, the vegetation in the Yaoundé urban area belongs to the semi-deciduous forest type, but

---

**Table 5.** Hierarchical analysis of molecular variance (AMOVA) with country as grouping factor (Cameroon, *n* = 304; Australia, *n* = 419; South Africa, *n* = 20).

| Source               | df | MS      | Percentage of variation (%) | F value | p   |
|----------------------|----|---------|----------------------------|---------|-----|
| Between countries (*FRT*) | 2  | 526.586 | 34%                        | 0.340   | 0.010 |
| Among populations (*FSR*) | 6  | 54.041  | 9%                         | 0.135   | 0.010 |
| Within populations (*FST*) | 1477 | 2.095  | 57%                        | 0.429   | 0.010 |
| Total                | 1485 | 100%    |                            |         |     |


currently is a mixture of forest relics on hill summits and garden crops (urban agriculture) along river and inland valleys. In this zone, *Pheidole megacephala* is opportunist; it nests under stones or dead branches, and is found in gardens, kitchen-gardens and dwellings. Only a few other species lived in the proximity of the *P. megacephala* nests, namely *Odontomachus troglodytes*, *Monomorium biclor*, *Crematogaster claviventris*, *Lepisiota sp.* and *Myrmicaria spicaeiventris*. Samples of workers and, when available, queens and males were collected from each nest and used for the behavioural assays. *Pheidole megacephala* has a pronounced worker caste polymorphism: major workers are considerably larger than minor and have disproportionately large heads. The minor are more active than the major, notably in exploring the ground and in alerting nestmates of the presence of intruders [as shown in *P. guilemai*, *P. pubiventris* [96], *P. morrisi* [97] and *P. pallidula* [98]]. A sub-sample of 50 minor from each nest was used for chemical extraction, and then stored in ethanol for subsequent genetic analyses.

**Morphological studies**

Morphological characteristics of specimens were assessed using a stereomicroscope and a scanning electron microscope. The maximum head width (across compound eyes) of 12 minors per population was determined at the nearest 0.005 mm by using a stereomicroscope (Wild M3, Wild Heerbrugg, Switzerland) at a magnification of 40×. Head width is an accurate estimate of size, commonly used as a dependent variable in studies of allometry [99,100]. Repeated measures differed on average by only 0.03 mm and were highly correlated (r = 0.803, p = 0.001). A scanning electron microscope (JSM-6480LV, JEOL; Tokyo, Japan) was used to study the shape and surface details of two minor and one major of each ecological zone. To this purpose, samples were dehydrated in ethanol, critical point air-dried and coated with gold using an ion sputter (JFC-1300 Fine Coater, JEOL).

**Behavioural assays**

Behavioural assays were conducted by confronting minor workers. Intraspecific aggressiveness was quantified between pairs of minor according to previously described protocols [34,101]. Standardized aggressiveness tests were conducted during 5 min. after the first interaction between two individuals placed together in a neutral arena (diameter = 2 cm, height = 1 cm, sides coated with Fluon™). Interactions were scored on a scale from 1 to 4: levels 1 (short antennations (<2 sec)) and 2 (prolonged antennations) were considered as non-aggressive behaviours, whereas levels 3 (hunger and attempts at biting) and 4 (prolonged biting and pulling) were considered as agonistic. Five trials, each involving different minor, were conducted for each pair of nests and the highest score was averaged across trials.

Interactions between individuals belonging to different nests, whether from the same or different sites, were considered as experimental assays. Interactions between individuals from the same nests were considered as control assays. In addition, we set up encounters between major from different nests to confirm that workers from both castes did not behave differently when confronted with non-nestmates.

**Diversity and variations in cuticular hydrocarbon profiles**

Protocols for the extraction of cuticular hydrocarbons and gas-chromatography, mass-spectrometry (GC-MS) analyses are described elsewhere [34]. In short, samples of ant cuticular hydrocarbons were extracted from 50 minor per nest and placed in 1 ml of cyclohexane during 5 min; the cyclohexane was evaporated and the extract was re-diluted in 5 μl of cyclohexane. We injected 1 μl of the extract into a Thermo Polaris Q™ electron impact ion trap mass spectrometer interfaced to a Thermo Trace GC Ultra™ gas chromatograph (Thermo Finnigan; Austin, Texas, USA). Qualitative and quantitative data were obtained by running the THermo XCalIBUR™ data system (Thermo Finnigan; Austin, Texas, USA). Cuticular profiles obtained for three nests of the population YM did not present clear patterns, and were therefore discarded from the analyses. We used the relative amount (*i.e.* area) of the peaks corresponding to cuticular lipids as a quantitative measurement for each profile. Compounds with a relative area of less than 2% in all profiles were excluded from the data. Cuticular lipids were identified by analysing their mass spectra produced by both electron impact and chemical ionization with methane.

We estimated the chemical diversity and similarity of cuticular hydrocarbon profiles using the Nei index I and Euclidian distances E, respectively [34]. To visualise the differences in cuticular hydrocarbon profiles, we conducted a canonical discriminant function analysis based on the relative amounts of all identified compounds.

**Genetic analyses**

DNA was isolated through phenol/chloroform extractions and ethanol precipitation by following standard protocols [102].

The phylogenetic relationships between collected samples were inferred from a 650 base-pair (bp) region near the 5′ terminus of the CO1 gene. CO1 sequences were generated from a subset of the individuals sampled (4 individuals per population) using barcoding protocols [39,103]. Full-length COI barcodes (658 bps) were amplified using the standard insectprimers LepF1/LepR1 (LepF1: 5′-ATTCACCAATCTCAAAAGATATTGG-3′; LepR1: 5′-TAAACCTTCGATGTTCAAAAATACTA-3′). PCRs were carried out in 25 μl reaction volumes containing 14 μl of distilled water, 5 μl of genomic DNA, 2.5 μl of Qiagen 10× buffer (providing a final concentration of 1.5 mM MgCl2, 2 μl 2.5 mM of dNTP, 0.625 μl 100 mM of forward and reverse primers and 0.250 μl of Qiagen Taq DNA polymerase. The PCR thermocycling profile consisted of one cycle of 2 min at 94°C, five cycles of 40 sec at 94°C, 40 sec at 45°C, and 1 min at 72°C; followed by 36 cycles of 40 sec at 94°C, 40 sec at 51°C, and 1 min at 72°C; with a final step of 5 min at 72°C. Products were visualized on a 2% agarose gel and samples containing clean single bands were sequenced using BigDye v3.1 on a 48-capillary 3730 DNA Analyzer (Applied Biosystems). The forward and reverse sequences for each sample were inspected and aligned using CodonCode Aligner 3.7.1.1 (CodonCode Corporation, Dedham, MA). Programs freely available from the phylogeny.fr web server [104] (**MUSCLE** for multiple alignment [105], **GBLOCKS** for alignment curation [106], **PhyML** for phylogeny [107] and **TreeDyn** for tree drawing [108]) were used to reconstruct a phylogenetic tree from our set of sequences. The sequences of *Pheidole sexspinosa* and *P. xerophila* were used as sister species and those of *Aphaenogaster seoulis* as outgroup [38].

Genetic diversity and population genetic structure were investigated by genotyping eight statistically independent microsatellite loci [34]. The PCR amplification was carried out in 10 μl volumes. PCR products were separated on a 48-capillary 3730 DNA Analyzer (Applied Biosystems), and an internal size standard (GeneScan™ 350 ROX™, Applied Biosystems) was run in every sample. The lengths of the PCR products were determined using GeneMapper software (Applied Biosystems) and used to construct a multi-loci genotype for each individual.

Genetic diversity parameters (*i.e.* the number of alleles, allelic richness, expected and observed heterozygosities) and fixation

---

**Evolutionary Ecology of Two Cryptic Ant Species**

PLoS ONE | www.plosone.org 13 February 2012 | Volume 7 | Issue 2 | e31480
indices were estimated using FSTAT 2.9.3 [109] and averaged across loci. Regression relatedness and the jackknife estimates of the standard errors were calculated with the program RELATEDNESS 5.60 [110,111]. The mean genetic relatedness among minor was estimated by including all individuals as reference. The effect of inbreeding on relatedness was corrected by using the estimator $r^b$ [42]. The number of singly-mated, related and equally reproducing queens per nest was inferred from relatedness values among nestmate minor [112]. The assumptions of single mating and equal partitioning of reproduction among queens are corroborated by a previous study [34], and that nestmate queens are related is shown herein (see Results).

The most likely number of genetic clusters ($K$) reflecting the population genetic sub-structure of our data set was characterised with the Bayesian model-based clustering method implemented in STRUCTURE v2.3.2 [113]. For this purpose, the program was run without population information under the admixture model (individuals may have mixed ancestry) and independent allele frequencies. The length of the burn-in period was 10,000 and the number of Markov chain Monte Carlo replications after the burn-in was 10,000. Ten independent chains were run for each $K$ from $K = 2$ to $K = 64$. The $ad hoc$ statistic $\Delta K$ based on the rate of change in the log probability of data between successive $K$ values was used to find the most likely value of $K$ [114,115].

**Associations between behavioural, genetic, chemical and spatial data**

Correlations between the geographical origin, genetics, chemical composition and behaviour of *P. megacephala* minor were considered through Mantel correlation tests [116] based on 999 permutations and by using GenAlEx 6 [117]. The independent influences of the genetic, chemical and spatial distances on workers’ aggressiveness were estimated through a stepwise multiple regression analysis [118]. If not stated otherwise, statistical tests were carried out with the computer program SPSS Statistics 19 (Spss Inc., 1989–2010).

**Contributions of various levels of population structure to patterns of chemical and genetic variation**

Hierarchical analyses of molecular variance (AMOVA) of the eight populations (i.e. collection sites) nesting within the two ecological zones (i.e. rainforest and human-modified habitats) were conducted to quantify the distribution of genetic and chemical variations between populations, zones and populations within zones. The significance of each variance component was tested using a random permutation test (999 permutations) using GenAlEx 6 [117].

**Supporting Information**

**Figure S1** Minor of *P. megacephala* var. 1 (A) and *P. megacephala* var. 2 (B) in lateral views. Magnification and scale bar are indicated for each scanning electron microscopic image. (TIF)

**Figure S2** Lateral views of the propodeum showing shorter spines in *P. megacephala* var 1. than in *P. megacephala* var. 2 (B). Magnification and scale bar are indicated for each scanning electron microscopic image. The terminal part of the hairs forms a point in *P. megacephala* var. 1 (A) and a brush in *P. megacephala* var. 2 (B). (TIF)

**Figure S3** Details in lateral views of hairs present on the petiole. Magnification and scale bar are indicated for each scanning electron microscopic image. The terminal part of the hairs forms a point in *P. megacephala* var. 1 (A) and a brush in *P. megacephala* var. 2 (B). (TIF)

**Acknowledgments**

We are grateful to C. Djieto Lordon, A. D. Fouguieng Saha, P. Godefroy and A. Ngome for assistance during field studies, to L. Grumiau for the chemical analyses, to P. Mandhyn, D. D. Shoemaker, N. Tsutsui and two reviewers for their helpful comments, and to A. Dejean for proofreading the manuscript.

**Author Contributions**

Conceived and designed the experiments: DF. Performed the experiments: DF MT MK PSMM VVB EDC. Analyzed the data: DF. Wrote the paper: DF SA.

**References**

1. Stachowicz JJ, Tilman D (2005) Species invasions and the relationships between species diversity, community saturation, and ecosystem functioning. In: Sax DF, Stachowicz JJ, Gaines SD, eds. Species invasions: insights into ecology, evolution, and biogeography. SunderlandMA: Sinauer Associates. pp 41–64.
2. Perrings C, Mooney H, Williamson M, eds. (2010) Bioinvasions and consequences of ant invasions. Annual Review of Ecology and Systematics 33: 131–155.
3. Wilson EO (1990) Success and dominance in ecosystems: the case of the social insects. Oldendorf/Luhe (Germany): Ecology Institute. 104 p.
4. McGlynn TP (1999) The worldwide transfer of ants: geographical distribution and ecological invasions. Journal of Biogeography 26: 535–548.
5. Lach I, Hooper-Bui LM (2009) Consequences of ant invasions. In: Lach I, Parr C, Abbott K, eds. Ant ecology. Oxford: Oxford University Press. pp 261–286.
6. Pasqua L (1994) Characteristics of tramp species. In: Williams DF, ed. Exotic ants: biology, impact, and control of introduced species. Boulder: Westview Press. pp 23–43.
7. Moller H (1996) Lessons for invasion theory from social insects. Biological Conservation 78: 125–142.
8. Holway DA, Lach I, Suarez AV, Tsutsui ND, Case TJ (2002) The causes and consequences of ant invasions. Annual Review of Ecology and Systematics 33: 181–203.
9. Krasnelycvich PD, Holway DA, LeBrun EG (2009) Invasion processes and causes of success. In: Lach I, Parr C, Abbott K, eds. Ant ecology. Oxford: Oxford University Press. pp 245–260.
10. Meer RK, Meer R, Lor I (1998) Nestmate recognition in ants. In: Vander Meer RK, Breed MD, Espelie KE, Winston ML, eds. Phermone
communication in social insects: ants, wasps, bees, and termites. Boulder: Westview Press. pp 79–103.

D’Ettorre P, Lenoir A (2009) Nestmate recognition. In: Lach L, Parr C, Abbott K, eds. Ant ecology. Oxford: Oxford University Press. pp 194–209.

van Zweden JS, D’Ettorre P (2010) Nestmate recognition in social insects and the role of hydrocarbons. In: Blomquist GJ, Bagnères A-G, eds. Insect hydrocarbons: biology, biochemistry, and chemical ecology. Cambridge: Cambridge University Press. pp 222–243.

Pedersen JS, Krüger MJR, Vogel G, Giraud T, Keller L (2006) Native supercolonies of unrelated individuals in the invasive Argentine ant. Evolution 60: 782–791.

Tsutsui ND, Suarez AV, Holway DA, Case TJ (2000) Reduced genetic variation and the success of an invasive species. Proceedings of the National Academy of Sciences, USA 97: 5948–5953.

Giraud T, Pedersen JS, Keller L (2002) Evolution of supercolonies: the Argentine ants of southern Europe. Proceedings of the National Academy of Sciences, USA 99: 6073–6078.

Steiner FM, Schlick-Steiner BC, Moser K, Stauffer C, Arthofer W, et al. (2007) Abandoning aggression but maintaining self/nonself discrimination as a first stage in ant colony formation. Current Biology 17: 1903–1907.

Holway DA, Suarez AV (2004) Colony-structure variation and irsocompetitive capability in the invasive Argentine ant. Oecologia 138: 216–222.

Orvel J, Granger J, Foucaud J, Le Breton J, Andrés F-X, et al. (2009) Ecologically heterogeneous populations of the invasive ant Wasmannia auropunctata within its native and introduced ranges. Ecological Entomology 34: 504–512.

Wheeler WM (1922) The ants collected by the American Museum Congo Expedition. Bulletin of the American Museum of Natural History 45: 39–269.

Bolton B (1995) A new general catalogue of the ants of the world. Cambridge: Harvard University Press. 504 p.

Wetterer JK (2007) Biology and impacts of Pacific Island invasive species. 3. The African big-headed ant, Pheidole megacephala (Hymenoptera: Formicidae). Pacific Science 61: 437–436.

Lowe S, Browne M, Boudjelas S (2001) 100 of the World’s Worst Invasive Species. London: ZSL. pp 186–199.

Hoffmann MJ, Dias AT, Gurgel-de-Souza M, Abeel J, et al. (2011) Molecular and biological evidence for the origin of the invasive ant Wasmannia auropunctata in Brazil. PLoS ONE 6: e2235–2242.

Facon B, Hufbauer RA, Tayeh A, Loiseau A, Lombaert E, et al. (2011) Inbreeding depression is purged in the invasive insect Hormia australis. Current Biology 21: 424–427.

Barkowskii G, Vargo EL, Silverman J (2004) The diminutive supercolony: the Argentine ants of the southeastern United States. Molecular Ecology 13: 2235–2242.

Suarez AV, Holway DA, Tsutsui ND (2008) Genetics and behavior of a colonizing species - The invasive Argentine ant. American Naturalist 172: 78–88.

Lenoir A, Freneau D, Errard C, Hefta A (1999) Individuality and colonial identity in ants: the emergence of the social representation concept. In: Drits C, Deneubourg JL, Pasteels JM, eds. Information processing in social insects. Basel: Birkhäuser. pp 219–232.

Drescher J, Bühnig N, Schmitt T, Bühler J, Feldhaar H (2010) Societies drifting apart? Behavioural, genetic and chemical differentiation between supercolonies in the yellow crazy ant, Anoplolepis gracilipes. PLoS ONE 5: e13581.

Martin SJ, Vinhäuser E, Helanterä H, Driftshøj FF (2008) Chemical basis of nest-mate discrimination in the ant Formica exsecta. Proceedings of the Royal Society of London, Series B 275: 1271–1278.

Greene MJ, Gordon DM (2007) Structural complexity of chemical recognition cues affects the perception of group membership in the ants Lasius niger and Mylitta dominica. Chemical Ecology 23: 987–995.

Reed MD (1998) Recognition phenomnes of the honey bee. Bio Science 48: 463–470.

Torres CW, Branch M, Tsutsui ND (2007) The role of cuticular hydrocarbons as chemical cues for nest recognition in the invasive Argentine ant (Lasius niger). Insectes sociaux 54: 363–373.

Newell W, Barber TC (1913) The Argentine ant. Washington, D.C.: U.S. Department of Agriculture - Bureau of Entomological Bulletin No. 122. 98 p.

Borchers P, Morin K (2005) The effect of protein expression and cuticular hydrocarbons on nest-mate discrimination in the ant Pheidole megacephala. Proceedings of the Royal Society of London, Series B 272: 1271–1278.

Greene MJ, Gordon DM (2007) Structural complexity of chemical recognition cues affects the perception of group membership in the ants Lasius niger and Mylitta dominica. Chemical Ecology 23: 987–995.

Reed MD (1998) Recognition phenomnes of the honey bee. Bio Science 48: 463–470.

Torres CW, Branch M, Tsutsui ND (2007) The role of cuticular hydrocarbons as chemical cues for nest recognition in the invasive Argentine ant (Lasius niger). Insectes sociaux 54: 363–373.

Newell W, Barber TC (1913) The Argentine ant. Washington, D.C.: U.S. Department of Agriculture - Bureau of Entomological Bulletin No. 122. 98 p.

Borchers P, Morin K (2005) The effect of protein expression and cuticular hydrocarbons on nest-mate discrimination in the ant Pheidole megacephala. Proceedings of the Royal Society of London, Series B 272: 1271–1278.

Clark DB, Guayasamin C, Pizzino O, Donoso C, Paez de Villacis Y (1982) Differentiation between native and introduced populations of the Argentine ant. Annals of the Entomological Society of America 81: 913–918.

Boomsma JJ, Brouwer AH, Van Loon AJ (1990) A new polygynous Lebiasina species (Hymenoptera: Formicidae) from central Europe. II. Allozymic confirmation of species status and social structure. Insectes sociaux 33: 363–375.

Wetterer JK, Miller SE, Wheeler DE, Olson CA, Polhemus DA, et al. (1999) Ecological dominance by Pantomima longiceps (Hymenoptera: Formicidae), an invasive tramp ant, in Biotropica 2 Florida Entomological Society 82: 897–905.

Barkowskii G (2010) Extreme life history plasticity and the evolution of invasive characteristics in a native ant. Biological Invasions 12: 3341–3349.

Kenny M, Dejean A (1999) Spatial distribution, size and density of nests of Myrmecia invicta Emery (Formicidae, Myrmicinae). Insectes sociaux 46: 179–185.

Folgarait PJ, D’adamo P, Gilbert LE (2004) A grassland ant community in Argentina: the case of Solenopsis richteri and Camponotus punctulatus (Hymenoptera: Formicidae), attaining high densities on a native range, Annals of the Entomological Society of America 97: 450–457.

Queller DC, Strassmann JE (1998) Kin selection and social insects. Bio Science 48: 165–175.
Evolutionary Ecology of Two Cryptic Ant Species

97. Patel AD (1990) An unusually broad behavioral repertoire for a major worker in a dimorphic ant species: Pheidole morrisi (Hymenoptera: Formicidae). Psyche 97:181–192.

98. Detrain C, Pasteels JM (1992) Caste polyethism and collective defense in the ant, Pheidole pallidula: the outcome of quantitative differences in recruitment. Behavioral Ecology and Sociobiology 29:405–412.

99. Wheel DE (1981) The development of worker caste polymorphism in ants. American Naturalist 106:1219–1226.

100. Fournier D, Battail G, Timmermans I, Aron S (2008) Genetic diversity, worker size polymorphism and division of labour in the polyandrous ant Camponotus castoris. Animal Behaviour 76:51–58.

101. Holway DA, Suarez AV, Case TJ (1998) Loss of intraspecific aggression in the success of a widespread invasive social insect. Science 282:949–952.

102. Sambrook J, Russell D (2001) Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory Press. 2231 p.

103. Dereeper A, Guignon V, Blanc G, Audic S, Buffet S, et al. (2008) Phylodynamic.fr: robust phylogenetic analysis for the non-specialist. Nucleic Acids Research 1:W465–W469.

104. Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32:1792–1797.

105. Pamilo P (1991) Evolution of colony characteristics in social insects. II. Number of reproductive individuals. American Naturalist 138:412–433.

106. Escalante R, Regnaut S, Goudet J (2005) Detecting the number of clusters of relatedness for the non-specialist. Nucleic Acids Research 1:W465–W469.

107. Queller DC (1994) Genetic relatedness in viscous populations. Evolutionary Ecology 8:232–240.

108. Crozier RH (1979) Genetics of sociality. In: Hermann HR, ed. Social insects. New York: Academic Press. pp 223–256.

109. Blowsley B, Notzold R (1995) Evolution of increased competitive ability in invasive nonindigenous plants: a hypothesis. Journal of Ecology 83:867–889.

110. Goudet J (2001) Fstat, a program to estimate and test gene diversities and fixation indices. version 2.9.3 ed: Institute of Ecology, University of Lausanne, Switzerland.

111. Queller DC, Goodnight KF (2000) Relatedness 5.0.8. 5.0.8 ed. Houston: Goodnight Software.

112. Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945–959.

113. Earl DA (2009) Structure Harvester v0.3. http://taylor07.niehs.nih.gov/structure/structureHarvester.html.

114. Pamilo P (1991) Evolution of colony characteristics in social insects. II. Number of reproductive individuals. American Naturalist 138:412–433.

115. Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of relatedness for the non-specialist. Nucleic Acids Research 1:W465–W469.

116. Mantel N (1967) The detection of disease clustering and a generalized regression approach. Cancer Research 27:209–220.

117. Peakall R, Smouse PE (2006) GenAlEx 6: genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes 6:288–295.

118. Sekal RR, Rehfeldt FJ (1995) Biometry: the principles and practice of statistics in biological research. New York: W.H. Freeman and Company. 867 p.