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SHORT COMMUNICATION

Very low genetic variability in the Indian queenless ant *Diacamma indicum*

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Abstract

We developed microsatellite markers and combined them with mitochondrial markers to analyse the population genetic structure of the queenless ant *Diacamma indicum*. This species, lacking winged queens, is likely to have a restricted female dispersal but exhibits various life history traits suggesting higher dispersal abilities than the other *Diacamma* species. Only 4 of 11 microsatellites were polymorphic and only 1 had more than 4 alleles over 166 individuals originating from 7 populations from the south of India. Only one mitochondrial DNA (mtDNA) haplotype was detected throughout India (including one population in the north) and Sri Lanka. Such a level of polymorphism is particularly low compared with other *Diacamma* species having much smaller ranges in the south of India. A strong genetic differentiation was observed between populations separated by more than a few kilometres. We also analysed the genetic differentiation between the Indian populations and two populations from the Japanese island of Okinawa, which are morphologically similar and might belong to the same species. The genetic differentiation was high for both markers, suggesting an absence of ongoing gene flow between these populations.

Keywords: *Diacamma indicum*, gamergate, microsatellites, mtDNA, population genetics

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Introduction

The population genetic structure of a species is affected by its population history, habitat characteristics and life history traits. Disentangling these effects is a difficult but central task of population genetic studies. Correlative studies have shown some association between the life history traits and population genetics of a species in plants and animals (Hamrick & Godt 1996; Arndt & Smith 1998). For instance, in ants with dependent colony foundation, the queen depends on the help of workers to establish a new colony, and dispersal is limited to ant walking distance (reviewed in Peeters & Ito 2001). Species or populations with dependent colony foundation show a higher level of genetic differentiation than species or populations with independent colony foundation by flying queens (Sundström 1993; Seppä & Pamilo 1995; Ross *et al*. 1997).

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The aim of this study was to examine the level of genetic diversity and pattern of population genetic structure in the queenless ant *Diacamma indicum*. All *Diacamma* species have no queens and are peculiar among ants by the occurrence of a pair of tiny appendages on the workers' thorax, the gemmae, which play an essential role in the regulation of reproduction (Peeters *et al*. 1992; Tsuji *et al*. 1999). Colonies are monogynous, with a single mated worker (the gamergate) retaining her gemmae and producing all the diploid eggs. The absence of winged females and the obligate occurrence of colony fission strongly restrict the dispersal and colonization abilities of these species, as shown in previous population genetic studies of two *Diacamma* species from the south of India (Doums *et al*. 2002; Baudry *et al*. 2003). *D. indicum* differs from these previously studied *Diacamma* species in various ecological characteristics that are expected to result in higher dispersal rates and/or colonization abilities.

1 Even though *Diacamma* ants typically nest underground in open areas, *D. indicum* is more opportunistic with regard

to its nesting preferences. During our field sampling, we observed typical underground nests, but also nests under stones, in abandoned rice paddies, in fissures of walls in an ancient fort and even in tree branches. Related to this opportunistic nesting habit, the nests of *D. indicum* are generally shallow, with little signs of construction.

2 Colonies of *D. indicum* are small (88 ± 62 workers, N = 11; unpublished data) and are prone to emigrate. Nest relocation can be triggered by slight physical disturbance of the nests (personal observation in India; Fukumoto & Abe 1983 in Okinawa), whereas in the other *Diacamma* species from the south of India, workers retreat to the deeper chambers when the nest is disturbed.

3 *D. indicum* has a larger distribution area relative to other species. Indeed, *D. indicum* has been found in a large part of southern India and in Sri Lanka, as well as in the north (near Calcutta), whereas the geographical distribution of the other *Diacamma* species from the south of India are, as far as we know, restricted to small and nonoverlapping areas. In contrast, *D. indicum* can be sympatric with the other *Diacamma* species. Furthermore, a species referred in the literature as *Diacamma* sp. from Japan occurs throughout the Ryukyus islands (south of Japan), and specimens from Okinawa were identified as *D. indicum* on the basis of male genitalia (W.L. Brown, unpublished monograph).

Based on these life history and ecological traits, we therefore expected a lower population genetic structure in *D. indicum* than that previously observed in other *Diacamma* (Doums *et al*. 2002; Baudry *et al*. 2003). To address this issue, we used a mitochondrial marker (a fragment of the COII gene) and developed microsatellite markers. Unexpectedly, both types of markers showed a very low level of polymorphism. However, the use of four variable microsatellites was sufficient to reveal a strong genetic structure at a large spatial scale. We also analysed individuals from two populations of Okinawa Island in order to test whether these *Diacamma* sp. from Japan are genetically very close to *D. indicum* from India, as postulated by W.L. Brown (unpublished monograph), and whether they also harboured such low level of polymorphism.

Materials and methods

Field sampling

Worker ants were collected from 166 colonies distributed in seven populations from the south of India (Fig. 1) in October 1998 and from 45 colonies distributed in two populations on Okinawa Island (Japan) in August 2002. As far as we know, *Diacamma indicum* has never been found on the mainland between India and Okinawa preventing us to obtain samples between these two distant set of populations. The mean (± SD) number of colonies studied per population was 17.3 ± 4.4. The exact location of each population, as determined by GPS, is available upon request. For each colony, a single individual was analysed using microsatellite markers developed specifically for this study (see below). For the mitochondrial DNA (mtDNA), we also analysed one individual from Sri Lanka, kindly collected by S. Yamane, and one individual from one population in the north of India (Bhubaneshwar, near Calcutta), collected by C. Peeters.

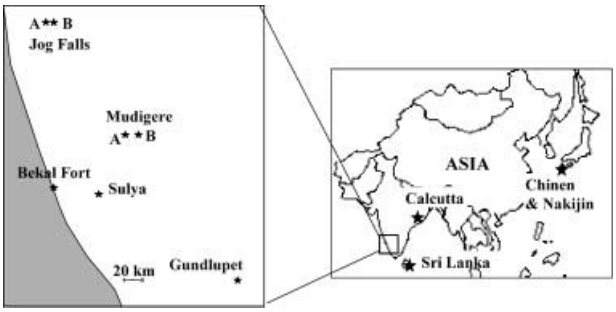

Fig. 1 Geographical position of the seven *D. indicum* populations from south India (Karnataka State) and the two populations from Japan (stars) analysed. The two Japanese populations, named Nakijin and Chinen, were distant from each other by 60 km. For the mtDNA, one individual was analysed for each of these populations as well as one individual from Sri Lanka and one near Calcutta.

Microsatellites cloning and screening

Genomic DNA was extracted from four larvae from Jog Fall A population by classic high salt procedure using NaCl. The digestion, cloning and screening of the microsatellites were performed following a standard protocol detailed in Doums (1999). Synthetic oligonucleotides $(TG)_{10}$ and $(TC)_{10}$ were used to screen ~1200 recombinant colonies. Of 88 positive clones, 40 were purified and sequenced by Genome Express (Meylan, France). Primers could be designed for 11 loci using PRIMER 3 software (Rozen & Skaletsky 2000) and all produced successful amplification. The sequences of these loci are available on GenBank with Accession nos AY258154 to AY258164. We first tested the level of polymorphism of each microsatellite marker by genotyping one individual originating from each of the seven Indian populations using a radioactive amplification. Each polymerase chain reaction (PCR) was carried out according to Doums (1999) with an annealing temperature of 53 °C. Only 4 of the 11 loci were polymorphic and used for the population genetic survey (Table 1).

Genetic analysis

Genomic DNA was extracted using a high salt procedure or an extraction kit Perfect gDNA Blood Mini (Eppendorf).

Table 1 Characteristics and number of alleles over all Indian populations for 11 microsatellite markers. The number of alleles (NaS) for all markers was assessed by genotyping one individual for each of the seven Indian populations. For the polymorphic markers, the total number of alleles (NaT) observed from the genotyping of 166 individuals originating from the seven Indian populations is also given

Loci	Motif	Primers (5′–3′)	NaS	NaT
DI5	[GA]^{21}	F: GCGAAGGTCGAGAAGTGC	1	—
		R: TCGGACTGGGAAGCAATC		
DI8	[GA]^{9}	F: GATGGTGGTAGTGGCAGTGG	1	—
		R: AAGCGCGTGCATAGCGTA		
DI11	[CA]^{8}CT[CA]^{2}	F: CGGCGAGTAAATTCGTAATAG	1	—
		R: CAGGCTTCGCAGGAGAAC		
DI13	[GA]^{11}	F: GTTTCGCAAAGGTGGTTCTC	1	—
		R: TTCACTTCGCTCCAGTCTCC		
DI16	[TC]^{10}	F: CCGATAGATAGGCGAACACA	1	—
		R: ACGTTCCGATTCCCGTTCT		
DI26	[CT]^{17}	F: TCAAGTTCGGCATCTCCTTA	1	—
		R: AACCGGACCTAAATGCGTTA		
DI28	[AG]^{11}	F: TGACGGAAGGACATCGTATC	1	—
		R: GTAATAAAGGCGGACGGAAG		
DI14	[TC]^{10}	F: TTATCGCGGCTTTCTATTCC	2	2
		R: GCATATCTTTCCGTGAGGTTG		
DI31	[CA]^{10}	F: TCTTCCTTTCACGCTCTAATTC	2	3
		R: TGGCAGTGAGCAAGTGTAAA		
DI32	[AC]^{14}	F: GTAGGATGACGGTGGTGGAA	2	2
		R: CGGCTAAGGTAGAGCTGGAA		
DI33	[GT]^{24}	F: CGCCACGTTAGAACTATACGAA	4	11
		R: GGCAATTTCGTCGTTGCT		

PCR were run in 10 μL with 40 ng of template DNA, 1 μL Buffer 10×, 0.25 U *Taq* DNA polymerase (Qiagen), 0.2 μM of each primer and 200 μM of each dNTPs using a GeneAmp 2700 thermal cycler (Applied Biosystems). The thermal cycle profile was as follows: 15 min at 94 °C; 10 amplification cycles of 12 s at 94 °C, 15 s at 53 °C and 30 s at 72 °C; 20 amplification cycles of 15 s at 89 °C, 15 s at 53 °C and 30 s at 72 °C; and a final step for 10 min at 72 °C. The loci *DI14*, *DI31*, *DI32* were co-amplified with the same PCR conditions. The genotypes were determined using a ABI prism 310 sequencer (Applied Biosystems).

A 675 bp fragment of the COII gene was amplified from the genomic DNA of one individual from each of the seven populations. The amplification was performed using the forward 5′-GTGCAATGGATCTAAAATCTA-3′ and reverse 5′-ATATATTATGTTTGATTTAA-3′ primers designed by L. Brazier. PCR were run in 50 μL with 40 ng of template DNA, 1 μL Buffer 10×, 1.25 U *Taq* DNA polymerase (Qiagen), 0.5 μM of each primer and 200 μM of each dNTPs using a GeneAmp 2700 thermal cycler (Applied Biosystems). The thermal cycle profile was as follows: 5 min at 94 °C; 30 amplification cycles of 45 s at 94 °C, 45 s at 48 °C and 1 min at 72 °C; and a final extension step for 8 min at 72 °C. PCR products were sequenced using the Bigdye Terminator V3.0 cycle sequencing kit (Applied Biosystems) and loaded in a ABI Prism 310 sequencer (Applied Biosystems).

Data analysis

Data were analysed using GENEPOP 3.3. (Raymond & Rousset 1995). Hardy–Weinberg equilibrium (HWE) was tested for the four loci at each locality using exact tests (Raymond & Rousset 1995). Fisher's method of combining independent test results (Sokal & Rohlf 1995) was used to determine the overall significance for each locality and each locus. Only 1 of the 19 Hardy–Weinberg tests was significant at the 0.05 level and showed a deficit in heterozygotes ($P = 0.001$) for locus *DI33* in Sulya. The two other loci variable in this population did not show any significant deviation from HWE. We have thus considered all populations to be at HWE. Linkage disequilibrium between pairs of loci was tested for each locality. Among 18 linkage disequilibrium tests, 2 were significant at the 0.05 level and none was significant after a Bonferroni correction. The significance of the genetic differentiation between populations was examined by conducting exact tests of allele frequency differentiation (Raymond & Rousset 1995). The level of genetic differentiation was estimated by *F*-statistics, assuming the infinite allele model (Wright 1978). To test for a pattern of isolation-by-distance, the significance of the Spearman rank correlation coefficient between genetic differentiation and geographical distance was assessed with a Mantel test.

We first compare the number of allele observed per locus (including the nonpolymorphic ones) during our first screening of seven individuals (one from each population) with the one observed in a similar sampling in *D. ceylonense*. To do so, we randomly selected one individual for each population (eight populations) studied by Doums *et al*. (2002) and estimated the number of alleles observed in this reduced data set. We perform this random selection 100 times using a modified SAS procedure of randomization kindly provided by P.L. Leberg (Leberg 2002) and estimated the mean number of allele per locus over the 100 randomly selected dataset. We then compared the number of alleles per locus (including the nonpolymorphic ones) between the two species using a Kolmogorov–Smirnov nonparametric exact test using SAS version 8. The same test was used to compare genetic diversity within populations between *D. indicum* and the others *Diacamma*. We compared both the mean number of alleles and the mean heterozygosity per population. Given that only three populations were analysed for *D. ceylonense*, we pooled these three populations with those of the closely related taxon *Diacamma* sp. from nilgiri from which the same microsatellite loci were screened (Baudry *et al*. 2003) under the species name *D. ceylonense*. In order to avoid comparing

populations with different sample size (Leberg 2002), we did not consider the population with a very large sample size in *D. cyaneiventre* (Kottigehara 1 in Doums *et al*. 2002). The different species had then similar sample size analysed per population (*D. indicum* = 17.3 ± 4.4 (± SD); *D. cyaneiventre* = 18.5 ± 6.6; *D. ceylonense* = 20.1 ± 3.65).

Results and discussion

Genetic diversity over all Indian samples

The level of polymorphism detected was very low. Indeed, only 4 of 11 microsatellite markers were polymorphic, with only one locus (*DI33*) having more than four alleles over all populations (Table 1). Note that the alleles observed in the single individual from Bhubaneshwar (near Calcuta) and Sri Lanka were not different from those already detected in the other Indian populations. Taking these individuals into account does not, therefore, change the number of polymorphic loci or number of alleles in the Indian sample. The percentage of polymorphic loci (36%) was much lower than in *Diacamma cyaneiventre* (80%) (Doums 1999), even though the screening procedure was the same. The absence of polymorphism in most microsatellites cannot be due to small numbers of repeats or impurities (see Table 1). In order to compare the diversity observed in both species, one individual per population (eight populations) was randomly selected in the data set of *D. cyaneiventre* (Doums *et al*. 2002; see Materials and methods). The number of alleles detected per locus (including the nonpolymorphic ones) was significantly lower in *D. indicum* (mean [range] = 1.5 [1–4]) than in *D. cyaneiventre* (mean of the 100 random selections [range] = 5.6 [1–10.5]; Kolmogorov–Smirnov [KS] exact test, $P = 0.004$).

No polymorphism was observed for the mtDNA in the 675 bp of the COII gene fragment over the nine individuals from India and Sri Lanka. It is difficult to explain such absence of mutations by a peculiarity of this mitochondrial gene, because again polymorphism was observed for the same gene in *D. cyaneiventre* and *D. ceylonense*, even within a single population (Doums *et al*. 2002; Baudry *et al*. 2003). Moreover, in the few mtDNA studies on ants, mtDNA polymorphism was observed within populations, suggesting that mtDNA generally harboured polymorphism in ants (Tay *et al*. 1997; Ross *et al*. 1999; Liautard & Keller 2001).

Two main explanations can be put forward to explain such low level of polymorphism for the two classes of markers observed in our species: the occurrence of recent population bottlenecks or a very low effective population size of this species. This last explanation appears less likely when comparing with the other species of *Diacamma* for two main reasons. First, the large distribution area and more opportunistic nesting habits of *D. indicum* do not really suggest a lower effective population size. Second, the level of population fragmentation, which might also affect the effective population size, appears to be similar in all the *Diacamma* species studied (see below).

The occurrence of bottlenecks could be due either to an extinction of most Indian populations followed by a recolonization, or to an introduction of this species into India. Unfortunately, we do not have any historical indication that could favour either of these scenarios. Both scenarios imply a history of rapid colonization of India and suggest that *D. indicum* has high colonization abilities. Its small colonies and unstructured nests might increase the rate of fission and also facilitate passive transport by human activities. It is interesting to note that most ants with high colonization abilities are polygynous, unlike *D. indicum*. Polygyny appears beneficial in association with fission because a reproductive is needed for the propagules to be successful (Holway *et al*. 2002). However, colony fission will succeed in monogynous *Diacamma* as long as cocoons or other brood are present; all emerging workers are capable of becoming reproductives (Peeters *et al*. 1992).

Whatever the causes of such low genetic diversity, this could have major consequences on the social organization of colonies. For instance, in the Argentine ant, internest aggression disappeared in introduced populations with a resulting switch to unicoloniality (Tsutsui *et al*. 2000 but see Giraud *et al*. 2002). In the fire ant, the decrease of allelic diversity at the sex-determining locus or loci generated a genetic load (production of sterile diploid males) in the introduced populations (Ross *et al*. 1993). Further studies are necessary to test whether such effects occur in *D. indicum*.

Within- and among-population genetic structure

When considering the level of polymorphism within populations, the number of alleles per locus varied from one to eight (Table 2). Note that more than two alleles can be observed only for locus *DI33* in three populations and in these cases, most of the alleles occur at low frequency (Table 2). The mean number [range] of alleles per population in *D. indicum* (2 [1.5–3]) is significantly lower than that detected in *D. cyaneiventre* (Doums *et al*. 2002; 6.4 [4.7–8.9]; exact KS test, $P = 0.012$) and *D. ceylonense* (Baudry *et al*. 2003; 3.6 [2.6–6.4]; exact KS test, $P = 0.002$) (Table 2). The pattern was the same when considering the mean expected heterozygosity per population which varied from 0.13 to 0.38 with a mean of 0.25 in *D. indicum*. This value is significantly lower than that detected in *D. cyaneiventre* (Doums *et al*. 2002; 0.63 [0.54–0.68]; exact KS test, $P = 0.012$), and *D. ceylonense* (Baudry *et al*. 2003; 0.44 [0.29–0.70]; exact KS test, $P = 0.002$). The level of polymorphism was also very low in the two Japanese populations (Table 2). Such low level of polymorphism within population was expected given the low level of polymorphism of the markers observed over all samples. This level of polymorphism was

Table 2 Allelic frequencies within Indian and Japanese (Chinen and Nakijin) populations of *Diacamma indicum* for four microsatellite markers. Alleles are named according to their size in base pairs. For each population, the number of individuals (one individual per colony) studied is given between parentheses

	DI14		*DI31*			*DI32*		*DI33*										
	108	110	121	123	125	183	185	209	215	217	219	229	231	233	235	237	239	241
JogFallsA (18)	1.00	—	0.80	—	0.20	—	1.00	0.14	—	—	—	0.08	0.14	0.06	0.44	0.08	0.03	0.03
JogFallsB (21)	1.00	—	0.71	—	0.29	—	1.00	0.19	—	—	—	—	0.31	0.07	0.43	—	—	—
MudigereA (20)	1.00	—	0.76	0.24	—	0.58	0.42	—	0.02	0.98	—	—	—	—	—	—	—	—
MudigereB (22)	1.00	—	0.77	0.23	—	0.64	0.36	—		0.91	0.09	—	—	—	—	—	—	—
BekalFort (10)	0.78	0.22	1.00	—	—	0.50	0.50	1.00	—	–	–	—	—	—	—	—	—	—
Sulya (17)	0.44	0.56	0.97	0.03	—	0.68	0.32	0.14	—	0.68	0.18	—	—	—	—	—	—	—
Gundlupet (13)	1.00	—	0.67	—	0.33	0.96	0.04	1.00	—	—	—	—	—	—	—	—	—	—
	108		136			181	183	217	224	228	232							
Chinen (22)	1.00		1.00			0.93	0.07	—	0.32	—	0.68							
Nakijin (23)	1.00		1.00			0.82	0.18	0.02	0.22	0.02	0.74							

of the order of that observed in the introduced populations of invasive ants such as the Argentine ant *Linepithema* humile (with an expected heterozygosity of 0.2 in introduced Californian populations; Tsutsui *et al*. 2000) and lower than that observed in introduced American populations of the fire ant *Solenopsis invicta* (with an expected heterozygosity of 0.62; Ross *et al*. 1999).

Exact tests of genic differentiation computed across all localities were highly significant at each microsatellite locus ($P < 10^{-5}$) with associated high fixation indices ($F_{ST} = 0.45$). Most of the values of fixation indices between each pair of Indian populations were high and the tests of genic differentiation were significant, except for the two pairs of neighbouring populations (Jog Falls A and B; Mudigere A and B). A Mantel test detected a significant correlation between geographical and genetic distances ($P = 0.03$). This pattern of isolation-by-distance should, however, be confirmed using more polymorphic markers.

Such high level of genetic structure has already been found in other *Diacamma* species (Doums *et al*. 2002; Baudry *et al*. 2003). Colony fission clearly restricts female dispersal in ants. Nothing is known about the ecological dynamics of male dispersal but the high population genetic structure observed at the scale of few kilometres in other *Diacamma* species also suggests restricted male dispersal (Doums *et al*. 2002; Baudry *et al*. 2003). Also, genetic differentiation between populations could be enhanced by habitat fragmentation and by high levels of genetic drift within populations, the number of nests per population being generally lower than a few hundreds. Moreover, if *D. indicum* colonized India, the founding events associated with the establishment of each population, probably involving a few individuals from a single colony, are likely to increase the divergence between populations (Wade & McCauley 1988).

Genetic differentiation between Indian and Japanese populations

The two Japanese localities were not significantly differentiated (exact test $P = 0.27$; $F_{ST} = -0.003$). We therefore pooled the two localities and considered a single Japanese population. The genetic differentiation between the Japanese population and the various Indian populations were significant with values of F_{ST} ranging from 0.72 to 0.78. These extremely high fixation indices reflect the few common alleles observed between Indian and Japanese populations (Table 2). For the mtDNA, the two Japanese populations shared the same haplotype, which differed from that observed in India by 53 variable sites, i.e. 7.8% of sites. This value is of the order of that found between the most differentiated haplotypes of *D. cyaneiventre* (7.2% of sites for the same gene, Doums *et al*. 2002).

The Indian and Japanese populations are thus highly differentiated. It is of course difficult from pure genetic data to conclude on the species status of these populations. Even though *D. indicum* is phylogenetically closest to *Diacamma* sp. from Japan (ump. data on mtDNA), the very high genetic differentiation observed between all the Indian populations studied and *Diacamma* sp. from Japan indicates a lack of gene flow. *D. indicum* belongs to the *D. vagans* complex; its most-closely related species is *D. pallidum*, which occurs in Hong Kong (differing from Japanese population by 13.6% of sites), and in Hainan island (14.2%; unpublished mtDNA data). As far as we know, *D. indicum* has never been found on the mainland between India and Okinawa. A phylogeographical study on this species complex would be necessary to clearly resolve the potential origin of *D. indicum* populations of India and Japan.

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This study is part of a research programme on the behavior and genetics of queenless *Diacamma* performed in collaboration with the group of R. Gadagkar in Bangalore (India). Claudie Doums is a molecular evolutionary biologist with a particular interest in social insects. At the time of this study, Barbara Viginier was a master student and Lionel Brazier provided technical help with the mtDNA screening. Christian Peeters studies the behavioral regulation of reproduction in queenless ants and conducted fieldwork in this study.
