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MECHANISMS OF REPRODUCTIVE ISOLATION BETWEEN AN ANT SPECIES OF HYBRID ORIGIN AND ONE OF ITS PARENTS

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The establishment of new species by hybridization is difficult because it requires the development of reproductive isolation (RI) in sympatry to escape the homogenizing effects of gene flow from the parental species. Here we investigated the role of two pre- and two postzygotic mechanisms of RI in a system comprising two interdependent *Pogonomyrmex* harvester ant lineages (the H1 and H2 lineages) of hybrid origin and one of their parental species (*P. rugosus*). Similar to most other ants, *P. rugosus* is characterized by an environmental system of caste determination with female brood developing either into queens or workers depending on nongenetic factors. By contrast, there is a strong genetic component to caste determination in the H1 and H2 lineages because the developmental fate of female brood depends on the genetic origin of the parents, with interlineage eggs developing into workers and intralineage eggs developing into queens. The study of a mixed mating aggregation revealed strong differences in mating flight timing between *P. rugosus* and the two lineages as a first mechanism of RI. A second important prezygotic mechanism was assortative mating. Laboratory experiments also provided support for one of the two investigated mechanisms of postzygotic isolation. The majority of offspring produced from the few matings between *P. rugosus* and the lineages aborted at the egg stage. This hybrid inviability was under maternal influence, with hybrids produced by *P. rugosus* queens being always inviable whereas a small proportion of H2 lineage queens produced large numbers of adult hybrid offspring. Finally, we found no evidence that genetic caste determination acted as a second postzygotic mechanism reducing gene flow between *P. rugosus* and the H lineages. The few viable *P. rugosus*-H hybrids were not preferentially shunted into functionally sterile workers but developed into both workers and queens. Overall, these results reveal that the nearly complete (99.5%) RI between *P. rugosus* and the two hybrid lineages stems from the combination of two typical prezygotic mechanisms (mating time divergence and assortative mating) and one postzygotic mechanism (hybrid inviability).

KEY WORDS: **Hybrid speciation, hybrid inviability, gene flow, genetic caste determination, *Pogonomyrmex*.**

Hybrid speciation refers to the establishment of novel hybrid lineages that are reproductively isolated from their parental species. The establishment of such hybrid species is difficult because it requires the development of reproductive isolation (RI) in sympatry to escape the homogenizing effects of gene flow from parental species (Rieseberg 1997; Buerkle et al. 2000; Schwarz et al. 2005; Gompert et al. 2006; Mavarez et al. 2006). It is thus not surprising that the majority of documented cases of hybrid speciation

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involve mechanisms such as chromosomal rearrangements or polyploidization which directly interfere with meiosis in F1 hybrids (e.g., Rieseberg et al. 1995; Rieseberg 1997; Chapman and Burke 2007). When the admixture of divergent genomes has no negative effects on meiosis in hybrids, genetic and ecological factors must drastically reduce gene flow between the parental species and the hybrid lineage for the hybrid to become reproductively isolated (Schwarz et al. 2005; Gompert et al. 2006; Mavarez et al. 2006).

An unusual case of hybrid speciation has been described in harvester ants of the genus *Pogonomyrmex*. Historical hybridization between *P. barbatus* and *P. rugosus* resulted in genetically distinct and mutually interdependent lineages, which always co-occur as a pair at a given location (Helms Cahan and Keller 2003; Schwander et al. 2007). Queens of both lineages are polyandrous and mate with males of their own and of the alternate lineage; offspring fathered by males of the alternate lineage (inter-lineage females) develop into workers, whereas offspring fathered by males of the same lineage (pure-lineage females) develop into queens (Helms Cahan et al. 2002; Julian et al. 2002; Volny and Gordon 2002b; Helms Cahan and Keller 2003). In the parental species, females can develop either into workers or queens independently of the females' genotype (Helms Cahan et al. 2002; Julian et al. 2002).

Although the lineages still show clear genetic signatures of their historical hybrid origin, there is no current genetic exchange between them and their two parental species, *P. rugosus* and *P. barbatus* (Helms Cahan and Keller 2003; Anderson et al. 2006; Schwander et al. 2007). RI is even complete at sites where the lineages co-occur in sympatry with either of the parental species (Anderson et al. 2006; Helms Cahan et al. 2006; Schwander et al. 2007), but the mechanisms that prevent gene flow between the parental species and the lineages remain elusive (Helms Cahan et al. 2006).

The aim of the present study is to identify mechanisms of RI between the parental species and the hybrid lineages. We focus on RI between *P. rugosus* and the H1 and H2 lineages [labeled according to the site in which they were first described (Helms Cahan and Keller 2003)] because there is a well-studied population in southeast Arizona where the three groups occur in sympatry (Helms Cahan et al. 2006). We tested four mechanisms that might explain the lack of gene flow between *P. rugosus* and the two H lineages and quantified the relative contribution of each mechanism to the total RI between the two groups. First, we assessed whether the timing for the nuptial flight differs between *P. rugosus* and the H lineages. Distinct flight timing is considered an important mechanism for prezygotic isolation in ants (Hölldobler and Wilson 1990). Second, we tested if matings are assortative within a mixed mating aggregation. Third, we measured the viability of *P. rugosus*-H hybrids by two different approaches. For one approach, we created reciprocal *P. rugosus*-H crosses through controlled matings in the field to test whether *P. rugosus*-H hybrid eggs can develop into adults. For the other approach, we used microsatellite analyses to identify *P. rugosus*-H hybrids in eggs and adult offspring produced by naturally mated queens collected from a mixed mating aggregation. Finally, we investigated whether gene flow may be reduced because *P. rugosus*-H hybrids are preferentially shunted into the worker caste rather than the queen caste (Helms Cahan et al. 2006). Because workers in *Pogonomyrmex* are functionally sterile, shunting of *P. rugosus*-H hybrids into the worker caste would result in the RI of *P. rugosus* and the H lineages and suggest genetic caste determination as a mechanism favoring speciation. We tested this by comparing the overall proportion of *P. rugosus*-H hybrids among workers to that among daughter queens.

Methods

In *Pogonomyrmex* ants, matings typically occur in large mating aggregations comprising thousands of males and females (Hölldobler 1976). To investigate the mechanisms preventing gene flow between *P. rugosus* and the H lineages, we studied a single mating aggregation at site "PC," located at the upper end of the San Simon Valley, Cochise County, Arizona (N32°17.555/W109°20.235) on 16 July 2004. A previous census of adult colonies at this site revealed that 68% of the colonies are headed by a *P. rugosus* queen, 3% by a H1 queen and 29% by a H2 queen, but the proportions can be variable at a microgeographic scale due to significant clustering of colonies of a given type (Helms Cahan et al. 2006). We divided the duration of the mating aggregation into five time intervals of 30 min each, from the beginning of the mating aggregation (1745 h) through dusk (2015 h). During each of the five time intervals we collected three types of samples: males and females randomly collected from the mating aggregation (290–376 per time interval), pairs collected in copula (50–61 pairs per interval), and mated queens walking on the soil surface in the vicinity of the swarm site after they dealated (33–88 queens per interval). The wingless queens were brought to the laboratory and raised under standard conditions (Schwander et al. 2006) whereas individuals from the two other samples were frozen at −20°C and stored in 75% ethanol until further analyses. For all individuals, we determined whether they belonged to *P. rugosus*, H1, or H2 by analyzing a 430-bp portion of the mitochondrial sequence COI as described in Helms Cahan et al. (2006) and/or by microsatellite genotyping at six loci informative for distinguishing the three groups [L-18 from Foitzik et al. (1997), Myrt-3 from Evans (1993), Pb-5 and Pb-7 from Volny and Gordon (2002a), Pr-1 from Gadau et al. (2003) and PO-8 from Wiernasz et al. (2004), Helms Cahan et al. (2006); Schwander et al. (2007)]. To avoid sacrificing

mated queens, we extracted DNA from a piece of the mid hind leg.

To test whether flight timing differed between *P. rugosus* and the H1 and H2 lineages, we ranked individuals from the random samples according to the time interval (1–5) during which they were collected. We then tested for differences in mean rank between *P. rugosus*, H1, and H2 separately for males and females using Kruskal–Wallis tests and appropriate post-hoc comparisons.

To test whether males and females of *P. rugosus* and of the two H lineages mate assortatively, we first estimated the expected proportion (p_e) of matings between *P. rugosus* and the H lineages for each time interval under the assumption of random mating. This proportion is given by $p_e = f_{rug} \times (m_{H1} + m_{H2}) + m_{rug} \times (f_{H1} + f_{H2})$ where f_{rug}, f_{H1}, and f_{H2} are the proportions of females of *P. rugosus*, H1 and H2, respectively and m_{rug}, m_{H1} and m_{H2} are the proportions of males of *P. rugosus*, H1 and H2, respectively. The proportions of males and females of the three groups were estimated from the random samples collected from the mating aggregation. We then compared the expected and observed proportion of copulations between *P. rugosus* and the H lineages across the five time intervals.

The level of *P. rugosus*-H hybrid viability was estimated by two different means. First, we crossed *P. rugosus* and the H lineages under controlled conditions to test whether *P. rugosus*-H hybrid eggs can develop into adults. For logistic reasons, these crosses were performed both at PC and at a second site located in El Paso, TX where *P. rugosus* also occurs in sympatry with the H1 and H2 lineages (site "AH"; [Schwander et al. 2007]). The protocol used at both sites was identical. To elicit mating flights, we watered colonies with 25 L of water late in the afternoon and again with 25 L on the following morning. Colonies from which sexuals did not emerge were watered again the following day. We captured males and females in traps consisting of conical wire mesh as they flew out of their mother colony during the late afternoon. When both sexes were captured from a single colony they were separated immediately to avoid inbreeding. We next released males and females from pairs of different colonies into 50 × 30 cm buckets with a ca 4:1 male biased sex ratio. We used individuals from 15 colonies to conduct these mating experiments. As soon as they separated from their mate, queens were transferred to glass vials and kept under standard laboratory conditions to allow them to initiate new colonies. Their insemination status was verified at the end of the experiment by dissecting the spermatheca and checking whether it contained sperm. Unmated females (8 of 62, 13%) were excluded from analyses. The remaining 54 females comprised 31 *P. rugosus* queens mated to a *P. rugosus* male (nine different colonies), 11 *P. rugosus* queens mated to a H2 male (seven different colonies), four H1 and four H2 queens mated to a *P. rugosus* male and four H2 queens mated to a H1 male (three different colonies; see also Table 2). Females of the H lineages were crossed only with a male of the alternate H lineage as within-lineage crosses produce only queen-destined eggs and virtually no workers (Helms Cahan et al. 2004). For each queen, we noted the most advanced developmental stage reached by her offspring twice per week during the 10 weeks following mating.

Second, we determined the fate of *P. rugosus-H* hybrid offspring of naturally mated queens collected from the mating aggregation at PC. Because *Pogonomyrmex* queens mate multiple times (Hölldobler 1976; Gadau et al. 2003), naturally mated queens should mate with both conspecific and heterospecific males. We used microsatellite genotyping to identify *P. rugosus*-H hybrids in the offspring of the naturally mated queens. We selected queens collected at the beginning and end of the mating aggregation because this is when the proportions of *P. rugosus* and the H lineages were most unbalanced (see Results). We did not include H1 queens for these experiments because they were too rare overall (only two H1 wingless queens were collected during the first time interval and none during the last time interval of the mating aggregation). Of the 121 dealate *P. rugosus* queens collected from the mating aggregation, we used all seven *P. rugosus* queens sampled during the first time interval and eight *P. rugosus* queens of the second time interval. Of the 199 wingless H2 queens collected from the mating aggregation, we used 15 H2 queens sampled during the last time interval of the mating aggregation. To identify *P. rugosus*-H hybrids among the offspring of each of these 30 queens, we genotyped 20 eggs and 20 workers at the six microsatellite loci and used the population assignment software Structure 2.1 (Pritchard et al. 2000) to identify *P. rugosus*-H2 and *P. rugosus*-H1 hybrids [see Helms Cahan et al. (2006) for details].

When genotyping the offspring of naturally mated queens we found that four of the 15 H2 queens sampled during the last time interval of the mating aggregation produced adult *P. rugosus*-H offspring (see Results). To obtain an overall estimation of the proportion of H2 queens producing viable *P. rugosus*-H hybrid offspring we used the 15 H2 colonies founded from queens collected during the last time interval and genotyped 374 additional workers from the 184 remaining colonies headed by H2 queens collected from the mating aggregation during all time intervals ($n_{tot} = 199$ colonies).

Although we observed that up to 16% *P. rugosus* queens in the mating aggregation mated with males of the H-lineages (see Results, Table 1), we detected no *P. rugosus*-H hybrids eggs or workers among offspring produced by *P. rugosus* queens. Because a previous study revealed that DNA from eggs that abort during their development cannot be successfully amplified (Schwander et al. 2006), we tested whether *P. rugosus*-H hybrid eggs could be detected by microsatellite genotyping, by measuring the amplification success of eggs from the controlled crosses (using

Table 1. Sample sizes for mating pairs collected from the mating aggregation. Pobs, observed proportion of matings between individuals of *P. rugosus* (Rug) and the H lineages (H); Pexp, expected proportion between individuals of *P. rugosus* and the H lineages under random mating and given the sex-specific proportions of each group in the mating aggregation.

			Intraspecific mating pairs			Interspecific mating pairs				
Interval	Time	Total	Total	Female Rug	Female H	Total	Female Rug	Female H	Pobs	Pexp
1	17.15	51	51	0	51	0	0	0	0.00	0.10
2	17.45	61	59	16	43	2	1	1	0.03	0.33
3	18.15	58	50	19	31	8	2	6	0.14	0.44
4	18.45	54	39	21	18	15	7	8	0.28	0.51
5	19.15	50	40	31	9	10	6	4	0.20	0.49

six markers allowing species identification). The genotyping of 24 eggs (1 to 3 eggs per queen) from the crosses *between* the H lineages and *P. rugosus* and 24 eggs (1 to 3 eggs per queen) from crosses *within* the H lineages and *within P. rugosus* revealed that *P. rugosus*-H hybrid eggs had a very low amplification success (0 eggs out of 24) whereas the amplification success for eggs from within species crosses was 91.7% (22 out of 24; Fisher's exact test $P < 0.0001$). Nonamplifying eggs laid by *P. rugosus* queens were thus considered as *P. rugosus*-H hybrids for further analyses.

To test for a preferential shunting of *P. rugosus*-H hybrids into the worker or queen caste, we raised the 320 colonies founded by naturally mated H2 (199 colonies) and *P. rugosus* queens (121 colonies) for over two years in the laboratory. Laboratory colonies occasionally produce a small number of daughter queens along with workers, which allowed us to collect a total of 86 *P. rugosus* daughter queens (produced by 27 out of the 121 *P. rugosus* colonies) and 13 H2 daughter queens (produced by 6 out of the 199 H2 colonies). We used these queens to compare the proportion of colonies producing *P. rugosus*-H hybrid queens to the proportion of colonies producing *P. rugosus*-H workers separately for *P. rugosus* and H2 colonies. To estimate the proportion of colonies producing hybrid workers, we used all colonies from the previous analyses (199 H2 colonies and 15 *P. rugosus* colonies) with additional genotyping of 200 *P. rugosus* workers from 10 colonies for a total of 25 *P. rugosus* colonies. Queen and worker hybrids were identified as described above by analyzing their genotypes at the six microsatellite markers with the software Structure 2.1 (Pritchard et al. 2000).

We found that three of the four investigated mechanisms contributed significantly to RI between *P. rugosus* and the H-lineages (see Results). To quantify the contribution of each of the three mechanisms to total RI we applied a method proposed by Coyne and Orr (1989) and extended by Ramsey et al. (2003). This method quantifies the cumulative RI between two groups as a multiplicative function of the individual components of RI at sequential stages in the life history. RI-values specify the strength of RI for a given pre- or postzygotic barrier, and generally vary between zero and one. A given reproductive barrier eliminates gene flow that has not already been prevented by previous stages of RI. The absolute contribution (AC) of a component of RI at stage n is calculated as [for further details see Ramsey et al. (2003)]

$$ACn = RIn\left(1 - \sum_{i=1}^{n-1} ACi\right)$$

The RI-value for differences in mating flight timing between *P. rugosus* and the H-lineages was computed as the expected proportion of *P. rugosus*-H matings given the observed timing difference, divided by the expected proportion of *P. rugosus*-H matings without a timing difference (assuming random mating in each case). The first value was calculated by averaging the expected proportion of *P. rugosus*-H matings over the five time intervals. The second was calculated as $2(p_{Rug})(1 - p_{Rug})$ whereby p_{Rug} is the overall proportion of *P. rugosus* individuals in the mating flight. The RI-value for assortative mating was computed as the observed proportion of *P. rugosus*-H matings (averaged over time intervals) divided by the expected proportion given the observed timing difference (see above). As an estimate for RI through hybrid inviability, we used the proportion of colonies producing viable hybrids. Because hybrid inviability differed between offspring produced by *P. rugosus* queens and the H-lineage queens (see Results), we computed it independently for each group as well as for both combined.

Results

The mating aggregation at PC was composed of individuals belonging to *P. rugosus* (31%), the H1 lineage (11%), and H2 lineage (58%) with an approximately 3:1 male-biased sex ratio (74% males). The shift in the relative proportions of the three groups over the five time intervals revealed a difference in flight timing between *P. rugosus* and the H1 and H2 lineages. *Pogonomyrmex rugosus* males and females appeared significantly later in the mating aggregation than males and females of the H1 and H2 lineages (Kruskal–Wallis tests; males: $\chi^2_2 = 188.7$, $P < 0.0001$, post hoc tests both $P < 0.0001$; females: $\chi^2_2 = 98.2$, $P < 0.0001$, post

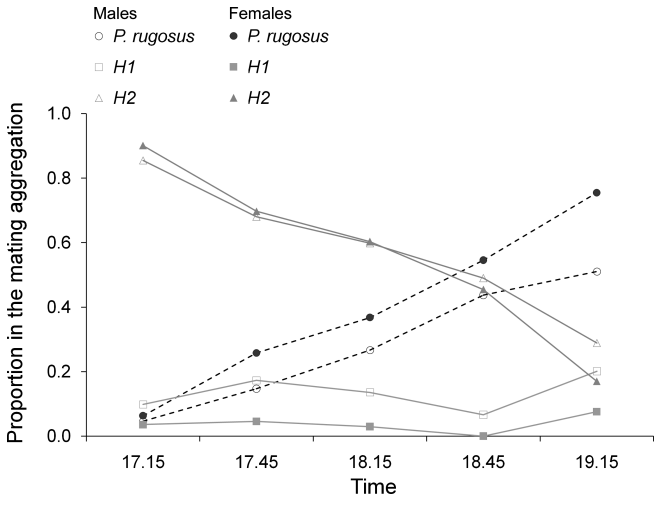

Figure 1. Proportion over time of randomly collected males and females of the H lineages and of *P. rugosus* in a mating aggregation at PC. For the proportions, each sex is considered separately; the overall sex ratio was male biased (3:1).

hoc tests both $P < 0.05$; Fig. 1). At the beginning of the mating aggregation, the majority of males (85%) and females (90%) were of the H2 lineage. Their frequency decreased over time and during the last time interval of the mating aggregation, H2 females (17%) and males (29%) were less frequent than *P. rugosus* females (75%) and males (51%, Fig. 1). H1 males (13% overall) and females (3% overall) were rare throughout the five time intervals of the mating aggregation. Similarly, the proportion of *P. rugosus*, H1, and H2 wingless queens ($n = 431$) was also very unbalanced with 173 (40%) *P. rugosus* queens, 240 (56%) H2 queens, and only 18 (4%) H1 queens. Because of the small sample size for individuals (especially females) of the H1 lineage we pooled data of the H1 and H2 individuals (thereafter H lineages) for further statistical analyses. Assuming random mating, the observed difference in flight timing between *P. rugosus* and the H-lineages would result in 35.7% *P. rugosus-H* matings across the five time intervals whereas 42.5% would be expected if the two groups had simultaneous mating flights.

The proportions of *P. rugosus* and H lineages males and females collected in copula showed that 12.8% of all collected pairs were between *P. rugosus* and the H lineages. Copulations between *P. rugosus* and the H lineages were significantly rarer than expected under random mating (χ^2_4: 47.21, $P < 0.0001$) with a 10–30% deficit of *P. rugosus*-H lineage copulations in each of the five time intervals of the mating aggregation (Table 1). Both reciprocal matings occurred at similar frequencies; *P. rugosus* female were found in copula with a male of the H lineages (16 out of 35 copulas; 45.7%) and queens of the H lineages were found in copula with a *P. rugosus* male (19 out of 25 copulas; 54.3%, binomial test $P = 0.73$; Table 1).

Table 2. Proportion of queens from controlled crosses producing adult worker offspring. The numbers in brackets indicate the number of colonies from which males and queens were collected and the total number of singly mated queens per cross.

Female \ Male	*P. rugosus*	H lineages
P. rugosus	94% (9, 31)	0% (7, 11)
H lineages	0% (7, 8)	100% (3, 4)

The controlled reciprocal *P. rugosus*-H crosses and the analysis of offspring of naturally mated queens revealed an extremely low viability of *P. rugosus*-H hybrids. In the controlled crosses, none of the 11 *P. rugosus* queens mated to a H2 male and none of the four H1 and four H2 queens mated to a *P. rugosus* male raised any larvae or workers. Although all 19 queens laid eggs (14.7 $\pm$ 8.5 eggs per queen), none of these eggs hatched. In contrast, *P. rugosus* queens mated to a *P. rugosus* male and H2 queens mated to an H1 male were significantly more successful in raising workers than queens from the crosses between *P. rugosus* and the H lineages (Fisher's exact test, $P < 0.0001$; Table 2). Twenty-nine of the 31 *P. rugosus* queens mated with a *P. rugosus* male and all four H2 queens mated with a H1 male produced adult worker offspring.

The analysis of offspring from the 15 naturally mated *P. rugosus* queens from the first time interval of the mating aggregation revealed that their *P. rugosus*-H hybrid eggs also aborted. The proportion of *P. rugosus* females in copula with a male of the H lineages was 6% in the second time interval (1 out of 16, Table 1) predicting at least 6% *P. rugosus*-H hybrid eggs in colonies headed by *P. rugosus* queens from the first and second time interval (no *P. rugosus* queens were collected in copula during the first time interval). Given the complete failure of amplification for *P. rugosus-H* hybrid eggs from the controlled crosses, we thus expected at least 6% amplification failure for eggs laid by *P. rugosus* queens. Of the 300 analyzed eggs laid by 15 of these queens, 34 (11.2%) failed to amplify and none (0%) of the remaining 266 eggs had a *P. rugosus*-H hybrid genotype. The proportion of nonamplifying eggs laid by the naturally mated *P. rugosus* queens did not differ significantly from the expected proportion of hybrid eggs (Fisher's exact test: $P = 0.99$). In line with the complete lack of *P. rugosus-H* hybrid genotypes among viable eggs from *P. rugosus* queens, we also did not find a single *P. rugosus*-H hybrid worker of the 300 workers (15 colonies) genotyped.

Most H2 queens also failed to produce *P. rugosus*-H hybrid eggs detectable by genotyping; however, a small number of H2 queens produced hybrid eggs and workers. Four of the 15 H2 queens collected during the last time interval from the mating aggregation produced almost exclusively hybrid workers (95–100%). One additional queen had a single *P. rugosus*-H hybrid

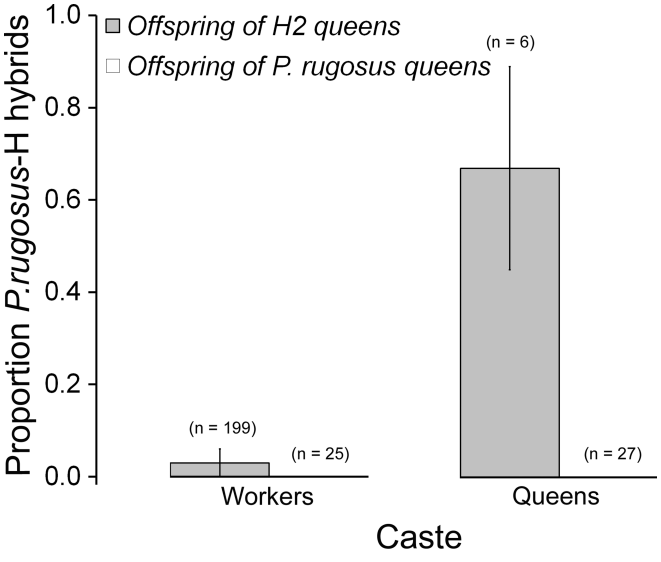

Figure 2. Overall proportion and binomial SD of *P. rugosus*-H hybrids in worker offspring and the proportion of *P. rugosus*-H hybrid queens for the subset of colonies that produced new queens. The numbers indicate the number of colonies used to estimate each proportion.

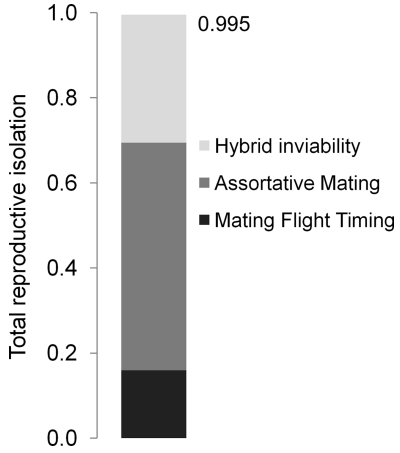

Figure 3. Total reproductive isolation (99.5%) between *P. rugosus* and the H-lineages decomposed into pre- and postzygotic components following the method described by Ramsey et al. (2003).

egg, while the remaining 11 queens had no hybrid eggs or workers at all. The abortion of *P. rugosus*-H hybrid eggs in the 10 colonies without any *P. rugosus*-H genotypes was revealed by significantly lower egg-amplification success than in the five colonies with hybrid eggs and/or workers ($W = 38$, $p = 0.045$). Genotyping of 374 workers produced by the remaining 184 H2 queens from the mating aggregation showed that overall 3% of H2 queens produced adult *P. rugosus*-H workers (four out of the 15 queens from the last time interval of the mating aggregation and two out of the remaining 184 queens).

There was no evidence for a preferential shunting of *P. rugosus*-H hybrids into workers rather than queens in colonies with a H2 mother queen. To the contrary, only 3% (six out of 199) of these colonies produced *P. rugosus*-H hybrid workers whereas among the H2 colonies that produced new queens ($n = 6$), 67% produced *P. rugosus*-H hybrid queens (Fisher's exact test $P = 0.0004$; Fig. 2). This difference stems from H2 colonies with *P. rugosus*-H hybrid workers being more likely (4 out of 6, 67%) to produce new queens (with a *P. rugosus*-H hybrid genotype) than colonies without *P. rugosus*-H hybrid workers (2 out of 193 colonies, 1%; Fisher's exact test $P < 0.0001$). We could not test for a preferential shunting of *P. rugosus*-H hybrids into workers rather than queens in *P. rugosus* colonies because none of these colonies produced *P. rugosus*-H hybrid workers (out of the 500 workers from 25 colonies genotyped) or queens (out of 86 queens from 27 colonies).

The quantification of the contribution of the addressed reproductive barriers revealed that prezygotic mechanisms explained almost 70% of the observed RI between *P. rugosus* and the H-lineages (Fig. 3). The difference in mating flight timing reduced the opportunities for interspecific matings by 15.9% as compared to simultaneous flight times. Assortative mating reduced interspecific crosses by additional 53.5%. The combination of these two prezygotic mechanisms with the high level of observed hybrid inviability (100% in *P. rugosus* offspring and 95% in H-lineage offspring, accounting overall for 30.1% of RI) resulted in nearly complete RI between *P. rugosus* and the H-lineages (99.5%; Fig. 3).

Discussion

We investigated four different possible mechanisms of RI between the H lineages of hybrid origin and one of their parental species, *P. rugosus*. We found that three of the four mechanisms, two prezygotic (differences in timing for the mating aggregation and assortative mating) and one postzygotic (hybrid inviability), strongly reduced gene flow between *P. rugosus* and the H lineages, whereas there was no evidence for the direct contribution of the second postzygotic mechanism (genetic caste determination).

Differences in mating flight timing between sister species is a typical pattern for contact zones in ants (Hölldobler and Wilson 1990). However, although males and females of the H lineages were present in the mating aggregation at site PC before *P. rugosus* males and females (Fig. 1) and this timing difference reduced interspecific mating opportunities by approximately 16% (Fig. 3), it is unlikely to have evolved as a mechanism of reinforcement. Observations of mating aggregations at sites comprising only *P. rugosus* or the H lineages revealed similar timing differences. Mating aggregations of the H lineages in Hidalgo County, NM [site H in Helms Cahan et al. (2006)] typically start around 16 h ($n = 3$ mating aggregations) whereas *P. rugosus* mating aggregations in Bowie, AZ [site B in Helms Cahan et al. (2006)]

start around 18h ($n = 2$). These patterns thus suggest that different flight timing of *P. rugosus* and the H lineages has evolved in allopatry, and that *P. rugosus* and the H lineages exhibited different circadian rhythms when they came into secondary contact.

Preferential mating among males and females of *P. rugosus* and among males and females of the H lineages acts as a second prezygotic mechanism for RI (Table 1) and accounted for the largest part of RI between the two groups (Fig. 3). Such assortative mating may be driven by both sexes that could use pheromones or hydrocarbon patterns to distinguish between con- and heterospecific mates. Pheromones are of primary importance for synchronization of nuptial flights and mate attraction in various ant species (e.g., Cherix et al. 1993; Buschinger 2003; Greenberg et al. 2004) and cuticular hydrocarbons are used as recognition cues to determine species and colony membership (e.g., Lahav et al. 1999; Wagner et al. 2000). Two genetic caste determining lineages not considered in this study (J1-J2 lineages) display lineage-specific hydrocarbon patterns in males, which could be used as cues for species discrimination (Volny et al. 2006). Alternatively, differences in male body size and shape, shown to influence male mating success in *P. occidentalis* (Abell et al. 1999) may also play a role in assortative mating. Interestingly, high levels of assortative mating are also critical in reducing gene flow between incipient hybrid species and their parents in *Heliconius* butterflies (Mavarez et al. 2006). Contrary to the majority of described animal hybrid species (Mallet 2007), *Heliconius* and *Pogonomyrmex* hybrids occupy similar habitats as their parents. In the absence of strong habitat divergence, mating cues that are more similar among hybrids than between hybrids and the parental species from the very first hybrid generation, may be fundamental for reticulate speciation.

In addition to the two prezygotic mechanisms, a high level of hybrid inviability also contributes to the RI between *P. rugosus* and the H1-H2 lineages. Hybrid inviability was expressed very early during the development as revealed by the controlled *P. rugosus*-H lineages crosses invariably resulting in inviable eggs. The vast majority of *P. rugosus*-H hybrid offspring of naturally mated queens also appeared to abort at the egg stage. Six to 25% of *P. rugosus* queens mated with a male of the H-lineages depending on the time of the mating aggregation (Table 1). These matings resulted in the production of inviable eggs, as indicated by the complete lack of *P. rugosus*-H hybrid genotypes and the amplification failure for many eggs laid by naturally mated queens. The majority of naturally mated H2 queens (97%) also failed to produce viable hybrids and only 3% of them produced significant numbers of *P. rugosus*–H hybrid eggs and workers.

The low viability of *P. rugosus*-H hybrids is somewhat surprising because analyses of sequence divergence suggest a relatively recent separation between the lineages and their parents, *P. rugosus* and *P. barbatus*. Both H-lineages have mitochondrial sequences derived from *P. barbatus* (Anderson et al. 2006; Schwander et al. 2007). The observed level of sequence divergence between *P. barbatus* from Texas populations and the H-lineages is 5.4–5.6% (Anderson et al. 2006) which, assuming the conventional insect divergence rate (DeSalle et al. 1987), suggests that the H-lineages originated approximately 2.8 million years ago. This divergence time is likely to be overestimated as the *P. barbatus* populations from Texas are probably highly differentiated from the parental source populations (Schwander et al. 2007). Indeed, *P. barbatus* mitochondrial sequences from Mexico would instead suggest 1.3–1.4 million years of divergence (2.6–2.7% sequence divergence) between *P. barbatus* and the H lineages calculated from (Anderson et al. 2006). The minimum time for total hybrid inviabililty to evolve has been estimated 2 million years in *Drosophila* (Coyne and Orr 1997), 4 million years in Lepidoptera (Presgraves 2002), 5.5 million years in birds (Lijtmaer et al. 2003) and even over 20 million years in Centrarchid fish (Bolnick and Near 2005). An important mechanism for hybrid nonviablility and postzygotic isolation between two different species are deleterious epistatic interactions between a recessive allele on a sex chromosome and an autosomal locus (Coyne and Orr 2004). Such a mechanism does not apply for ants and other Hymenoptera because of the haplo–diploid sex determination mechanism (there are no sexual chromosomes). Further studies are thus needed to investigate why hybrid inviability has evolved so rapidly in *Pogonomyrmex*.

The fact that some H2 queens but no *P. rugosus* queens produced adult *P. rugosus*-H hybrids suggests a maternal effect on hybrid viability. Additional support for this view comes from anecdotal data of a previous field study (Helms Cahan et al. 2006). Two colonies at a site comprising almost exclusively *P. rugosus* colonies [site "F" in Helms Cahan et al. (2006)] were headed by an F1 *P. rugosus*-H hybrid queen; both had a mitochondrial haplotype typical for the H2 lineage, suggesting they were offspring of a H2 queen mated to a *P. rugosus* male. Viability asymmetries depending on which species is the female (or male) parent have been documented in a wide range of organisms, including *Drosophila* and various plants (Wu and Davis 1993; Tiffin et al. 2001). The asymmetrical viability of reciprocal hybrids argues for a major role of interactions between haploid (e.g., mitochondrial loci) and diploid genes, or cytonuclear interactions in postzygotic isolation.

We found no evidence for genetic caste determination as a second, postzygotic mechanism reducing gene flow between *P. rugosus* and H lineages as *P. rugosus*-H hybrids were not shunted specifically into the worker caste. *Pogonomyrmex rugosus*-H hybrids that survived the egg stage appeared to be phenotypically plastic. When considering all H2 colonies, the proportion of *P. rugosus*-H hybrid daughter queens was even significantly larger than the proportion of hybrid workers. The higher proportion of *P. rugosus*-H hybrid queens than workers stems from the fact that colonies with *P. rugosus*-H hybrid individuals were more likely to produce queens (with *P. rugosus*-H hybrid genotypes)

than colonies without *P. rugosus*-H hybrids. Because queens were also produced in a larger proportion in *P. rugosus* colonies (22%, 27 out of 121) than H colonies (3%, 6 out of 199), these results suggest that caste determination in *P. rugosus*-H hybrids might be nongenetic, hence resulting in the production of a small number of daughter queens in the laboratory.

The measured reproductive barriers are sufficient to cause complete or nearly complete isolation between *P. rugosus* and the H-lineages (Fig. 3). Although introgression through backcrossing can occur even when F1 hybrids are rare (e.g., Cruzan and Arnold 1993; Rieseberg 1997; Arnold et al. 1999), the opportunity for introgressive hybridization in these two groups is severely limited by both pre-and postzygotic barriers. This nearly complete RI is in accordance with extremely high levels of genetic isolation revealed in previous studies (Helms Cahan and Keller 2003; Anderson et al. 2006; Schwander et al. 2007) and may contribute to the maintenance of variation in the caste determination mechanisms in *Pogonomyrmex*.

In conclusion, this study shows that differences in flight timing, assortative mating, and relatively high levels of hybrid nonviability all contribute to the nearly complete RI between the H lineages and their parent *P. rugosus*. *Pogonomyrmex rugosus*-H hybrids are not shunted preferentially into the worker caste so that caste determination does not play a direct role in the current isolation between the environmental caste determining species *P. rugosus* and the lineages with genetic caste determination. The current RI between *P. rugosus* and the lineages can thus be explained by a combination of two typical prezygotic and one postzygotic mechanism.

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