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RESEARCH ARTICLE

The conundrum of the yellow crazy ant (*Anoplolepis gracilipes*) reproductive mode: no evidence for dependent lineage genetic caste determination

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Abstract Asexual reproduction and hybridisation are often found among highly invasive plants and marine invertebrates. Recently, it has been suggested that clonality may enhance the success of invasive ants. In contrast, obligate hybridisation (dependent lineage genetic caste determination or DL GCD in ants) may decrease the chances of population persistence if one lineage is less prevalent than the other (asymmetry in lineage ratio). Genetic data available for the invasive yellow crazy ant (*Anoplolepis gracilipes*) suggest that it has an unconventional mode of reproduction that may involve asexual reproduction by workers or queens, or a form of genetic caste determination. Here, we investigated whether *A. gracilipes* reproduction involved DL GCD. The potential for worker reproduction was also assessed. We used microsatellite markers to assess the population structure of *A. gracilipes* workers, males, queens and sperm in queen spermathecae, from field collections in Arnhem Land. We found that a single queen lineage is present in Arnhem Land. The presence of a single lineage of queens discounts the possibility of DL GCD. Population structure separated queens and workers into different lineages, suggesting that these castes are determined genetically in *A. gracilipes*, or the mode of reproduction differs between workers and queens. Evidence for worker reproduction was weak. We conclude that the reproductive mode of *A. gracilipes* does not involve DL GCD. The resolution of the reproductive mode of *A. gracilipes* is complicated by a high prevalence of diploid males. The determination of the *A. gracilipes* reproductive mode remains a fascinating research question, and its resolution will improve our understanding of the contribution of the reproductive system to invasion success.

Keywords Dependent lineages · Invasive species · Reproduction · Clonality

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Introduction

Biological invasions are one of the chief threats to biodiversity, and affect ecosystem function, native organisms and economic well-being (Mack et al., 2000; Pimentel, 2005). Although there is little consensus on the mechanisms and predictability of invasiveness, clonal reproduction and hybridisation are commonly observed traits in successful invaders of terrestrial plant and aquatic communities (Facon et al., 2006; Ren et al., 2005; Mergeay et al., 2006). It has become apparent that ant reproductive systems are more diverse than previously thought (Heinze, 2008; Keller, 2007; Schwander et al., 2010), and recently it has been suggested that the ecological success of some invasive ants may also be facilitated by clonal reproduction (Kellner and Heinze, 2010; Foucaud et al., 2009; Pearcy et al., 2011). In clonal reproductive systems workers are produced sexually by mated queens, while queens and males are produced clonally. Variations on this system have been found in four species, *Cataglyphis cursor* (Pearcy et al., 2004), *Wasmannia auropunctata* (Fournier et al., 2005), *Vollenhovia emeryi* (Kobayashi et al., 2008; Ohkawara et al., 2006), and

Paratrechina longicornis (Pearcy et al., 2011), although the exact mechanisms differ between species. In addition, unmated workers (gamergates) of many species reproduce clonally (reviewed in Heinze, 2008).

As well as clonality, other unusual reproductive systems found among ant species involve forms of hybridisation, which can have different biological implications to clonality. Hybridisation among ant species can often have negative fitness consequences for the population (Feldhaar et al., 2008). However, some ant species have evolved a reproductive system that requires hybridisation (dependent lineage genetic caste determination or DL GCD, Anderson et al., 2006). The DL GCD system requires obligate hybridisation of two lineages (typically species) to produce workers, while matings within lineages result in queens (Volny and Gordon, 2002; Helms Cahan et al., 2002; Helms Cahan and Vinson, 2003). Queens must mate with multiple males to produce both workers and other reproductives. Thus, the ratio of the two lineages present in the population has consequences for colony fitness. The chance of a queen acquiring sperm from only one lineage increases as the lineage ratio increases in skew, resulting in higher colony failure rates (Anderson et al., 2006; Schwander et al., 2006). Clearly, while clonal reproduction could enhance the chance of successful colonisation and persistence, DL GCD in invasive ants could result in a lower likelihood of invasion success if one lineage is under-represented.

The yellow crazy ant (*Anoplolepis gracilipes*), considered to be one of the most damaging invasive ant species (Holway et al., 2002), has an unresolved reproductive mode. Although the reproductive mode remains elusive, and the ants native range is not known (Wetterer, 2005), studies in the invaded range have consistently found that workers are typically heterozygous, queens are typically homozygous, and diploid males are common (Christmas Island: Thomas et al., 2010; Borneo: Drescher et al., 2007; Arnhem Land, Australia: Gruber et al., 2012). High heterozygote frequencies in workers, and a prevalence of homozygous queens has been interpreted as possibly owing to genetic caste determination (Drescher et al., 2007), or asexual reproduction by unmated workers (Heinze, 2008). In addition, on Christmas Island at least two mitochondrial lineages of the ant are known to co-exist, and they appear to be reproductively isolated from each other (Thomas et al., 2010). In Arnhem Land in the Northern Territory of Australia, *A. gracilipes* worker populations are of a single mitochondrial lineage (Gruber et al., 2012), which provided the opportunity to determine if the mode of reproduction involved a DL GCD system.

Here, we use molecular markers to test the hypothesis that the reproductive mode of *A. gracilipes* involves DL GCD. If DL GCD was involved we would expect the population genetic structure to reflect workers arising from hybrid lineages, and queens and males resulting from pure lineages. It would have been desirable to also investigate the possibility of clonal production of queens and males, however, field samples alone are insufficient to conduct the parentage analyses necessary to distinguish clonal versus sexual production of the different castes. We did, however, dissect workers and their ovaries to investigate the possibility of worker reproduction.

Methods

Study site and sampling

Anoplolepis gracilipes are patchily distributed throughout 16,000 km^2 in Arnhem Land (Fig. 1). Workers were collected from 10 sites throughout the ant's geographic range in October 2007, and queens, workers and brood were collected from an additional ten sites between August 2008 and March 2009 (Fig. 1). Samples were collected from a single nest at each site except site SC where we sampled seven nests, spaced 5–100 m apart, on different dates. This sampling regime allowed analysis at a broad (regional) scale, as well as the finer scale at which groups of ants are likely to interact. Collected nests contained between one

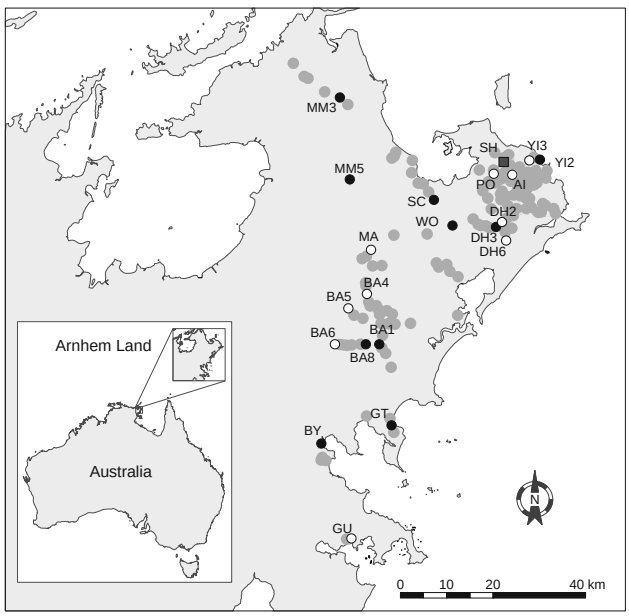

Fig. 1 Sampling sites of *Anoplolepis gracilipes* in Arnhem Land. *Grey circles* indicate known locations of *A. gracilipes*. *Open circles* indicate sites where workers only were sampled in October 2007. *Black circles* indicate sites where queens, workers and male brood were sampled in August 2008 and March 2009. One nest was sampled at each site, with the exception of SC, where seven nests were sampled. The *black square* indicates the site (SH) where workers for dissection and males were collected in October 2009

and 16 queens (mean 4.1 ± 0.9 SE). Ants were stored in 95 % ethanol at 4 °C until DNA extraction. No adult males were found during the sampling, but four male brood were collected from a nest at site YI3 and one from a nest at site SC. In October 2009 (coinciding with the emergence of *A. gracilipes* sexuals), 24 males from a single nest (site SH) were sampled for genotyping. Workers from 13 nests at this site were also sampled for dissection to investigate the possibility of worker reproduction.

Genetic analyses

To determine the genotypes of males contributing to reproduction, we extracted sperm from the spermathecae of queens. Queen gasters were dissected, and the contents extracted from the spermathecae and placed in 95 % ethanol. Contamination by queen tissue from the spermatheca was considered unlikely as the sperm DNA content was likely to be greater than that of the spermathecal wall. Thus, sperm cell DNA would have preferentially amplified under PCR (Krieger and Keller, 2000). This assumption was upheld by an earlier test of the method for another project on four queens and their spermathecal contents from Christmas Island (unpublished data). Three of the four sperm samples had one allele (i.e., were likely to be haploid), which did not match an allele of the queen from which the spermatheca had been dissected. The fourth sperm sample had two alleles, one of which matched an allele of the queen from which it had been dissected. Thus, for the present study we cannot entirely rule out the possibility that some alleles amplified from sperm samples were in fact maternal alleles.

We extracted DNA using a modified Chelex protocol (Sepp et al., 1994). Spermathecal contents were placed in 1.7-ml microcentrifuge tubes with 5 µl of 10 mg/ml Proteinase K and 60 µl of 10 % w/v Chelex-100 resin solution and heated for 2 h at 60 °C. Individual workers, brood or heads of queens were ground using sterile plastic pestles in microcentrifuge tubes containing 150 µl of a 10 % w/v Chelex-100 resin solution. Tubes were centrifuged briefly, boiled for 15 min, chilled on ice for 5 min and then centrifuged for 15 min at 15,000×*g* at 4 °C. The supernatant containing DNA was stored at 4 °C.

We used five microsatellite markers, *Ano1*, *Ano3*, *Ano4*, *Ano7* and *Ano8* (Feldhaar et al., 2006), to investigate population structure and identify multiple lineages. Each 15 µl PCR consisted of ~20 ng template DNA, 10× PCR buffer, 0.4 mg/ml of bovine serum albumin (BSA), 1.5–2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1 mM of forward primer, 0.4 mM of reverse primer, 0.4 mM of universal fluorescent dye-labelled M13(−21) primer (Schuelke, 2000) and 0.1 U of *Taq* DNA Polymerase (New England Biolabs). Thermal cycling was performed on an Eppendorf 2700 thermal cycler using conditions specified by Feldhaar et al. (2006), with modification for M13(−21) primers (Schuelke, 2000). Amplified products were analysed on a 3730 Genetic Analyzer and genotypes scored using Genemapper v 3.7 (Applied Biosystems). Successful genotyping of 16 nests included 3–12 workers (mean ± SE = 6.1 ± 0.7, $n = 96$), 1–10 spermathecal contents (mean ± SE = 3.6 ± 0.6, $n = 68$) and 1–11 queens (mean ± SE = 4.1 ± 0.9, $n = 73$). Fifteen to 20 workers (mean ± SE = 19.4 ± 0.4, $n = 214$) were genotyped from 11 nests without queens. Four male brood from one nest and one from a second nest were also genotyped, along with the 24 adult males from a single nest. We used Micro-Checker v 2.2.3 (van Oosterhout et al., 2004) to validate genotyping quality using data for all workers. Scoring errors, presence of null alleles and large allele dropout were not apparent.

Sperm samples with homozygous multi-locus genotypes (MLGs) were considered to be haploid (hemizygous). Sperm samples with heterozygous genotypes at one or more loci were either diploid, and/or the queen was multiply mated, or the sperm sample was contaminated by queen tissue. We visually inspected the sperm genotypes to detect features that could indicate contamination. It was decided that sperm samples with relatively low volume that had a MLG matching the genotype of the queen from which they were dissected could be unreliable results and should be excluded from analysis, although we excluded only one sperm sample on this basis. In addition, sperm samples with genotype scoring peaks that were substantially lower for maternal alleles were also considered unreliable (i.e., we would expect maternal alleles to be non-preferentially amplified if the queen contribution of DNA from the spermatheca was much lower than the sperm contribution). No sperm samples were excluded on this basis.

Data analyses

Population genetic parameters

We tested for Hardy–Weinberg proportions (HW) and linkage disequilibrium (LD) using GENEPOP v 4.0.10 (Raymond and Rousset, 1995; Rousset, 2008). The close relatedness of workers within nests may result in non-independence of genotypes and thus pseudoreplication in HW and LD analyses (Drescher et al., 2007). We corrected for this by using a single randomly selected ant per nest.

We used Genclone v 2.0 (Arnaud-Haond and Belkhir, 2007) to enumerate the MLGs of the ants sampled. To enable easier visualisation of MLGs, we grouped and encoded alleles according to their observed distribution among queens, workers and spermathecal contents. Alleles that were not found in homozygous queens, but found in male brood, workers or sperm samples were designated as paternal alleles (i.e., putatively paternally inherited). The

other of the pair of alleles found in heterozygous workers (i.e., putatively maternally inherited), which were found in homozygous queens, we designated as maternal alleles. We enumerated allele frequencies using GenAlEx v 6.3 (Peakall and Smouse, 2006).

Population structure

We used AWclust (Gao and Starmer, 2008) to estimate population structure. Unlike other methods (e.g., STRUCTURE, Pritchard et al., 2000), AWclust makes no assumptions about HW proportions or LD, and thus appears appropriate to use when an unusual reproductive mode is possible. In addition, the results enable easier identification of individuals on the resulting tree than STRUCTURE.

Results

Population genetic parameters

Microsatellite diversity was consistent with the levels of diversity seen in single supercolonies of *A. gracilipes* (Thomas et al., 2010). Microsatellite genetic diversity among sites was low in two loci (*Ano1* and *Ano4*), which had the same two alleles in all heterozygotes, and one allele in all queens (Table 1). The remaining loci (*Ano3*, *Ano7* and *Ano8*) had 4, 6 and 16 alleles, respectively (Table 1).

Departures from HW proportions were significant and consistent with an unusual reproductive mode (Table 1). Heterozygote deficiency could not be determined for queens for *Ano1* and *Ano4* as these loci were fixed at two alleles, but was significant for *Ano7* and *Ano8*. Heterozygote excess was significant for workers for all loci (Table 1). LD could not be estimated for all 10 pairwise locus combinations, because of a lack of genetic variation in *Ano1* and *Ano4*. Only one comparison was possible for queens, and LD was not significant ($P = 1.000$). For workers, LD could only be determined for three of ten pairwise comparisons and was not significant for any of these comparisons.

Genotypic patterns

If the reproduction mode of *A. gracilipes* was a DL GCD system, we would have expected to see genotypic patterns among workers that reflected a combination of queen and male genotypes, or complementary queen genotypes. Genotype and allele frequencies did differ considerably among queens, workers, spermathecal contents and male brood. Queens were typically homozygous at most loci (Fig. 2; Table 2), and shared the same (maternal) allele at each of the two invariant loci (Fig. 2). Queens were also never heterozygous for different maternal alleles. Queen MLGs from a single nest were not always identical. In 10 of the 12 nests for which two or more queens were genotyped, queen genotypes differed (Table 3). No queens were homozygous for putatively paternal alleles at any locus, which indicates a single lineage of queens is present in Arnhem Land.

In almost all cases, workers harboured both a paternal and maternal allele at all loci. Paternal alleles that were found in workers were rarely found in queens at loci *Ano3*, *Ano7* and *Ano8*. In all nests except BA8 and BY, multiple worker MLGs were present, and often worker genotypes could not be assigned back to a queen from the same nest, which suggests that either worker exchange among nests is common (which is consistent with ecological observations, and with expectations of a polydomous species), or unassigned worker genotypes were produced by queens that were not sampled.

We found no evidence of males belonging to a distinctly different or complementary lineage to queens. Sperm samples displayed genotypic patterns that were similar to workers, typically being heterozygous at all loci, and possessing both maternal and paternal alleles (Fig. 2; Table 2). More than 60 % of males and 90 % of spermathecal content genotypes were heterozygous. No males had genotypes that would be expected if paternal MLGs were complementary to maternal MLGs (i.e., *aabbccddee* MLGs, Table 2). In addition, all heterozygote genotypes showed a bimodal distribution, with putatively paternal and maternal alleles clearly distinct from each other

Table 1 Genetic diversity estimates for *Anoplolepis gracilipes* queens (Q), workers (W) and males (M) in Arnhem Land

Locus	*N*	Size range (base pairs)	# of alleles	H_O (H_E)			Heterozygote excess or deficiency		
				Q	W	M	Q	W	M
Ano1	424	97–101	2	0.000 (0.000)	0.997 (0.500)	0.448 (0.338)	NA	<0.01 (E)	NS
Ano3	432	162–178	4	0.234 (0.207)	0.997 (0.550)	0.414 (0.400)	NS	<0.01 (E)	NS
Ano4	431	156–174	2	0.000 (0.000)	0.997 (0.500)	0.276 (0.285)	NA	<0.01 (E)	NS
Ano7	423	228–246	6	0.026 (0.121)	0.984 (0.628)	0.517 (0.438)	0.03 (D)	<0.01 (E)	NS
Ano8	432	208–282	16	0.067 (0.662)	0.990 (0.853)	0.207 (0.248)	< 0.01 (D)	<0.01 (E)	NS

Values given for heterozygote excess (E) or deficiency (D) represent *P* values. NA indicates values could not be determined as the locus showed no variation for the group

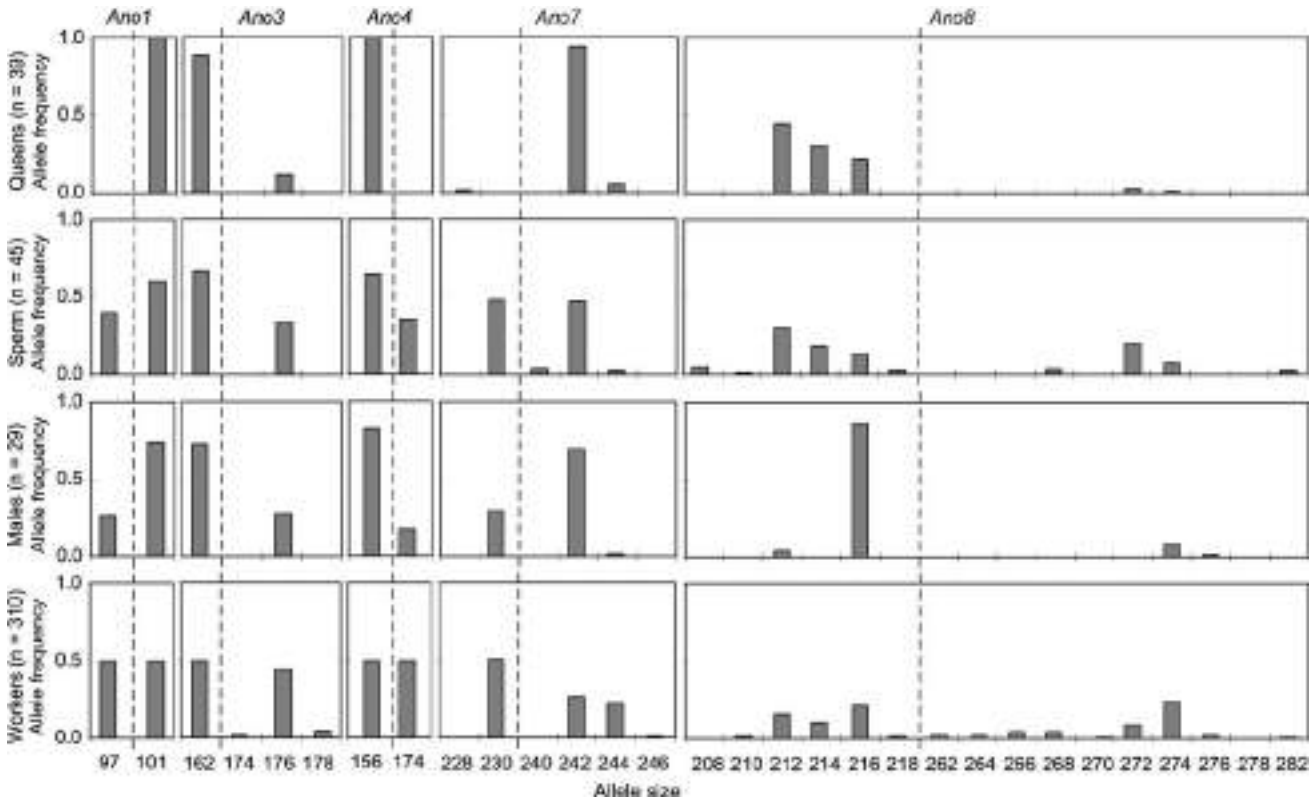

Fig. 2 Allele frequencies for the five microsatellite loci (*Ano1*, *Ano3*, *Ano4*, *Ano7* and *Ano8*) used in this study of *A. gracilipes* queens, sperm, males and workers. The *vertical dashed lines* indicate potentially disjoint allele distributions that typically separated putatively paternal and maternal alleles

(Fig. 2). Eighteen of 46 sperm samples had alleles that did not match the maternal allele of the queen from which they had been dissected in at least one locus, which indicates the genotype of the mother of the males that produced the sperm differed from the queen genotype from which they had been dissected. The genotypic patterns of male brood and adult males were typically similar to that of sperm samples (i.e., typically heterozygous at 1 or more loci, Table 2). However, a higher proportion of male genotypes than sperm sample genotypes were similar to queen genotypes (Table 2).

Population structure

The population structure among queens, workers, males and sperm samples was consistent with the genotypic patterns we observed. The population was divided into two main clusters: one consisting primarily of workers, and the other primarily of queens and males (Fig. 3). However, sperm samples and males were found in both clusters. Samples within sites did not cluster together (Supplementary Fig. 1).

Worker reproduction

Dissections of workers revealed no evidence that workers were reproducing at the time they were collected. Our dissections of 470 workers from 13 nests (mean = 36 ± 5 SE) found ovaries in 2 –15 % of workers from 7 nests (Fig. 4). No spermathecae were found, which indicates the workers did not mate with males. Vitellogenesis, yolky oocytes and yellow bodies that are characteristic of fertile eggs (Peeters, 1987) were not apparent.

Discussion

Recently, it has been suggested that the ecological success of some invasive ants may be facilitated by clonal reproduction (Kellner and Heinze, 2010; Foucaud et al., 2009; Pearcy et al., 2011). In contrast, populations of ant species with DL GCD could have reduced chances of invasion success if one lineage is under-represented. We found clear evidence to reject a hypothesis of DL GCD for *A. gracilipes*. We found evidence of only one lineage of queens, to which most male

Table 2 Multi-locus genotypic patterns among *A. gracilipes* queens, sperm, males (and male brood) and workers

Multi-locus genotype										Queens	Sperm	Males	Workers
A	*A*	*B*	*B*	*C*	*C*	*D*	*D*	*E*	*E*	25[a,b,c]	1[a,b,c]	11[a,b]	
A	*A*	*B*	*B*	*C*	*C*	*D*	*D*	*E*	*e*	2			
A	*A*	*B*	*B*	*C*	*C*	*d*	*D*	*E*	*E*	1	5		
A	*A*	*B*	*B*	*C*	*c*	*d*	*D*	*E*	*E*		1		
A	*A*	*B*	*b*	*C*	*C*	*D*	*D*	*E*	*e*	1		1	
A	*A*	*B*	*b*	*C*	*C*	*d*	*D*	*E*	*E*		1		
A	*A*	*B*	*b*	*C*	*C*	*D*	*D*	*E*	*E*	10	1		1
a	*a*	*b*	*b*	*c*	*c*	*d*	*d*	*E*	*e*			1	
a	*A*	*B*	*B*	*C*	*c*	*d*	*D*	*E*	*e*		5		
a	*A*	*B*	*b*	*C*	*C*	*d*	*D*	*E*	*E*		3		
a	*A*	*B*	*b*	*C*	*c*	*d*	*d*	*E*	*e*		1		4
a	*A*	*B*	*b*	*C*	*c*	*d*	*D*	*E*	*E*			1	1
a	*A*	*B*	*b*	*C*	*C*	*D*	*D*	*E*	*E*		1		
a	*A*	*B*	*B*	*C*	*c*	*d*	*D*	*E*	*E*		2		1
a	*A*	*B*	*b*	*C*	*c*	*d*	*D*	*E*	*e*		23[a]	34[d]	303[d]
a	*A*	*B*	*B*	*C*	*C*	*d*	*D*	*E*	*E*		1	1	
−1	*−1*	*B*	*B*	*C*	*C*	*−1*	*−1*	*E*	*E*	6			
−1	*−1*	*B*	*B*	*C*	*C*	*−1*	*−1*	*−1*	*−1*	2			
a	*A*	*B*	*b*	*−1*	*−1*	*−1*	*−1*	*E*	*E*		1		
A	*A*	*B*	*B*	*C*	*C*	*D*	*D*	*e*	*e*			1	
A	*A*	*B*	*B*	*C*	*c*	*d*	*D*	*E*	*e*			1	
a	*A*	*B*	*B*	*C*	*c*	*D*	*D*	*E*	*e*			4	
a	*A*	*B*	*B*	*C*	*c*	*D*	*D*	*E*	*E*			2	
a	*A*	*B*	*b*	*C*	*c*	*D*	*D*	*E*	*e*			1	
a	*A*	*B*	*b*	*C*	*c*	*D*	*D*	*E*	*E*			1	
A	*A*	*B*	*b*	*C*	*c*	*d*	*D*	*E*	*e*			1	

Alleles have been encoded as letters for ease of interpretation. *A,a*, *Ano1*; *B,b*, *Ano3*; *C,c*, *Ano4*; *D,d*, *Ano7*; *E,e*, *Ano8*; *−1*, failed to amplify. Lower case denotes alleles typically found in males, male brood, and workers and only occasionally in queens (putatively paternal). Upper case denotes alleles found in queens, workers and sperm (putatively maternal)

[a] Homozygous multi-locus genotypes with maternal alleles at all loci

[b] Putative haploid male multi-locus genotypes. Heterozygous genotypes are assumed to be diploid

[c] Putative haploid sperm multi-locus genotypes with alleles that differed from the genotype of the queen from which they were extracted

[d] Heterozygous multi-locus genotypes with paternal and maternal alleles at all loci

genotypes also belonged. Although we found no physiological or genetic evidence of asexual worker reproduction, we cannot entirely discount this possibility, as workers may reproduce seasonally during times we did not assess.

Dependent lineage genetic caste determination

We found no evidence of multiple lineages of reproductives to support DL GCD. Our sampling covered a very broad area, so that it was likely that we would have discovered at least a few examples of multiple lineages if they existed. If heterozygous workers were produced through DL GCD, we would have expected our data to show three overlapping populations, with homozygous queen genotypes matching each of the alleles found in the heterozygous worker genotypes. These features were not apparent in the population structure of *A. gracilipes* in Arnhem Land. The absence of any queens homozygous for the putatively paternal alleles in workers, despite extensive sampling, strongly suggests that the reproductive mode of *A. gracilipes* does not involve DL GCD. However, the divergent genotypic pattern between workers and queens supports the suggestion that caste determination may be genetic (Drescher et al., 2007), although it does not involve dependent lineages, or may indicate that the mode of reproduction differs between workers and sexuals (i.e., clonal vs. sexual reproduction). The disjoint distribution we found among microsatellite alleles are possible indicators of ancient hybridisation events, or clonal reproduction.

Table 3 Examples of genotypes of males, queens, workers and sperm for three *A. gracilipes* nests sampled in Arnhem Land, indicative of the genotypic patterns found among nests

Site	Type	*Ano1*		*Ano3*		*Ano4*		*Ano7*		*Ano8*	
SC3	Male	101	101	162	162	156	156	242	242	212	212
SC3	Queen	101	101	162	162	156	156	242	242	216	216
SC3	Queen	101	101	162	176	156	156	242	242	216	216
SC3	Queen	101	101	162	176	156	156	242	242	216	216
SC3	Queen	101	101	162	162	156	156	242	242	216	216
SC3	Queen	101	101	162	176	156	156	242	242	216	216
SC3	Sperm	101	101	162	162	156	156	230	242	216	216
SC3	Sperm	101	101	162	176	156	156	230	242	216	216
SC3	Sperm	97	101	162	176	156	156	230	242	216	216
SC3	Sperm	97	101	162	176	156	156	242	242	226	226
SC3	Worker	97	101	162	176	156	174	230	242	216	274
SC3	Worker	97	101	162	176	156	174	230	242	212	274
SC3	Worker	97	101	162	176	156	174	230	242	216	274
SC3	Worker	97	101	162	176	156	174	230	242	212	274
SC3	Worker	97	101	162	176	156	174	230	242	212	274
YI2	Male	97	101	162	176	156	174	230	242	216	276
YI2	Male	97	101	162	162	156	156	230	242	216	216
YI2	Male	97	101	162	176	156	174	230	244	216	216
YI2	Queen	101	101	162	162	156	156	242	242	214	214
YI2	Sperm	97	101	162	176	156	174	230	242	214	274
YI2	Worker	97	101	162	176	156	174	230	242	212	274
YI2	Worker	97	101	162	176	156	174	230	242	212	274
YI2	Worker	97	101	162	176	156	174	230	242	214	274
YI2	Worker	97	101	162	176	156	174	230	242	216	274
MM3	Queen	101	101	162	162	156	156	242	242	212	212
MM3	Queen	101	101	162	162	156	156	244	244	212	212
MM3	Queen	101	101	162	162	156	156	242	242	212	212
MM3	Sperm	101	101	162	162	156	156	230	242	212	212
MM3	Sperm	97	101	162	176	156	174	230	244	212	272
MM3	Sperm	101	101	162	162	156	174	230	242	212	212
MM3	Worker	97	101	162	176	156	174	230	244	216	274
MM3	Worker	97	101	162	176	156	174	230	242	212	272
MM3	Worker	97	101	162	176	156	174	230	244	212	276

Potential alternative reproductive modes

The pattern of heterozygous workers, predominantly homozygous queens and often heterozygous males found in this study is consistent with the results of other studies of this species (Christmas Island: Thomas et al., 2010; Borneo: Drescher et al., 2007), which suggests that the mode of reproduction of *A. gracilipes* is likely to be similar throughout the invaded range. However, the actual reproductive mode remains unclear. The consistently observed patterns of fixed heterozygote genotypes among workers suggest a number of possible underlying genetic mechanisms, including asexual reproduction of other workers by unmated workers (Balloux et al., 2003). Queen genotypes that are primarily homozygous may also indicate asexual reproduction among queens (Pearcy et al., 2006; Simon et al., 2003).

Asexual production of workers

Our dissections of workers provided little support for worker reproduction. We found ovaries in a minority of *A. gracilipes* workers, but no spermathecae, which indicates that *A. gracilipes* workers are not capable of sexual reproduction. The presence of ovaries in workers is not sufficient evidence for asexual reproduction because workers in many ant genera produce unfertilised trophic eggs that do not

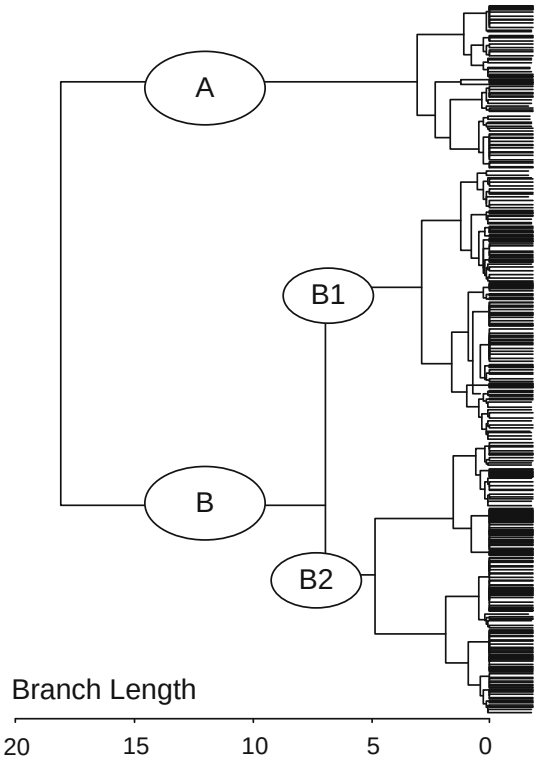

Fig. 3 Population structure of *A. gracilipes* queens, workers, sperm and males in Arnhem Land. The hierarchical plot was based on Allele Sharing Distance calculated with Ward's minimum variance algorithm. Queens were found in cluster *A*, and workers were found in cluster *B*. Sperm and males were found in both clusters. Details of cluster membership are provided in Supplementary Fig. 1

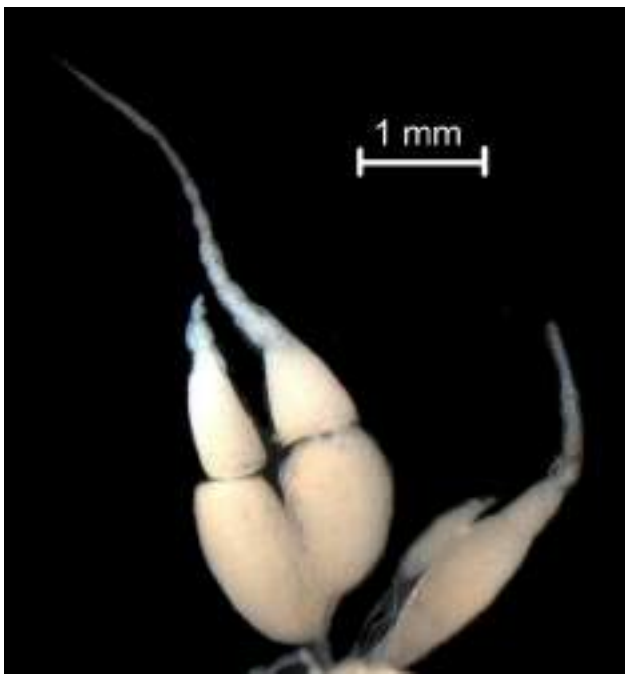

Fig. 4 Gaster dissection of *A. gracilipes* worker, showing ovarioles with no evidence of yolky oocyte development or yellow bodies

develop (Hölldobler and Wilson, 1990), or eggs that develop into haploid males (Bourke and Franks, 1995). Despite the presence of ovaries, there were no oocytes or evidence that viable eggs had been laid. If the ovaries were functional, this would suggest that worker reproduction is either rare, or may occur seasonally differing to our collection time.

Consistent with the morphological evidence, asexual worker reproduction was also not supported by the genotypic patterns observed. The two possible forms of asexual production of female castes in ants (thelytoky) are ameiotic (apomixis) and meiotic (automixis). Apomixis results in offspring genotypes that are identical to the parent (barring mutations and gene conversions), and overall levels of heterozygosity in the offspring are maintained (Suomalainen et al., 1987). In contrast, automixis can result in a steep loss of heterozygosity over a few generations (Pearcy et al., 2006). Thus, as all the loci assessed in *A. gracilipes* workers were heterozygous it is unlikely that workers produce other workers via automixis. The alternative mode of clonality, apomixis, would appear to be the more likely mode of parthenogenetic production of *A. gracilipes* workers by other workers (if it occurs) because apomixis retains heterozygosity and offspring are identical to their mother. Low recombination rates, reflected in departures from HW expectations and significant LD, are typical indicators of asexual reproduction by apomixis (Balloux et al., 2003). Although we found departures from HW among workers, the degree of LD was not clearly resolved. In summary, there appear to be too many uncertainties to conclude that workers reproduce asexually. The disjoint bimodal allele distributions among heterozygous workers, together with the variability of loci *Ano3*, *Ano7* and *Ano8* could equally be interpreted as owing to sexual reproduction between queens and males with complementary alleles.

Asexual production of queens

Queen genotypes were almost exclusively homozygous with significant heterozygote deficiency, a pattern consistent with asexual reproduction of queens by other queens via automixis (Pearcy et al., 2006; Simon et al., 2003), or matched mating of queens with males possessing the same alleles. Most modes of automixis cause a rapid loss of heterozygosity over a few generations, but loci distant from the centromere can retain heterozygosity (Pearcy et al., 2006; Suomalainen et al., 1987). We found heterozygote genotypes at the *Ano3*, *Ano7* or *Ano8* loci in 14 of 39 queens. The polymorphism in these loci and occasional heterozygote genotypes suggests that if queen production of other queens was occurring via automixis, these loci might undergo recombination. In contrast, if workers produced other workers asexually, their heterozygous genotypic patterns

suggest it would most likely be via apomixis (or via automixis with very low recombination rates). However, it is problematic to interpret how a system would evolve with different modes of asexuality or different recombination rates between workers and queens (queens producing queens via automixis and workers producing workers by either apomixis or automixis with low recombination rates). It seems simpler to conclude that if one of these castes is produced asexually, it is likely the other is not.

The enigma of diploid males

In haplo-diploid species, diploid males are diagnostic of complementary sex determination (CSD) (van Wilgenburg et al., 2006). Under CSD, individuals that are heterozygous at one or more sex loci develop into females, while individuals that are haploid or homozygous at one or more sex loci develop into males (Crozier, 1971). Diploid males arise owing to a loss of variation at a CSD locus, are an indicator of inbreeding, are typically inviable or sterile, and are therefore rare (Hedrick and Parker, 1997; Zayed and Packer, 2005). However, a number of recent studies have found that diploid male Hymenopterans (including ants) can mate and produce viable offspring, although workers are triploid as a result (Cournault and Aron, 2009; Liebert et al., 2005). If this was the case for *A. gracilipes*, we would perhaps expect to have found genotypes that indicated triploid workers. However, if matched matings occur, the maternal allele of the queen may mask the maternal allele of the diploid male and triploid worker genotypes would not be detectable.

The existence of diploid genotypes in our sperm samples may not reflect diploid males. An alternative explanation for multiple sperm genotypes from a single queen is the gametes are haploid and that multiple matings occur. This pattern is similar to that seen in ants with DL GCD, where queens mate once with a male of their own lineage and once with a male of the second lineage (Helms Cahan et al., 2002; Volny and Gordon, 2002). While *A. gracilipes* clearly does not require a second lineage, both paternal and maternal alleles are likely to be required to produce queens, workers and males, and caste fate may be controlled by a caste-determining locus. While sperm genotypes may be haploid, and queens may be mated to multiple males, this does not explain the presence of diploid male genotypes, or the origin of paternal alleles in workers and diploid males.

Another explanation for the presence of diploid male genotypes is that males may be mosaics of both maternal and paternal cells. Such sex mosaics (gynandromorphs) have been reported for more than 40 ant species among 22 genera (Jones and Phillips, 1985), and matings involving mosaics can produce viable non-mosaic offspring (Yoshizawa et al., 2009). Importantly, sex mosaicism is known to occur in species with clonal production of males and females (Dobata et al., 2012). *A. gracilipes* males could thus be haploid but with cells from both paternal and maternal sources. These haploid males could produce sperm with alleles inherited from either the maternal cells or from the paternal cells. The production of viable offspring, and their caste, could be determined by a combination of male and female alleles. The occurrence of sex mosaics in *A. gracilipes*, together with caste determination based on the allele combination inherited may provide an explanation for the presence of male and sperm genotypes in both worker and queen clusters.

Conclusion

The range of reproductive systems among Hymenopterans is likely to be far more varied than currently known (Heinze, 2008; Keller, 2007), and despite the reproductive mode of *A. gracilipes* remaining unresolved, it is clear that when it is determined it will be novel. Of the many uncertainties, the prevalence of diploid males is most problematic. The production of males, and the source and fate of paternal alleles remain enigmatic. However, the resolution of this enigma will also help determine the mode of production of queens and workers. While it is interesting to speculate on the reproductive system based on our data, experimental laboratory nests that allow the control of the parental relationships between individuals are clearly required to understand this unusual reproductive mode. Whether the unusual reproductive mode affects the abundance, fitness and invasion success of this ant remains an intriguing question.

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