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# Supporting Online Material for

### **Ancestral Developmental Potential Facilitates Parallel Evolution in Ants**

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### SUPPORTING ONLINE MATERIAL

### **Materials and Methods**

*Animal collection and culturing.* We collected queen-right colonies of *P. morrisi*, *P. tysoni*, *P. pilifera* from Long Island, New York, USA, and *P. rhea*, *P. obtusospinosa*, *P. hyatti*, *P. spadonia*, and *P. vallicola* from the southwest of Arizona, USA, and *P. dentata*, *P. megacephala*, and *P. moerens*, from Tallahassee, Gainesville, and Fort Lauderdale, Florida, USA. Colonies were maintained in plastic boxes with glass test tubes filled with water constrained by cotton wool, and were fed a combination of mealworms, cockroaches, crickets, sunflower seed, and Bhatkar-Whitcomb diet (*31*). All colonies were maintained at 27 °C, 70% humidity, and 12 hour day:night cycle.

*Isolation of sal homologues.* We isolated fragments of *sal* from *P. morrisi* (1), as well as from *P. obtusospinosa* and *P. rhea* using PCR on cDNA that was generated from reverse transcription of their larval mRNA. We cloned and sequenced these fragments to confirm their identity, and performed gene tree analyses to confirm their orthology with *Nasonia*, *Apis*, and *Drosophila sal*. PCR primers and conditions are described in (1). GenBank accession numbers are JN205075 for *P. obtusospinosa sal* and JN205076 for *P. rhea sal*.

*Whole mount in situ hybridization and immunohistochemistry.* We fixed and processed last instar larvae just prior to the prepupal stage (*32*). We carefully dissected larvae under a Zeiss Discovery V12 stereomicroscope to remove obstructive tissues surrounding imaginal discs. We then synthesized a digoxigenin-labelled riboprobe (Roche Diagnostics Canada) for *P. morrisi sal (33)*. We used this *P. morrisi sal* probe for all *in situ* 

hybridizations because: (i) *sal* sequences in *P. morrisi*, *P. obtusospinosa*, and *P. rhea* are 96% similar to each other (fig. S15); and (ii) the *P. morrisi sal* probe cross-reacts and reveals a conserved pattern of *sal* expression in the wing discs of winged castes of the *Pheidole* species used in this study (fig. S16).

Phylogenetic analyses. To infer the relationships of the 11 Pheidole species included in our study, we performed a Bayesian analysis using MrBayes v3.1.2 (34). Following Moreau (10), Myrmica incompleta was used as an outgroup. We isolated, cloned, and sequenced the following fragments using PCR (Table S1): (i) a  $\sim$ 616bp fragment of mitochondrial (mt) protein-coding cox1; (ii) a ~744bp fragment of mt protein-coding cytb; (iii) a ~535bp fragment of nuclear protein-coding lwr. GenBank accession numbers for these sequences are listed in Table S2. Additionally, we retrieved sequences from GenBank for: (i) a ~340bp fragment of nuclear protein-coding H3; (ii) a ~360bp fragment of mt ribosomal 12S; and (iii) all sequences for Myrmica incompleta. In total, we used approximately 2.6kb of concatenated sequence spanning 5 genes. We initially aligned these sequences using ClustalX v1.83.1 (35), and subsequently adjusted these alignments by eye using McClade v4.06 (36). To determine the optimal model of sequence evolution for each gene, we used AIC criteria within ModelTest v3.7 (37, 38). As a result, we used GTR+ $\Gamma$ +I for *cox1* and *cytb*, GTR+I for *H3*, and GTR+ $\Gamma$  for *lwr* and 12S. Bayesian analysis involved two independent runs and used four Markov chains with an MCMC (Monte Carlo Markov Chain) length of 10,000,000 generations. The chains were sampled every 200 generations following a burn-in period of 1,000,000 generations. Data used for the analysis was partitioned by gene, each of which was assigned a separate substitution

model. The analysis resulted in a sample of trees with a mean likelihood score of  $-\ln L = 12156.91$  for run 1 and  $-\ln L = 12156.95$  for run 2. The average standard split frequencies of the chains after 10,000,000 generations was 0.002391, suggesting that the chains had reached convergence. The trees from both analyses were thus combined and summarized with a fully resolved majority-rule consensus tree.

Hormonal applications. We topically applied methoprene (Wellmark International), a juvenile hormone (JH) analogue, to the dorsal abdomens of Pheidole larvae. We applied methoprene to *P. morrisi larvae* (range of longest larval lengths was 0.9-1.8mm), *P.* spadonia larvae (1.5-2.7mm), and P. hyatti larvae (1.0-1.5mm) prior to their determination as either minor workers or soldiers. For P. obtusospinosa, a species that naturally produces supersoldiers, the final size range (longest larval length) is 1.7-2.18mm for minor worker larvae, 3.3-3.6mm for soldier larvae, and 4.1-4.6mm for supersoldier larvae. We therefore applied methoprene to P. obtusospinosa larvae that had already passed the soldier-minor worker switch point (2.2-3.3mm), but whose caste fate as either soldiers or supersoldiers had not yet been determined. We applied methoprene at a concentration of 5 mg/ml in acetone to P. morrisi larvae, 3 mg/ml in acetone to P. hyatti larvae, and both 3.5 mg/ml and 5 mg/ml in acetone to P. spadonia and P. obtusospinosa larvae. For P. morrisi, we followed Wheeler (16) and set-up multiple replicates of 40 methoprene-treated larvae raised by 40 workers and no soldiers. We fixed half of the surviving larvae just prior to the prepupal stage, and we let the other half of surviving larvae continue to develop. As controls, we set up multiple replicates in P. morrisi of 40 acetone-treated or untreated larvae raised by 40 workers and no soldiers. To

confirm that methoprene can induce supersoldier larvae in other species that lack supersoldiers, we treated a large number of P. spadonia and P. hyatti larvae with methoprene and raised them in replicate boxes each of which contained 50-100 minor workers. To test whether a second JH-sensitive period mediates the switch between soldiers and supersoldiers larvae in P. obtusospinosa, we treated 200 larvae with methoprene and 200 larvae with acetone only. We raised these methoprene and acetonetreated larvae in replicate boxes, each of which contained 50-100 minor workers. For all experiments, we identified "supersoldier" larvae by finding the smallest larva (shortest larval length in mm) in the sample with two pairs of vestigial wing discs (a defining feature of supersoldier larvae). We then considered any larva longer than this as "supersoldier" larvae. Furthermore, adult Pheidole supersoldiers are defined as showing discrete differences from their soldiers in: (i) their head size and/or; (ii) the allometric scaling relation between the size of their heads and size of their bodies (12, 13). Naturally produced supersoldiers in *P. obtusospinosa* have significantly larger heads than their soldiers, whereas in *P. rhea* they have both significantly larger heads and their heads are smaller relative to their bodies than in soldiers (12, 13). We therefore considered adults that were produced by methoprene treatment to be "induced supersoldiers" if their heads were significantly larger than the heads of untreated controls.

### Statistical analyses and measurement

We used a Zeiss Discovery V12 stereomicroscope and Zeiss Axiovision software to measure the larval size (body length in mm) and adult size (head width in mm). We used an unequal variances t-test (*39, 40*) to determine whether in *P. morrisi* the mean size of:

(i) adult minor workers from anomalous colony is equal to that of normal minor workers; (ii) adult anomalous supersoldiers is equal to that of normal soldiers; (iii) methoprenetreated larvae is equal to that of untreated controls; and (iv) adults resulting from methoprene-treated larvae is equal to that of untreated controls. We used a Fisher's exact test (40) to determine whether in *P. obtusospinosa* the proportions of supersoldier relative to soldier larvae in methoprene treatments are equal to the proportions of acetone-treated controls. Finally, we calculated the coefficient of variation to compare the relative amounts of variation (40) in the surface area of fore- and hindwing vestigial discs in normal and methoprene-induced supersoldier larvae in *P. obtusospinosa*, as well as induced supersoldier larvae in *P. morrisi*, *P. hyatti*, and *P. spadonia*.

### **Supplementary Figures and Figure Legends**

**Fig. S1. Anomalous** *P. morrisi* supersoldiers are significantly larger than normal soldiers. X-axis showing the mean (bars) and standard deviation (error bars) for head widths (mm) of normal minor workers (gray bar, n=20), minor workers from the anomalous colony (black bar, n = 5), normal soldiers (SD; gray bar, n = 20), and anomolous supersoldier-like individuals (aXSD; black bar, n = 8). An unequal variance t-test (two-tailed) between minor workers from the normal and from the anomalous colony shows that they are not significantly different from one another (t = 2.083, df = 12, P = 0.06). In contrast, an unequal variance t-test (two-tailed) between normal SD and aXSD shows that they are significantly different from one another (t = 6.265, df = 9, P = 0.0002). This shows that aXSD are significantly larger in size than normal soldiers.





Fig. S2. Definition and frequency distributions of the supersoldier subcaste in P. obtusospinosa and P. rhea. Pheidole supersoldiers (XSD) are defined as showing discrete differences from their soldiers (SD) in: (i) their head size and/or; (ii) the allometric scaling relation between their head and body size (12, 13). In P. obtusospinosa, naturally produced XSD have significantly larger heads than their SD (compare SD to XSD in A), whereas in *P. rhea* XSD have both significantly larger heads and their heads are smaller relative to their bodies than in SD (compare SD to XSD in **B**) (12, 13). Histograms showing the frequency distributions of soldiers (black bars) and supersoldiers (dark gray bars) based on total fresh body mass for (A) P. obtusospinosa and (B) P. rhea. Y-axis: indicates frequency of the soldier and supersoldier subcaste. Xaxis: scaled fresh body mass. To facilitate comparing the distributions of P. obtusospinosa and P. rhea, we scaled fresh body masses of each species by dividing every fresh body mass (mg) by a mean fresh body mass (mg) that was calculated over all supersoldiers and soldiers. All images are to the same scale. These distributions are consistent with those of (12, 13) in showing that the distribution of soldiers in P. rhea is much broader than that of *P. obtusospinosa*.





scaled fresh body mass

Fig. S3. Development of supersoldiers in *P. rhea*. Comparison of adults of (A) SD and (E) XSD as well as larvae of (B) SD and (F) XSD. Comparison of vestigial wing discs (DAPI) and *sal* mRNA expression (purple) of (C and D) SD and (G and H) XSD. White arrowheads indicate presence of mesothoracic wing vestiges or vestigial wing discs, whereas asterisks indicate their absence. Black arrowheads indicate *sal* expression in wing pouch. Adult, larval and vestigial wing disc images are all to scale.





# **Fig. S4. Expression of** *sal* **in vestigial wing discs of soldier and supersoldier larvae in** *P. obtusospinosa. sal* mRNA expression (purple) in the vestigial wing discs of *Pheidole obtusospinosa* (*Po*): (**A**) soldiers (SD) and (**B**) supersoldiers (XSD). Black arrowheads indicate wing pouch expression of *sal*. Note that, as in *P. morrisi, Po* SDs have (**A**) a single pair of vestigial forewing discs in which *sal* expression is conserved in the hinge region, but is highly downregulated in the wing pouch. (**B**) *Po* XSDs have both fore- and hindwing discs, in which *sal* expression is conserved in the hinge and elaborated in the wing pouch relative to the expression of *sal* in SD. Both images are to scale.

Fig. S4.



Fig. S5. Bayesian phylogenetic analysis of the 11 Pheidole species we used in this study confirms the independent evolution of supersoldiers of *P. rhea* and *P.* obtusospinosa and provides strong support for their inferred relationships. Although Moreau (10) reconstructed a genus-level phylogeny using 142 *Pheidole* species, the phylogenetic relationships of the 11 species that we used in this study remained largely unresolved. This greatly decreases our ability to reconstruct the evolutionary history of supersoldiers. We therefore performed a detailed Bayesian phylogenetic analysis of all 11 species to further resolve their relationships and confirm the independent evolution of supersoldiers in *P. rhea* and *P. obtusospinosa*. Our analysis yielded high posterior probabilities for almost all nodes (gray circles) in the tree, which provides substantial confidence in the inferred phylogenetic relationships. Our results are consistent with (10) in that *P. rhea* is supported as a sister taxon of almost all *Pheidole* species as well as one of the most basal species in the genus. Our phylogenetic analysis supports P. obtusospinosa as a derived species confirming the independent evolution of supersoldier subcastes (green lines) (10). Our analysis also shows that there are six species that are "basal" and four species that are "derived" relative to P. obtusospinosa.

Fig. S5.



**Fig. S6. Interruption of** *sal* **expression in soldier forewing discs differs between basal and derived** *Pheidole* **species.** (**A** to **I**), all images are to scale and represent vestigial forewing discs from late final instar soldier larvae. (**A** to **E**), *sal* mRNA expression (purple) in vestigial soldier forewing discs of basal *Pheidole* species (fig S5): relative to its expression in the hinge, black arrows indicate *sal* is expressed as a well-defined patch of expression in the wing pouch (see fig S3D for *P. rhea*). (**F** to **I**) *sal* expression in vestigial soldier forewing discs of derived *Pheidole* species (fig S5): relative to its expression in the hinge, black arrows indicate *sal* is expressed as a well-defined patch of expression in the wing pouch (see fig S3D for *P. rhea*). (**F** to **I**) *sal* expression in vestigial soldier forewing discs of derived *Pheidole* species (fig S5): relative to its expression in the hinge, black arrows indicate *sal* is expressed as a barely visible and diffuse patch of expression in the wing pouch (see figS4A for *P. obtusospinosa*). These differences in *sal* expression, which occur regardless of the size of vestigial forewing discs, reflect genetically fixed differences that have evolved between these basal and derived *Pheidole* species.





Fig. S7. Application of methoprene induces the development of supersoldier larvae and adults in P. morrisi. Graph on left shows mean (circles) and standard deviation (error bars) for larval length (mm) of untreated (white circle; n = 50) and methoprenetreated (black circle; n = 36) larvae. The mean for methoprene-treated larvae represents larvae from all replicates. An unequal variances t-test (one-tailed) shows that the mean of methoprene-treated larvae is significantly larger than that of untreated larvae (t = 4.887, df = 44, P < 0.0001). All larvae that survived the acetone-treated controls (n = 24; from all replicates) developed into minor worker larvae. Graph on right shows mean and standard deviation for adult head width (mm) of adults that developed from untreated larvae (white circle; n = 30) and adults that developed from methoprene-treated larvae (black circle; n = 7). The mean for adults that developed from methoprene-treated larvae represents adults from all replicates. An unequal variance t-test (one-tailed) shows that the mean of adults that developed from methoprene-treated larvae is significantly larger than those that were untreated (t = 3.742, df = 7, P = 0.0036). All adults that survived the acetone-treated controls (n = 42 from all replicates) developed into adult minor workers.

Fig. S7.



Fig. S8. The relative size ranges of methoprene induced supersoldiers overlap with those of anomalous and naturally produced supersoldiers. The x-axis shows the relative size range of supersoldiers as indicated by horizontal black bars, which was calculated in three steps: (i) the mean supersoldier (XSD)/soldier (SD) size ratio was taken by dividing the mean size (head width in mm) of either induced, anomalous, or evolved XSD adults or larvae by the mean size (head width in mm) of their respective normal SD adults or larvae; (ii) the maximum XSD/SD size ratio was calculated by dividing the largest induced, anomalous, or naturally produced XSD adult or larva in the sample by the mean size of their respective normal SD adults or larvae; and (iii) the minimum XSD/SD size ratio was calculated by dividing the smallest induced, anomalous, or naturally produced XSD adult or larvae; and (iii) the minimum XSD/SD size ratio was calculated by dividing the smallest induced, anomalous, or naturally produced XSD adult or larvae in the sample by the mean size of their respective normal SD adults or larvae; and (iii) the minimum XSD/SD size ratio was calculated by dividing the smallest induced, anomalous, or naturally produced XSD adult or larva in the sample by the mean size of their respective normal SD adults or larvae and those of anomalous and naturally produced supersoldiers.





Fig. S9. Variation in the number, size, and expression of *sal* in vestigial wing discs of normal soldier (SD) and induced supersoldier (iXSD) larvae in P. morrisi (Pm), P. hyatti (Ph), and P. spadonia (Ps). (A to X), asterisks indicate absence of visible wing primordia, while white arrows indicate variation in the number of vestigial wing discs stained with DAPI. Black arrows indicate variation in wing pouch expression of sal. (A to X), images are to scale, with the exception of (L) and (X) that were taken at lower magnification to show asymmetry in the number of vestigial wing discs between the left and right sides of an induced supersoldier larva. Note that this induced variation in iXSD larvae is not due to the incomplete induction of JH, but rather is due to the release of hidden variation that has not yet been subject to selection. We detected variants (B and K and T) that we consider to be iXSD larvae, but which have only one pair of visible extralarge vestigial wing discs. This is because we define iXSD larvae by finding the smallest larva (shortest larval length in mm) in our sample that had two pairs of vestigial wing discs, and then considered any larva longer than this as iXSD larvae. That these are iXSD larvae rather than incompletely induced larvae is further supported by the existence of asymmetrical iXSD larvae: some iXSD have supersoldier-like vestigial fore- and hindwing discs on one side but only a forewing disc on the other side (L and X).

Fig. **S9**.



Fig. S10. Actualization of supersoldiers in *P. obtusospinosa* through the re-evolution of a second JH-sensitive period that mediates the switch (green) between soldiers and supersoldiers. See fig. S11 for data supporting this conclusion and fig. S12 for models that may explain the mechanisms through which this second switch point evolved. Scale bars indicate the relative size of queen, soldier, and minor worker larvae. All adult images are to scale. Purple represents *sal* expression, whereas asterisks indicate absence of visible wing primordia and *sal* expression.

# Fig. S10.



Fig. S11. A second JH-sensitive period mediates the switch between the soldier and the supersoldier subcaste in *P. obtusospinosa*. (A to C) All images are to scale. Asterisks indicate absence of visible wing primordia, whereas white arrows indicate their presence. In P. obtusospinosa (Po), DAPI staining (blue) shows that, unlike (A) soldiers (SD) that have a single pair of vestigial forewing discs, (**B**) supersoldiers (XSD) and (**C**), methoprene-induced supersoldiers (iXSD) have two pairs of large vestigial wing discs. Furthermore, (**D**) y-axis shows the percent of XSD produced relative to SD in methoprene-treated larvae (black circle), acetone-treated control larvae (white circle), and in wild P. obtusospinosa colonies (12, 13) (dotted circle). A Fisher's exact test (onetailed) shows that methoprene treatment produced a significantly greater proportion of XSD larvae than in acetone treated controls (P < 0.0001). Methoprene treatment also produced a greater proportion of XSD larvae than that normally observed in wild colonies. This indicates that the development of SD and XSD subcastes in P. obtusospinosa are determined through a second JH-sensitive period that mediates the switch between SD and XSD. Methoprene- or acetone-treated larvae that did not develop into XSD went on to develop as either SD or minor workers.





Fig. S12. Two heuristic models showing how natural selection on the JH system may lead to the re-evolution of a continually produced supersoldier subcaste in P. *obtusospinosa*. The re-evolution of a supersoldier subcaste may have occurred either by a decrease in threshold sensitivity (as indicated by the green arrow in model A) or increase in JH level (as indicated by the green arrow in model **B**). In both models, the switch between minor workers (MW) and soldiers (SD), as well as between SD and supersoldiers (XSD), requires an intact JH-sensitive period. The JH-sensitive period is the period during which all of the key elements, including JH level and threshold, are present for the transduction of an environmental cue into a binary developmental decision (41). The threshold is made up of tissue responsiveness via a receptor complex, whereas JH level is determined by a balance of synthesis and degradation (42). Note that in both models the threshold and JH level is shown as static for simplicity – the threshold can be modulated, for example, by proportion of adult soldiers in the colony (43), whereas JH levels can be modulated, for example, in response to variation in environmental cues (41). In both models, if JH levels are below threshold during the JH-sensitive period, larvae initiate metamorphosis upon reaching the critical size for minor workers (16). If JH levels are above threshold, larvae continue to grow, their vestigial forewing imaginal discs appear, and the critical size is set (16). Metamorphosis at the soldier critical size is controlled by a sensitive period that precedes it (16). We propose that in the vast majority of *Pheidole* species there is a cryptic threshold that is set higher than the JH level, and therefore, all individuals initiate metamorphosis only at the soldier critical size (SD). In the case of anomalous supersoldier-like individuals, interaction between the ancestral developmental potential and induction by the environment causes the larva to

surpass this cryptic threshold by triggering the setting of a larger critical size (XSD). The re-evolution of supersoldiers in *P. obtuspspinosa* involves the actualization of the induced ancestral developmental potential such that a regular proportion of larvae, which surpassed both the minor worker and soldier threshold, are determined to be supersoldiers. The actualization can be achieved by natural selection on an evolved decrease in threshold sensitivity in model **A** or by natural selection on an increase in JH level as in model **B**.





Fig. S13. A signature of natural selection on the vestigial wing discs in naturally produced supersoldiers in *P. obtusospinosa*. The naturally produced supersoldiers (XSD) and methoprene induced supersoldiers (iXSD) in *P. obtusospinosa* (*Po*) (fig. S11) generally show less variation in their size of (**A**) forewing and (**B**) hindwing vestigial discs than that of (**A**) forewing and (**B**) hindwing vestigial discs of the iXSD larvae of *P. morrisi* (*Pm*), *P. hyatti* (*Ph*), and *P. spadonia* (*Ps*), all of which lack a XSD subcaste. Furthermore, in *Pm*, *Ph*, and *Ps*, we also observed variation in the number of vestigial wing discs, asymmetry of vestigial wing discs as well as variation in the expression of *sal* (fig. S9). Greater variation is expected in an induced response that has not yet been subject to selection, which suggests that the developmental response of vestigial wing discs to JH has been canalized by selection in *P. obtusospinosa*, but not in *Pheidole* species that lack XSD.





Fig. S14. Dynamic expression of *sal* during wing disc development in *P. morrisi* queens suggests that the winglessness of adult supersoldiers (XSD) in *P*.

obtusospinosa evolved through selection on environmentally induced supersoldier variants with novel interruption of *sal* expression. (A to F) All images are to scale. The wing discs of queens appear and begin to grow early in development during the first larval instar. sal mRNA expression (purple) in these discs remains constant in the hinge, but is dynamic in the wing pouch where it increases in length as development of the disc progresses. In normal soldier (SD) forewing discs, interruption of *sal* expression (Fig. 1) is similar to (A) sal expression at the earliest stages of wing disc development in queens, which results in the development of completely wingless soldiers (Fig. 1). In contrast to normal SD, vestigial wing discs in methoprene induced supersoldiers (iXSD) are larger in size and their sal expression (fig. S9) is interrupted at a later time, similar to that of (**B** to **D**) queen wing disc development. These results, together with the fact that wing vestiges appear on the mesothorax of iXSD (Fig. 3C) and anomalous XSD adults (Fig. 2D), suggest that wing development has been reactivated in iXSD and anomalous XSD. Wing development, however, is not completed because unlike queen wing discs, SD wing discs only appear in last instar SD larvae and their growth is prematurely halted as they have to undergo metamorphosis sooner than queen wing discs. Collectively, these observations suggest that the evolution of wingless adult XSD in P. obtusospinosa (Fig. 2I) evolved through selection on environmentally induced supersoldier variants with a novel interruption of *sal* such that any maladaptive development of wings is completely halted.

Fig. S14.



### Fig. S15. Sequence alignment of *sal* orthologs in *P. morrisi*, *P. rhea*, and *P.*

# *obtusospinosa* shows a 96% sequence similarity between their sequences. Sequence identity (dark green bar) is indicated for each nucleotide position above sequence alignment. 1078 nucleotides are conserved between the sequences of all three species, and there are only 46 unique sequence differences, which are marked by different colors (unique changes to G are in yellow, C in purple, T in light green, A in red).

Fig.	S15.
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1. Po_spalt	ΤG	СС	A :	ΓA	ΤТ	G	ΑA	Т	ΤС	Т	СG	Т	CΑ	ΤI	C	СТ	Т	сс	A	СG	A	CΑ	ΑΊ	C	ΤС	C I	ΑT	СA	ΑC	A A	A C	ΑT	С	ΑA	A A	A G	СA	GC	A.	ΑΤΊ
2. Pm_spalt 3. Pr_spalt	T G T G	СС	A 1 A 1	ГА ГА 4	Τ Τ Τ Τ 70	G G	A A A A	T	ТС ТС	T T	C G C G 480	Т ( Т (	C A C A	Т I Т I	C	СТ	Τ Τ 4	C C T C	A A	C G C G	A ( A (	C A C A	Г А Г А	C C 500	ТС ТС	C I C I	АТ АТ	C A C A	C C A C	A A A A 510	A C A C	A T A T	C C	A A A A	A A A A	A G A G 520	C A C A	G <mark>G</mark> G C	A A	АСТ АСТ
1. Po_spalt	ΤG	ΑG	CI	A G	A G	A	GΑ	G(	GΑ	Gi	АТ	CI	A	G	Т	СG	A	АC	A	GΑ	G I	ΑT	СA	A C	сс	G(	ΞA	СA	GG	A	ЗC	ΑG	Т	АТ	CI	A A	сC	A C	G	АСС
<ol> <li>Pm_spalt</li> <li>Pr_spalt</li> </ol>	T G T G	A G A G	C I C I	A G A G	A G A G	A	G A G A	G (	G A G A	G	A T A T	C A	AA	A C A C	Т	C G C G	A A	A C A C	A	G A G A	G A G A	ΑT ΑT	C A C G	C	ССС	G ( G (	G A G A	C A C A	G G G G	A ( A (	G C	A G A G	Т	A T A T		A A A A	САСА	A C A C	G	A C C A C C
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3. Pr_spalt	A G	A G	G I	A A	G A	G	A G	T (	GA	A (	GA	TI	ΓT	ΤG	A	GG	A.	A A	C	C A	CI	A A	G A	C	G A	Ţ,	A C	СТ	ΤA	A '	Г Т 710	CA	C	сс	CI	A A	TC	CG	T	GAA
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2. Pm_spalt 3. Pr_spalt	G A G A	G I G I	A C	G C	G G G G	Т	A G A G	Т	СТ СТ	A A	C A C A	G A G A	A C	A 1 A 1	C C	Т Т Т Т	T	T G C G	C C	C G C G	G A G A	A A A A	T T T T	C C C	T G T G	T 1 T 1	ГТ ГТ	СС СС	A G A G	G	ГС ГС	C C C C	C C	CA C <mark>G</mark>	G ( G (	СТ СТ	СА СА	Т С Т С	A	ТСА ССА
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3. Pr_spalt	A A	ТТ	CC	ΞT	C A	T	СТ	T	c c	T (	GT	TI	C	T A	C	A C	A	GG	C	A G	G	C	C A	AA	CC	A		A C	AI	C	CG	GA	A	СТ	G I	A A	AT	GA	T	CGA
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3. Pr_spait	GG	A G	A (	, C	т Т 130	G	A T	T (	GΑ	AI	A T 1,14	A /	A C	GA	A	A A	1	т Т 150	C	G G	A (	э А	СG	, T 1,160	C C	Al	4 G	сŤ	GC	A (	3 C	A A	C	TA	G !	1,180	GΑ	СА	A	TAT
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3. Pr\_spalt

Fig. S16. *In situ* hybridization using a *P. morrisi sal* antisense probe detects a conserved expression pattern of *sal* in the wing discs of winged castes in six different *Pheidole* species. (A to F) *sal* mRNA expression (purple) in the wing discs of winged castes showing a conserved pattern of expression in the hinge and wing pouch (Fig. 1)(1) in both basal and derived *Pheidole* species (fig. S5).

Fig. S16.



P. valicola

P. hyatti

P. pilifera



# **Supplementary Tables**

# Table S1. PCR primers used for genes in phylogenetic analysis of 11 Pheidole

species.

Gene	Primer	Sequence	Reference
cox1	Forward: LCO1490	5'- GGTCAACAAATCATAAAGATATTGG - 3'	(44)
cox1	Reverse: HCO2198	5'- TAAACTTCAGGGTGACCAAAAAATCA - 3'	(44)
cytb	Forward: CB-J-10933	5'- TATGTACTACCATGAGGACAAATATC - 3'	(45)
cytb	Reverse: TS1-N-11683	5'- TATTTCTTTATTATGTTTTCAAAAC - 3'	(45)
lwr	Forward: LR143F	5'- GACAAAGTKCCACCRGARATGCT - 3'	(46)
lwr	Reverse: LR639ER	5'- YTTACCGRTTCCATCCCRAACA - 3'	(46)

# Table S2. Genbank accession numbers for genes sequenced for phylogenetic analysis

# of 11 Pheidole species.

Species	GenBank	GenBank	GenBank						
	Accession # for	Accession # for	Accession # for						
	mtDNA COxI	mtDNA cytb	nDNA LW-Rh						
Pheidole megacephala	JN205088	JN205099	JN205077						
Pheidole moerens	JN205089	JN205100	JN205078						
Pheidole spadonia	JN205090	JN205101	JN205079						
Pheidole tysoni	JN205091	JN205102	JN205080						
Pheidole rhea	JN205092	JN205103	JN205081						
Pheidole obtusospinosa	JN205093	JN205104	JN205082						
Pheidole hyatti	JN205094	JN205105	JN205083						
Pheidole vallicola	JN205095	JN205106	JN205084						
Pheidole pilifera	JN205096	JN205107	JN205085						
Pheidole morrisi	JN205097	JN205108	JN205086						
Pheidole dentata	JN205098	JN205109	JN205087						

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