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Mating system evolution and worker caste diversity in *Pheidole* ants

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Abstract

The efficiency of social groups is generally optimized by a division of labour, achieved through behavioural or morphological diversity of members. In social insects, colonies may increase the morphological diversity of workers by recruiting standing genetic variance for size and shape via multiply mated queens (polyandry) or multiple-breeding queens (polygyny). However, greater worker diversity in multi-lineage species may also have evolved due to mutual worker policing if there is worker reproduction. Such policing reduces the pressure on workers to maintain reproductive morphologies, allowing the evolution of greater developmental plasticity and the maintenance of more genetic variance for worker size and shape in populations. *Pheidole* ants vary greatly in the diversity of worker castes. Also, their workers lack ovaries and are thus invariably sterile regardless of the queen mating frequency and numbers of queens per colony. This allowed us to perform an across-species study examining the genetic effects of recruiting more patrilines on the developmental diversity of workers in the absence of confounding effects from worker policing. Using highly variable microsatellite markers, we found that the effective mating frequency of the soldier-polymorphic *P. rhea* (avg. $me_N = 2.65$) was significantly higher than that of the dimorphic *P. spadonia* (avg. $me_N = 1.06$), despite a significant paternity skew in *P. rhea* (avg. B = 0.10). Our findings support the idea that mating strategies of queens may co-evolve with selection to increase the diversity of workers. We also detected patriline bias in the production of different worker sizes, which provides direct evidence for a genetic component to worker polymorphism.

Keywords: mating system, paternity, patriline bias, polyandry, social insects, worker size

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Introduction

A key characteristic of societies is the division of labour between members (Bourke & Franks 1995). For example, when social *Dictyostelium* slime moulds experience starvation, some cells altruistically form a nonreproductive stalk bearing multicellular fruiting bodies (Strassmann *et al.* 2000), whereas naked mole rats show a division of labour between colony members with some foraging and others reproducing (O'Riain *et al.* 2000). One of the best known examples of such a reproductive division of labour is the social Hymenoptera (ants, many bees and wasps) where some females (queens) are devoted to reproduction while helper females (workers) carry out nest work, foraging and defense (Wilson 1971). In some species, division of labour goes beyond reproduction and involves a subdivision of worker roles with subcastes that show distinct morphologies and/or behaviours (Oster & Wilson 1978; Hölldobler & Wilson 1990; Bourke & Franks 1995). Reproductive division of labour imposes a differential selection on individuals and results in some colony members having a greater individual fitness through their own fecundity and others deriving indirect fitness

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mainly or exclusively by rearing reproductive sisters with which they share genes. In contrast, a subdivision of nonreproductive roles affects the selection on colonies, where colonies with a better partitioning of labour enjoy a higher fitness (Oster & Wilson 1978). These two levels of selection can work in opposite directions, leading to conflicts and the evolution of mechanisms to resolve them. The iconic example of resolution of conflict between levels of selection is in the process of meiosis, which fairly partitions maternal and paternal chromosomes during gametogenesis (Maynard Smith & Szathmáry 1995).

Increased diversity of social insect workers due to selection for a better division of nonreproductive labour can come about in two ways: (i) through modification of development to produce a greater variety of worker sizes and shapes and (ii) through an increase in a colony's genetic diversity by queens having more mates or colonies containing more queens, assuming that genetic variance for worker size and shape occurs in the population (Wheeler 1986, 1991; Fjerdingstad & Crozier 2006). Increased genetic diversity has a strong impact on the balance between selection at the individual and colony levels. Workers in colonies with singly mated queens (monandry) share the father's single haploid set of genes, so relatedness among them is high, and workers benefit from focusing on rearing the queen's offspring (Bourke & Franks 1995). In contrast, workers in colonies with multiply mated queens (polyandry) have a lower relatedness. Low relatedness can tip the scales to the point that there is a greater payoff from direct worker reproduction. Near the tipping point, worker reproduction can disrupt the integrated functioning of colonies and reduce colony fitness. The conflict can be resolved and colony level efficiency restored by worker policing, that is, the destruction of eggs laid by other workers (Ratnieks 1988; Crozier & Pamilo 1996). Without worker fecundity, variation in worker size and shape may benefit colonies, and selection may further increase the genetic variance for worker caste diversity in populations (Fuchs & Moritz 1999). Workers, however, do retain ovaries in a large number of worker polymorphic species (Dijkstra & Boomsma 2006, 2007; Fjerdingstad & Crozier 2006; Smith *et al*. 2007; Kronauer *et al*. 2010). In these species, it is difficult to determine whether worker policing allows a release of developmental plasticity in workers through weaker stabilizing selection on worker morphology, or whether direct benefits of recruiting genetic variance for size and shape alone explains the greater worker diversity seen in species with polyandry and polygyny (Fjerdingstad & Crozier 2006).

One way to overcome this problem is to study whether the numbers of mates per queen or the numbers of breeding queens per colony are greater in caste-diverse species where worker policing does not occur. Policing is absent when there is no worker reproduction due to the complete lack of ovaries in workers. In this case, selection acting on genetic variance and developmental plasticity for worker size and shape in colonies would be unaffected by either worker reproduction or policing. *Pheidole* ants are suitable taxon for such tests because workers have no functional ovaries (Hölldobler & Wilson 1990; Khila & Abouheif 2010). Moreover, although nearly all *Pheidole* ants are dimorphic, with two monomorphic castes (minor workers and soldiers), several species have polymorphic soldiers with a wide range of sizes (Wilson 2003; Huang & Wheeler 2011; see Table 1). Although the ecology and division of labour of this genus has been well studied (Hölldobler & Wilson 1990), their mating strategies have been studied for only a few dimorphic species (Helms 1999; Fournier *et al*. 2002, 2009; Tripet *et al*. 2006).

Here, we use microsatellite DNA analyses of worker offspring from monogynous laboratory colonies to test whether the degree of polyandry is higher in *Pheidole* species with a wider range of worker sizes and whether different males contribute differentially to worker sizes, providing direct evidence for genetic influence over worker morphological diversity. Specifically, we study *P. rhea*, one of the most basal lineages in the *Pheidole* genus, and *P. spadonia*, a more derived lineage (Moreau 2008). These two species show a great contrast in the morphological diversity of workers: *P. rhea* has one of the broadest worker size ranges in its genus with a particularly great diversity of soldiers (majors and supermajors), while the dimorphic *P. spadonia* has a typically narrow size range (Huang & Wheeler 2011).

Materials and methods

Queen collection & colony rearing

All *Pheidole rhea* workers used in this study were sampled from 15 mature laboratory colonies reared from founding queens collected on the same hillside in the Pajarita Mountains (Santa Cruz Co.: Peña Blanca, AZ). Four cohorts of queens were collected in July of the following consecutive years: 2006 (six queens), 2007 (two queens), 2008 (six queens) and 2009 (one queen). All *P. spadonia* workers used were sampled from 15 laboratory colonies reared from founding queens collected at blacklights in Tucson, AZ within an area of 1 × 1 mile. Four queen cohorts were collected in July of the following successive years: 2004 (five queens), 2005 (three queens), 2007 (two queens) and 2008 (five queens). As the focus of our study is on possible polyandry, each founding queen was forced to establish a laboratory

Table 1 A review of life-history traits of some of the most well-studied *Pheidole* species. We determined the caste size range and worker coefficient of variation (CV) for *Pheidole desertorum*, *P. megacephala*, *P. xerophila*, *P. pallidula*, *P. morrisi*and *P. dentata* from measurements of museum and donated specimens (n = 10–20 per caste for these six species). Please note that polyandry in *P. xerophila* is uncertain because of confounding effects of polygyny on the analyses of Tripet *et al.* (2006)

Pheidole species	Worker diversity	Caste ratio (Min*, Sold†, Sup‡)	Size Range§ [with median(M)]	Worker CV¶	Mating & breeding system	Paper citations
Pheidole obtusospinosa	Soldier-polymorphic	Min = 84% Sold = 12% Sup = 4%	Min = 0.6–0.7 (M = 0.60) Sold = 1.2–1.6 (M = 1.5) Sup = 1.7–2.4 (M = 1.9)	Min = 4.43 Sold = 16.7 & Sup	— —	Wilson (2003) Huang & Wheeler (2011)
Pheidole rhea	Soldier-polymorphic	Min = 98.5% Sold = 1.2% Sup = 0.3%	Min = 0.6–0.9 (M = 0.80) Sold = 1.1–2.7 (M = 2.1) Sup = 2.8–3.5 (M = 3.0)	Min = 6.49 Sold = 22.6 & Sup	Polyandry —	Wilson (2003) Huang & Wheeler (2011)
Pheidole tepicana	Soldier-polymorphic	Min = 85.4% Sold = 13.7% Sup = 0.9%	Min = 0.5–0.6 (M = 0.53) Sold = 0.8–1.2 (M = 1.1) Sup = 1.3–1.7 (M = 1.4)	Min = 4.70 Sold = 11.4 & Sup	— —	Wilson (2003) Huang & Wheeler (2011)
Pheidole dentata	Dimorphic	Min = 91% Sold = 9%	Min = 0.5–0.65 (M = 0.6) Sold = 1.1–1.4 (M = 1.2)	Min = 6.41 Sold = 6.84	— —	Wilson (1984, 2003) Moreau (2008)
Pheidole desertorum	Dimorphic	Min = 83% Sold = 17%	Min = 0.55–0.65 (M = 0.6) Sold = 1.3–1.5 (M = 1.4)	Min = 4.61 Sold = 5.44	Monandry Monogyny	Droual (1983) Helms (1999) Wilson (2003) Moreau (2008)
Pheidole megacephala	Dimorphic	Min = 93% Sold = 7%	Min = 0.5–0.55 (M = 0.5) Sold = 0.9–1.4 (M = 1.2)	Min = 4.49 Sold = 12.1	Monandry Polygyny	Wilson (1984, 2003) Fournier *et al.* (2009)
Pheidole morrisi	Dimorphic	Min = 88% Sold = 12%	Min = 0.55–0.6 (M = 0.6) Sold = 1.1–1.3 (M = 1.2)	Min = 3.17 Sold = 4.17	— —	Wilson (2003) Yang *et al.* (2004) Moreau (2008)
Pheidole pallidula	Dimorphic	Min = 89% Sold = 11%	Min = 0.54–0.64 (M = 0.6) Sold = 1.2–1.4 (M = 1.4)	Min = 5.69 Sold = 4.84	Monandry Mono/Polygyny	Fournier *et al.* (2002) Wilson (2003); Sempo & Detrain (2004); Moreau (2008)
Pheidole spadonia	Dimorphic	Min = 91% Sold = 9%	Min = 0.5–0.6 (M = 0.55) Sold = 1.3–1.8 (M = 1.6)	Min = 4.72 Sold = 5.08	Monandry —	Wilson (2003) Moreau (2008) Huang & Wheeler (2011)
Pheidole xerophila	Dimorphic	– —	Min= 0.5–0.65 (M = 0.55) Sold = 1.4–1.6 (M = 1.6)	Min = 7.58 Sold = 5.41	Mono/Polyandry(?) Polygyny(?)	Wilson (2003) Tripet *et al.* (2006) Moreau (2008)

*Min = minors.
†Sold = soldiers.
‡Sup = supersoldiers.
§Size Range = minimum and maximum size range (head width, mm) for each caste, with their respective median (M) shown in parentheses.
¶CV = Coefficient of Variation (head width in mm).

colony on her own to eliminate potential confounding effects linked to polygyny that may occur in field colonies. All laboratory colonies were also reared in constant darkness, humidity and temperature (30°C). The colonies were fed twice a week with a diet consisting of frozen insects (cockroaches, lepidopteran larvae and crickets) and sunflower seeds. This rich diet was supplemented with drops of a liquid solution consisting of honey, water, Wesson salts and Vanderzant vitamins (Sigma-Aldrich Corporation).

Sampling protocol & storage

All workers were randomly sampled from the colonies within a 4-month period. Equal numbers of workers were collected to represent each of the worker castes for the two *Pheidole* species: *P. spadonia* (minors and soldiers) and *P. rhea* (minors, small soldiers and supersoldiers), as described by Huang & Wheeler (2011). Only adult workers were used for this study. Individual tubes for each sample were placed in a −20°C freezer for temporary storage. All frozen specimens were extracted for DNA within a week of collection.

DNA analyses

Whole-body extractions were performed on individual ants using the DNeasy Blood and Tissue kit (Qiagen Inc.) and their insect protocol to obtain genomic DNA. A motorized pestle was used to mechanically grind the samples in their respective tubes. All DNA extractions were stored in a −20°C freezer. For the paternity analyses, we initially extracted 21 workers for each of our 15 *P. rhea* colonies and 20 workers for each of our 15 *P. spadonia* colonies, but added 21 workers more for each *P. rhea* colony after analyses showed the presence of multiple patrilines in all *P. rhea* colonies. Also, for population genetics tests on the resolution power of our markers, we extracted DNA from one worker per colony for an additional 15 colonies per species, bringing the total data set for these tests to 30 colonies for each species. For *P. spadonia*, all 30 colonies were laboratory colonies that were reared from founding queens collected within five square miles of each other. For *P. rhea*, the 15 additional colonies were samples of field colonies that were approximately 80 square miles of each other (Southern AZ: Peña Blanca, Gardner Canyon and Catalina Mountains) and the location where the founding queens of the 15 laboratory colonies were collected (Southern AZ: Peña Blanca); there was no evidence that the 30 *P. rhea* colonies were from different populations.

Next, we performed PCR amplification of two dinucleotide-repeat microsatellite loci, using the primers set Ppal77 and Myrt3, previously developed for *P. pallidula* (Fournier *et al.* 2002) and *Myrmica tahoensis* ants (Evans 1993), respectively. These primer sets were optimized for cross-priming in *P. rhea* and *P. spadonia* (PCR protocol details below). The forward primer of Ppal77 was fluorescently labelled with 6-FAM (Eurofins MWG Operon) while that of Myrt3 was labelled with NED (Applied Biosystems Inc.). The two loci were amplified together (multiplexed). The following PCR mix was used for a 1× reaction: 5 μL of 5× Taq Master PCR Enhancer, 2.5 μL of 10× buffer containing 1.5 mM $MgCl_2$, 0.5 μL of 10 mM dNTP, 0.6 μL of 10 μM Myrt3 primer (F and R), 0.4 μL of 10 μM Ppal77 primer (F and R), 0.25 μL of 5 U/μL Taq DNA Polymerase, 2 μL of DNA template and ultra-purified water to a total reaction volume of 25 μL. All PCR components were included in a kit purchased from 5-Prime, except for the dNTP that was purchased from Fermentas. PCR amplification was performed on a MJ Research PTC-200 Peltier Thermal Cycler using the following protocol: (i) 3 min initial denaturing step at 94°C, (ii) followed by a 30 s denaturing step at 94°C, touchdown annealing sequence from 58 to 51°C (decrease of 0.5°C/cycle at 30 s/temperature hold), 30 s elongation step at 72°C (repeated for 15 cycles), (iii) then 30 s denaturing step at 94°C, 25 cycles of annealing at 50°C for 30 s, and 30 s of elongation at 72°C (repeated for 25 cycles), (iv) ending with a 10 min elongation at 72°C.

After PCR, the products (one tube per individual ant) were diluted by transferring aliquots of 8 μL from each tube to a new tube and mixing with 72 μL of sterilized distilled water and then sent to the Nevada Genomics Center (http://www.cabnr.unr.edu/genomics/) for fragment analysis on an ABI3730 DNA Analyzer, to permit separation and sizing of alleles. From the peak diagrams produced, we designated alleles using Genemarker 1.70 (SoftGenetics Corp.). At the end of peak diagram analyses, all alleles were cross-checked across colonies for our entire data set, to ensure that calling errors would not affect subsequent estimates of the relatedness of nestmates relative to the background population.

Population genetics

For these analyses, we selected one worker per colony among the colonies used for paternity studies using the random number generator at http://www.random.org/ and added the workers genotyped for field colonies. This gave us a total of 30 individual worker genotypes per species representing 30 colonies. Using these data, we estimated allelic richness and heterozygosity and tested whether genotype distributions deviated from those expected under random mating and specifically whether there was any evidence of inbreeding (deficit

of heterozygotes). These analyses were carried out using the software Genepop version 4.0 (Raymond & Rousset 1995). Next, we used the same software to test for evidence of null alleles that could confound the population genetics analyses (leading to an excess of apparent homozygotes). Additionally, we calculated the so-called nondetection error which represents the risk that unrelated males carry the same two-locus genotype and so cannot be distinguished in a pedigree analysis (Pamilo 1993). Pamilo's (1993) nondetection error estimation relies on the assumptions of (i) no linkage between marker loci, (ii) random mating and (iii) no relatedness between males mated to the same queen. We verified the first assumption (no linkage) through chi-square tests on the segregation of alleles of inferred heterozygous queens in their offspring, the second was verified as described earlier, and the third assumption was verified through regression relatedness analyses on inferred mate genotypes for colonies determined to have multiple fathers through pedigree analyses. Relatedness analyses were performed using the software Relatedness 5.0.8 (Queller & Goodnight 1988; Goodnight 1992).

Queen mating frequency and nestmate relatedness

We here used the data set of 15 colonies per species, comprising 42 workers per colony for *P. rhea* and 20 workers per colony for *P. spadonia*. For each colony, we inferred the parental genotypes and estimated the numbers of mates per queen as in Fjerdingstad *et al.* (1998, 2002) and Corley & Fjerdingstad (2010). This was facilitated by the fact that our laboratory colonies were monogynous (one queen per colony) and by the male-haploid sex determination system of ants (Crozier & Pamilo 1996). Male ants thus carry only one allele at each locus and pass it to all their offspring. We next verified that worker genotypes were consistent with Mendelian segregation of alleles in queens inferred to be heterozygous [exact binomial tests for each locus and species, no tests significant after False Discovery Rate control (Verhoeven *et al.* 2005)].

The genetic effects of multiple mating depend also on the degree to which siring success of the different mates of a given queen is skewed. We directly estimated the genetically effective paternity (me_{ped}) (Boomsma & Ratnieks 1996) from the number of offspring sired by each father for each colony. Additionally, we applied Nielsen *et al.* (2003) correction to our calculated effective paternities to achieve sample-size unbiased estimators for each colony of each species. Finally, we calculated the expected *nestmate relatedness* of workers in each colony (r_{ped}) based on the *Nielsen-corrected effective paternity* (me_N) and taking into account the male haploidy of ants (Crozier & Pamilo 1996). Lastly, we obtained independent estimates of the genetic diversity of colonies through regression relatedness analyses that directly examine the genetic similarity of nestmate workers relative to the background population (Queller & Goodnight 1988). We also used these nestmate relatedness data to reverse-infer the effective paternity for each colony.

Patriline bias in worker size

Patriline bias in the production of different worker sizes was only determined for *P. rhea*, a species that showed consistent multiple matings by queens. Fifteen colonies were used in this analysis, with 42 workers sampled for each colony. All workers were randomly selected to represent the full size range (including minors, soldiers and supersoldiers) for *P. rhea*. The head width of each individual sampled was measured according to methods of Huang & Wheeler (2011). The identity of the patriline siring each individual sampled was inferred based on pedigree analysis, as was carried out in our mating frequency study above. Head measurements were log transformed to normalize our data before running our statistical analysis. The transformed data set was visually inspected for normality, and the Levene test was used to determine unequal variance. As the worker caste sizes for *P. rhea* were not discretely separated between soldiers and supersoldiers [see Huang & Wheeler (2011)], a statistical model that analyses continuous data was used. To determine whether there was a significant difference in the sizes of workers produced by different patrilines, we used a nested ANOVA test, with the *Patriline* factor nested within Colony (both factors were designated as random effects in the model). Patrilines that sired three or fewer total offspring were excluded from the test because they had low statistical power. The exclusion of these patrilines only sacrificed approximately 3% of the total data collected. Also, a strong significant difference was detected regardless of whether these outlier patrilines were included ($P = 0.0011$) or excluded ($P = 0.0010$).

Results

Population genetics

The data set of one worker per colony for 30 colonies per species allowed us to determine that our two microsatellite loci were highly polymorphic in both species with 16 and 13 alleles at Myrt3, and 13 and 17 alleles at Ppal77 for *P. rhea* and *P. spadonia*, respectively (Table 2). As a consequence, the observed heterozygosities were high for both loci in both species (83–87%)

Table 2 Variability at the two microsatellite DNA marker loci for the two *Pheidole* species, and the resulting nondetection error [risk that two unrelated males carry the same two-locus genotype and so cannot be distinguished in our pedigree analyses, as defined in Pamilo (1993)]. Significant *P* value given in bold

	$n_{alleles}$*	H_{obs}*	H_{exp}*	P_{HWE}	F_{is}	$P_{Het\text{-}def}$	Nondetection error†
Pheidole rhea							
Ppal77‡	13	0.87	0.92	0.12	0.056	0.25	1.03%
Myrt3§	16	0.87	0.91	0.41	0.048	0.37	
Pheidole spadonia							
Ppal77‡	17	0.83	0.94	**0.02**	−0.01	0.13	1.24%
Myrt3§	13	0.87	0.86	1.00	0.11	0.56	

*Based on a sample size of one worker from each of 30 colonies for each species.
†Calculated following Pamilo (1993).
‡Primers developed for *P. pallidula* by Fournier *et al*. (2002).
§Primers developed for *Myrmica tahoensis* by Evans (1993).

(Table 2). Using a data set consisting of one worker per colony for 30 colonies, we found no evidence for null alleles at Myrt3 in *P. rhea* or *P. spadonia*. However, null alleles at Ppal77 were significant for both species (estimated frequencies: *P. rhea* = 0.08, 95% c.i. = 0.02–0.18; *P. spadonia* = 0.11, 95% c.i. = 0.04–0.21). The presence of null alleles may lead to an overestimation of the number of mates, if the queen is heterozygous at a given locus and one of her mates carries a null allele.

A detailed scrutiny, however, showed that none of our pedigree estimates are likely to have overestimated polyandry. We had across-locus confirmation for inferred patrilines and their allele designation for most of the colonies where some workers appeared homozygous for a queen allele. This suggests they were not carrying a paternal null allele and that a male sharing a queen allele did exist. Also, in almost all cases, the paternal allele shared with the queen (i.e. A) was associated with both of the queen's alleles (i.e. AA and AB offspring were observed). This again proves a correct allele designation for the male and makes null alleles unlikely. In one case, the queen was inferred to homozygous and only one mate was deduced, although he may have carried a null allele. Lastly, no cases of heterozygous queens (AB) with both AA and BB offspring were found, although this would have been expected had such a queen mated with a mate carrying a null allele.

The great allelic diversity at our two genetic marker loci meant that the risk of unrelated males carrying the same two-locus genotype (Pamilo 1993) was very low in both species (<1.3%) (Table 2). The assumptions for Pamilo's (1993) nondetection error (no linkage between marker loci, random mating and no relatedness between males mated to the same queen) were all upheld. First, chi-square tests on the 15 laboratory colonies per species from the pedigree analysis data set (see Fig. S1, Supporting information) showed that the segregation of alleles of the inferred queens did not deviate from expectations under free diallelic Mendelian segregation (all tests nonsignificant for all colonies, *P. rhea*: *P*-values between 0.10 and 0.99, *P. spadonia*: *P*-values between 0.07 and 0.94). Direct tests for linkage disequilibrium using the data set of one worker per colony (see Fig. S2, Supporting information) and the software Genepop version 4.0 (Raymond & Rousset 1995) were not possible as most two-locus genotypes were detected only once (a consequence of the high allelic diversity of our microsatellite markers). Second, genotype distributions did not deviate from those expected under random mating (Hardy–Weinberg proportions) in *P. rhea* (Table 2). Although the single-locus test for Ppal77 was significant for *P. spadonia* (Table 2), this did not hold when controlling for multiple tests using False Discovery Rate Control. Also, no deviations from Hardy–Weinberg proportions were found for either species in a two-locus test (*P. rhea*: $\chi^2 = 1.5$, d.f. = 4, $P = 0.83$, *P. spadonia*: $\chi^2 = 1.2$, d.f. = 4, $P = 0.88$). For both species, also, there was no evidence for a deficit of heterozygotes in single-locus tests (Table 2) (F_{is} was here estimated using the method of Weir & Cockerham (1984) and tests done using 20 batches of 5000 iterations), and this held when calculating two-locus estimates (*P. rhea*: $P = 0.73$; *P. spadonia*: $P = 0.43$, using Genepop version 4.0 (Raymond & Rousset 1995)). This suggested that queens and their mates are not related in any of our study species, and we confirmed this in independent analyses estimating the regression relatedness among inferred queen and paternal genotypes using Relatedness 5.0.8 (Goodnight 1992). The estimated relatedness among queens and their mates did not deviate significantly from zero in either *P. rhea* or *P. spadonia* (Table 3). Also, the average queen-mate relatedness for *P. rhea* (based on inferred genotypes from the pedigree

Table 3 Regression relatedness among queens and their mates ($r_{Q\text{-}M}$) and among males mated to the same queen ($r_{M\text{-}M}$), obtained using Relatedness 5.0.8 (Goodnight 1992) on the genotypes inferred for queens and their mates through pedigree analyses. Standard errors (SE) on relatedness estimates were obtained by jackknifing over colonies. A one-sided *t*-test was used to determine whether relatedness values were significantly different from zero within each species (n = 15 colonies for both species)

	$r_{Q\text{-}M}$	SE	d.f.	$t_{d.f.}$	P	$r_{M\text{-}M}$	SE	d.f.	$t_{d.f.}$	P
Pheidole rhea	−0.024	0.06	14	0.40	0.70	0.04	0.05	14	0.82	0.43
Pheidole spadonia	−0.064	0.10	14	0.64	0.53	0.18	0.29	1	—	—

analyses) was not significantly different from that found in *P. spadonia* (Mann–Whitney Test, W = 191, n_{RHEA} = 15, n_{SPAD} = 15, P = 0.09).

Finally, males mated to the same queen were unrelated in both *P. rhea* and *P. spadonia*, as shown by regression relatedness analyses on inferred paternal genotypes for each colony (Table 3). Note, however, that only two colonies were polyandrous for the mainly monandrous *P. spadonia*. As a result, the uncertainty in our estimate of mate–mate relatedness for this species would likely be great, and we could not statistically test whether it was greater than zero. As a note of caution, brothers on average will carry a two-locus type that is identical by descent in 25% of the cases and so be genetically indistinguishable in our pedigree analyses. Nevertheless, our results suggest that any relatedness among mates of the same queen is probably low for both species.

Queen mating frequency and nestmate relatedness

Our results showed that the species with a broader worker size range (*P. rhea*) has a significantly greater number of mates per queen (m_{abs}) than the species with a narrower worker size range (*P. spadonia*) (Mann–Whitney Test, median numbers of mates, $m_{abs,\ RHEA}$ = 3, $m_{abs,\ SPAD}$ = 1, n_{RHEA} = 15, n_{SPAD} = 15, $P < 0.0001$) (Fig. 1, Table 4). Effective mating frequency (me_N) estimates the genetic effect of multiple paternity on systems where each patriline does not sire an equal proportion of offspring. For the highly polyandrous *P. rhea*, the slope of effective vs. observed mating frequency was significantly less steep than 1:1 (*t*-test, slope = 0.345, t = −29.4, d.f. = 13, $P < 0.00001$) (Fig. 2). Using Skew Calculator to calculate the B skew index (Nonacs 2000, 2003), we found that 10 of 15 *P. rhea* colonies showed significant paternity skew ($P < 0.03$ for all tests) with a mean B = 0.104 (range: 0.039–0.20). However, the effective mating frequency remained high (Avg. me_N = 2.65, range: 1.53–3.95) and was significantly higher than that found for *P. spadonia* (Avg. me_N = 1.06, range: 1.00–1.79) (Table 4) (Mann–Whitney Test, median numbers of mates, $m_{abs,\ RHEA}$ = 2.67, $m_{abs,\ SPAD}$ = 1.00, n_{RHEA} = 15, n_{SPAD} = 15, $P < 0.0001$). Finally, the average relatedness between nestmates in *P. rhea* colonies (0.42 ± 0.038 = Standard Error) was significantly lower than in *P. spadonia* colonies (0.72 ± 0.001) (Mann–Whitney Test, W = 339, n_{RHEA} = 15, n_{SPAD} = 15, $P < 0.0001$)(Fig. 3).

Generally, the estimates of nestmate relatedness derived from pedigree inferences were similar to those based on the regression relatedness analyses (Table 4).

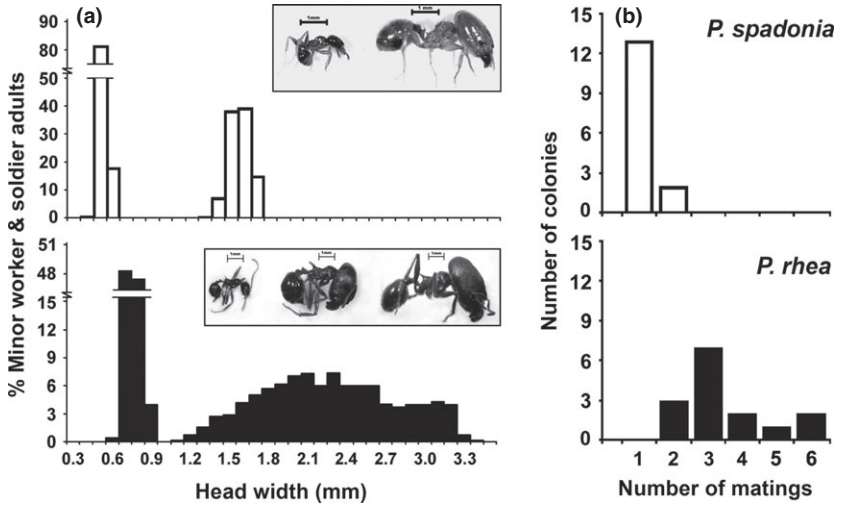

Fig. 1 (a) Size frequency distributions of adult minor workers and soldiers for *Pheidole spadonia* (white) and *P. rhea* (black) [adapted from Huang & Wheeler (2011)]. (b) Observed queen mating frequencies of *P. spadonia* (white) (n = 15 colonies; 300 workers) and *P. rhea* (black) (n = 15 colonies; 630 workers).

Table 4 Results of paternity analyses on *Pheidole rhea* and *P. spadonia*

	m_{abs}	me_{ped}	me_N	r_{ped}	r_{regr}	me_{regr}
Pheidole rhea						
RH1	4	2.91	3.05	0.41	0.32	6.73
RH2	3	2.58	2.62	0.44	0.33	6.39
RH3	3	2.93	3.07	0.41	0.30	10.14
RH4	3	2.57	2.67	0.44	0.58	1.50
RH5	6	3.12	3.28	0.40	0.50	2.01
RH6	5	2.36	2.44	0.45	0.39	3.69
RH7	2	1.51	1.53	0.58	0.49	2.11
RH8	3	2.65	2.75	0.43	0.33	6.21
RH9	2	2.00	2.04	0.50	0.64	1.28
RH10	3	2.57	2.67	0.44	0.37	4.09
RH11	4	2.59	2.69	0.44	0.47	2.30
RH12	3	2.82	2.94	0.42	0.39	3.63
RH13	6	3.71	3.95	0.38	0.34	5.76
RH14	2	1.96	2.00	0.50	0.38	4.00
RH15	3	2.00	2.05	0.49	0.48	2.17
Pheidole spadonia						
SP1	1	1	1	0.75	0.90	0.76
SP2	1	1	1	0.75	0.70	1.11
SP3	1	1	1	0.75	0.64	1.27
SP4	1	1	1	0.75	0.84	0.85
SP5	1	1	1	0.75	0.67	1.19
SP6	2	1.10	1.11	0.70	0.80	0.91
SP7	1	1	1	0.75	0.68	1.15
SP8	2	1.72	1.79	0.53	0.45	2.56
SP9	1	1	1	0.75	0.71	1.09
SP10	1	1	1	0.75	0.69	1.13
SP11	1	1	1	0.75	0.79	0.93
SP12	1	1	1	0.75	0.67	1.18
SP13	1	1	1	0.75	0.69	1.15
SP14	1	1	1	0.75	0.69	1.14
SP15	1	1	1	0.75	0.67	1.20

m_{abs} = observed number of matings.
me_{ped} = effective number of matings based on pedigree analysis.
me_N = Nielsen-adjusted effective number of matings based on pedigree analysis.
r_{ped} = Nestmate relatedness based on pedigree analysis.
r_{regr} = Nestmate relatedness based on regression analysis.
me_{regr} = effective number of matings based on regression analysis.

However, the greater uncertainty of regression relatedness estimates due to the chance occurrences of alleles shared by queens and their mates, or between mates, sometimes made these values different from the relatedness values obtained via pedigree analyses (Table 4). In turn, this caused estimates of effective paternity obtained from regression relatedness analyses to show greater variation among colonies than those based on pedigree analyses (Table 4).

Although some founding queens were collected in different years, we pooled the samples across years.

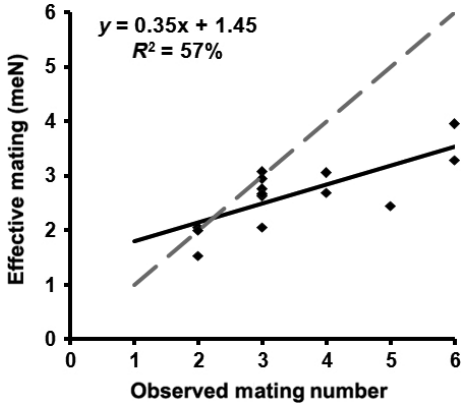

Fig. 2 Effective and observed mating frequencies were positively correlated for *Pheidole rhea*. A dashed line representing a perfect 1:1 relationship was shown for slope comparisons. The effective paternity used here was corrected for sample-size bias following Nielsen *et al*. (2003).

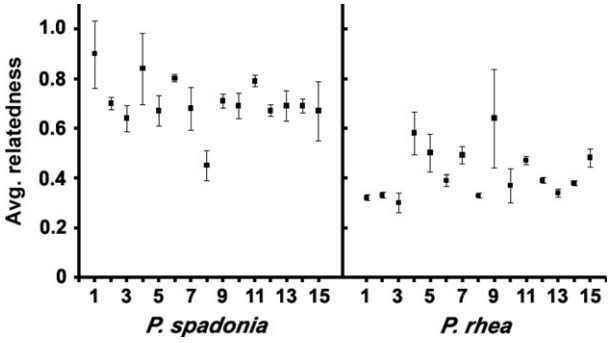

Fig. 3 Average nestmate relatedness for *Pheidole spadonia* (left) (15 colonies; 300 workers) and *P. rhea* (right) (15 colonies; 630 workers), with standard error bars (Jackknife/loci).

This was performed for the observed number of matings for both species (Table 4), because it did not differ significantly between yearly cohorts for either species (Kruskal–Wallis Test, *P. rhea*: $n_a = 6$, $n_b = 2$, $n_c = 6$, $n_d = 1$, $P = 0.728$; *P. spadonia*: $n_a = 5$, $n_b = 3$, $n_c = 2$, $n_d = 5$, $P = 0.749$). This was also carried out for the effective mating frequency for the polyandrous *P. rhea* because there was no significant difference between queens collected during different years (Kruskal–Wallis Test, $n_a = 6$, $n_b = 2$, $n_c = 6$, $n_d = 1$, $P = 0.80$).

Patriline bias in worker size

The partiline bias data were normally distributed after log transformation, and no significant difference in variance between patrilines was detected (Levene Test, d.f. = 39, $P = 0.051$). There were significant differences in the sizes of the workers produced by different patrilines after accounting for colony effects (Nested Multi-Factor ANOVA, log-transformed, $F_{25,571} = 2.159$, $P = 0.001$)

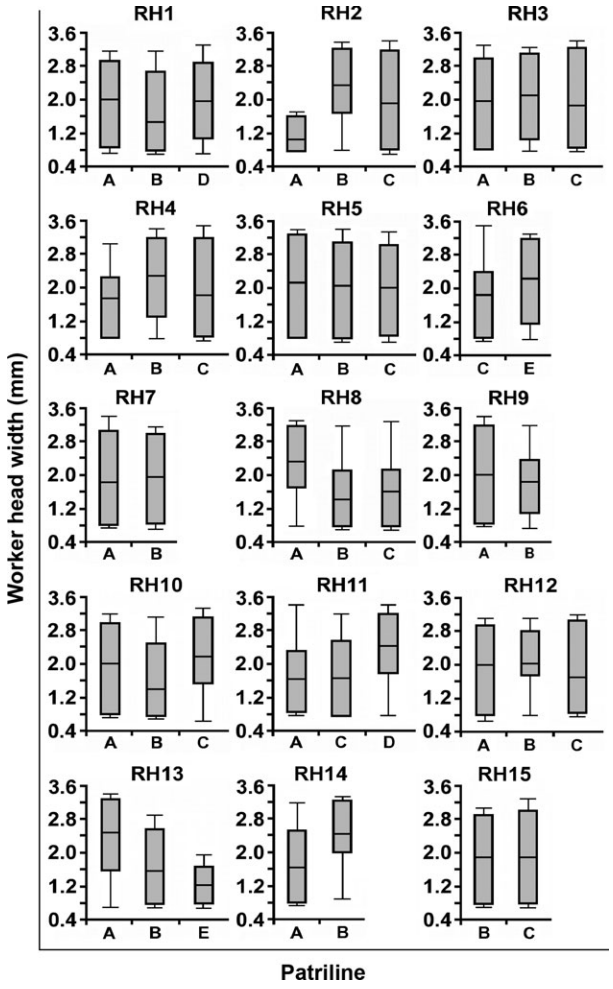

Fig. 4 Patriline contributions to different worker sizes for 15 colonies of *Pheidole rhea* (42 workers per colony) (nontransformed data are displayed). The average worker size is displayed as a horizontal line within each box plot for each patriline, which are noted as letters A–E. Note that the same letters between colonies do not represent the same patrilines. Also, patrilines that sired 3 or less total offspring are not displayed here because they are excluded in the statistical analysis due to low statistical power. Values within the grey box of the box plot represent the interquartile range (IQR, 25–75th percentile), and the whiskers represent 1.5 times IQR of the upper and lower quartiles. No outliers were present. A nested ANOVA test (*Patriline* nested within *Colony*) on log-transformed data show that there are significant differences between the patrilines in the sizes of workers they sired when colony effects are accounted for ($P = 0.001$).

(Fig. 4). In our statistical model, patriline A and B from colony RH2, patriline A from RH8, patriline B from RH10, patriline A from RH13 and patriline A in RH14 were the most different from the other patrilines in worker sizes sired ($P = 0.014$, 0.007, 0.012, 0.032, respectively); note that the same letters between colonies do not represent the same patrilines.

Discussion

Our comparison of mating systems showed that *Pheidole rhea*, which has a broad worker size range with two soldier castes, has significantly more mates per queen than *P. spadonia*, which is characterized by a narrow worker size range and only one soldier caste. These findings are consistent with the idea that mating systems and worker caste diversity co-evolve in social insects. *Pheidole* ants are invariably sterile (no functional ovaries) (Hölldobler & Wilson 1990; Khila & Abouheif 2010), and therefore our findings cannot result from worker policing differentially affecting the expression of developmental plasticity or the standing genetic variance of worker morphology in the two species. Instead, our results fit with the idea that multiple mating by queens may evolve in species when greater worker diversity brings benefits to colonies. Selection for such benefits will protect against the loss of existing genetic variance for size and shape in populations through genetic drift and result in an increase in the standing genetic variance for worker size diversity. Once multiple mating is installed in a species, this selection process may favour the evolution of lineages specializing in the production of even greater worker diversity (Fuchs & Moritz 1999; Fjerdingstad & Crozier 2006).

Our findings that the more worker diverse *P. rhea* has more mates per queen cannot be an artefact related to our genetic markers. Specifically, the results cannot arise from differences among species in the power to detect multiple paternity through offspring genotype analyses. For both species, the number of alleles at our two microsatellite DNA loci was high (≥ 13), observed heterozygosities were all greater than 80%, and nondetection errors (Pamilo 1993) were low (approximately 1%). For *P. spadonia*, the sample size of 20 genotyped workers per colony was adequate for detecting double-matings even in species with a high paternity skew (Pedersen & Boomsma 1999). The robustness of the sampling method was also supported by the fact that applying Nielsen *et al*. (2003) correction for sample-size bias did not change the effective paternity estimates for *P. spadonia* (Table 4). The finding that *P. spadonia* is almost exclusively monandrous is therefore reliable. It is equally well substantiated that *P. rhea* is highly polyandrous. Observed and effective paternities as well as sample-size bias-corrected values (following Nielsen *et al*. (2003) calculations) were very similar (Table 4), showing that our sample sizes of 42 workers per colony were suitable. Finally, nestmate relatedness based on pedigree inferences were generally close to the values based on regression relatedness in both species. This held although the latter showed more variance, which is expected as they are affected by chance sharing of

alleles between queens and their mates. Hence, the two independent ways of assessing the genetic structure of colonies gave consistent results, confirming the soundness of our findings.

Worker polymorphism and polyandry

Polyandry, and the genetic diversity it confers, is linked to worker polymorphism in *Pheidole* ants at two levels. First, higher mating frequency is associated with increased soldier size diversity. Such a relationship was apparent when we compare *P. spadonia* (one soldier morph, narrow worker caste size distribution) vs. the highly polymorphic *P. rhea*, which is consistent with the pattern found in other ants (Fjerdingstad & Crozier 2006). For example, army ants (*Eciton burchelli*) (Jaffé *et al.* 2007) and leafcutter ants (*Acromyrmex echinatior*) (Hughes *et al.* 2003) each have a broad range of worker sizes and have multiple matri- or patrilines (Fjerdingstad & Crozier 2006). Second, the effect of increased genetic diversity on worker polymorphism is reflected in different patrilines contributing unequally to different worker sizes. For the polyandrous *P. rhea*, this patriline bias is exhibited by some but not all colonies and many of the patrilines produced a broad range of worker sizes (Fig. 4), which suggests that this trait is plastic. Patriline bias in worker production has also been detected in other highly polymorphic ants in the genera *Acromyrmex* (Hughes *et al.* 2003), *Atta* (Evison & Hughes 2011), *Eciton* (Jaffé *et al.* 2007) and *Pogonomyrmex* (Rheindt *et al.* 2005). The presence of patriline bias in worker production may help highly polymorphic species effectively maintain the broad range of worker caste sizes present in colonies. Factors other than direct genetic variance of males have been found to be associated with patriline bias in caste determination. For example, variation in genetic compatibility between mates can affect the proportion of queen vs. worker offspring in *Pogonomyrmex rugosus* (Schwander & Keller 2008). In *Pogonomyrmex occidentalis*, apparent patriline biases are explained by variation in sperm usage by the queen over time (Wiernesz & Cole 2010). The extent to which genetic compatibility and shifting sperm usage contribute to patriline bias in *Pheidole rhea* remains unknown.

The degree of polyandry found in *P. rhea* (2–6 mates per queen, obligate polyandry) is similar to that of other ants with polymorphic workers [*Atta colombica* = 2–5 mates, avg. = 3 (Fjerdingstad *et al.* 1998, 2002); *Acromyrmex octospinosus* = 4–10 mates, avg. = 6 (Feener *et al.* 1988; Boomsma *et al.* 1999; Wetterer 1999)]. There are also extreme cases where highly polymorphic species have up to 17 mates per queen (avg. = 13) (*Eciton burchellii* (Franks 1985; Kronauer *et al.* 2006)) or up to 27 mates per queen (avg. = 11) [*Pogonomyrmex badius* (Tschinkel 1998; Rheindt *et al.* 2004)]. Collecting mating system data on additional soldier-polymorphic *Pheidole* species (*P. obtusospinosa*, *P. tepicana* and *P. polymorpha*) would also be valuable. This is particularly the case as these other soldier-polymorphic species do not form a monophyletic clade (Moreau 2008), which permits testing whether polyandry consistently evolves in this genus whenever high worker caste diversity occurs.

The genetic diversity resulting from polyandry can be related to benefits other than increasing worker polymorphism, such as reduced diploid male production and improved colony immunity (Crozier & Page 1985; Tarpy & Page 2002). It is possible that these benefits contribute additively to the fitness advantage provided by polyandry. Also, in species that produce split sex ratios, the queen can increase her fitness by mating multiply. Lower relatedness between nestmates due to polyandry removes the inclusive fitness advantage to workers for producing new gynes instead of males (Ratnieks & Boomsma 1995). In the case of our two *Pheidole* species, correlation between split sex ratios and mating number is unlikely as it has been shown that *Pheidole desertorum* has split sex ratios regardless of whether queens have mated once or twice (Helms 1999).

Monandry seems to be dominant in the dimorphic *Pheidole* species studied to date (percentage monandrous: *P. pallidula* = 100% (Fournier *et al.* 2002), *P. desertorum* = 99% (Helms 1999), *P. megacephala* = 87% (Fournier *et al.* 2009) and *P. spadonia* = 100% = this study) (see Table 1). It is unlikely that this link between monandry and low worker phenotypic diversity observed in the four dimorphic species above is due to phylogenetic constraints as they are distantly related on the *Pheidole* phylogeny (Moreau 2008). Polyandry has only been tentatively shown so far to occur in one dimorphic *Pheidole* species (*P. xerophila*) (Tripet *et al.* 2006), but the evidence is confounded by the potential co-occurrence of polygyny. This strong association between dimorphic species and monandry, however, does not imply that low genetic diversity in colonies is always associated with low worker diversity in species with sterile helpers. This is because a few of these species possess multiple breeding queens per colony (*P. pallidula* and *P. megacephala*) although this increase in genetic diversity via polygyny is apparently not linked to an increase in worker diversity (Fournier *et al.* 2002, 2009; see Table 1). No data are available thus far on the gyny-level for the dimorphic *P. spadonia* studied here. If *P. spadonia* should be found to be polygynous, it will not invalidate the idea that multiple mating evolves to recruit more genetic variance for worker size and shape into colonies (Fjerdingstad & Crozier 2006). Instead it would support that taxa where

workers do not need to be particularly diverse are monandrous and may be selected to exhibit polygyny for other reasons. Many such reasons have in fact been proposed and corroborated in studies [e.g. patchiness of resources, risks of queen loss during colony migration, sex ratio conflicts between queens and workers (Keller 1993)]. Although we found strong evidence for polyandry in the soldier-polymorphic *P. rhea*, which have high worker diversity, it is not yet certain whether polygyny also plays a role in increasing their colony genetic diversity. On one hand, polyandry and polygyny generally trade-off in social insects, as shown by comparative analyses (Hughes *et al*. 2008), which suggests that polygyny is less likely to occur in *P. rhea*. On the other hand, the presence of queen clusters of up to five individuals during early founding and the maintenance of numerous satellite colonies have been noted in the field (MH Huang, personal observation), which suggests that at least primary polygyny exists. Pedigree analyses of *P. rhea* field colonies need to be performed to determine how much of a role polygyny plays, if any, on colony genetic diversity and worker diversity. We are currently completing a genetic study on matriline and patriline effects on both *P. rhea* and *P. spadonia* field colonies (Huang & Fjerdingstad, in prep.).

Conclusions

In summary, the increase in genetic variance derived from multiple paternity cannot be tied to selection to enforce worker policing due to lack of worker ovaries in *Pheidole* species. For the same reason, the greater morphological diversity of workers in the polyandrous species cannot result from a release from selection to retain a reproductive morphology. Therefore, the increase in genetic and morphological diversity is likely due to colony level selection on benefits derived directly from having workers of multiple patrilines whose diversity enhances division of labour. Our detection of bias in the sizes of workers sired by different patrilines provides direct evidence that the increase in accumulation of colony genetic diversity via polyandry contributes to the diversity of worker castes. Comparative analyses that control for phylogenetic effects will permit further testing of how mating systems co-evolve with ecological characteristics likely to select for greater genetic and morphological diversity in colonies.

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The study was designed by M.H., E.J.F. and D.E.W. Sample collection, laboratory cultures, DNA extractions and PCRs were performed by M.H. Peak diagram reading and genetic analyses were carried out by E.J.F. M.H. and E.J.F. wrote the paper, D.E.W. discussed ideas and results and commented on the manuscript. M.H. and E.J.F. contributed equally to the work presented in this manuscript.

Data accessibility

Microsatellite data and head measurements (phenotypic data) are archived as supplemental online material. Two microsatellite loci (Ppal77 and Myrt3) are used to determine the pedigree of two *Pheidole* species (*P. rhea* and *P. spadonia*). Our primary data set includes samples that are used to analyse pedigree, nestmate relatedness and patriline bias (Fig. S1.xls, Supporting information). A second smaller data set is used for population genetics analyses (Fig. S2.xls, Supporting information).

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Dataset of worker genotypes used to analyze pedigree, nestmate relatedness, and patriline bias using microsatellite loci Myrt3 and Ppal77.

Fig. S2 Dataset of worker genotypes used for our population genetics analyses, using microsatellite loci Myrt3 and Ppal77.