RESEARCH ARTICLE

The Wing-Patterning Network in the Wingless Castes of Myrmicine and Formicine Ant Species Is a Mix of Evolutionarily Labile and Non-Labile Genes

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ABSTRACT

Wing polyphenism in ants is the ability of a single genome to produce winged or wingless castes in a colony in response to environmental cues. Although wing polyphenism is a universal and homologous feature of ants, the gene network underlying wing polyphenism is conserved in the winged castes, but is labile in the wingless castes, that is, the network is interrupted at different points in the wingless castes of different ant species. Because the expression of all genes sampled so far in this network in the wingless castes is evolutionarily labile across species, an important question is whether all "interruption points" in the network are evolutionarily labile or are there interruption points that are evolutionarily non-labile. Here we show that in the wingless castes, the expression of the gene *brinker* (*brk*), which mediates growth, patterning, and apoptosis in the *Drosophila* wing disc, is non-labile; it is absent in vestigial wing discs of four ants species. In contrast, the expression of *engrailed* (*en*), a gene upstream of *brk* is labile; it is present in some species but absent in others. In the winged castes, both *brk* and *en* expression are conserved relative to their expression in *Drosophila* wing discs. The differential lability of genes in the network in wingless castes may be a general feature of networks underlying polyphenic traits. This raises the possibility that some genes, like *brk*, may be under stabilizing selection while most others, like *en*, may be evolving via directional selection or neutral drift. *J. Exp. Zool. (Mol. Dev. Evol.) 320B:74–83, 2013.* © 2012 Wiley Periodicals, Inc.

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Dissociations between the evolution of phenotypes and their underlying genotypes are emerging as a general feature of biological systems (Abouheif, '97; Wray and Abouheif, '98; True and Haag, 2001; Bowsher et al., 2007). One type of dissociation is known as developmental system drift (DSD; True and Haag, 2001) or phenogenetic drift (Weiss and Fullerton, 2000), where homologous traits in closely or distantly related species can be produced by non-homologous cell precursors, and/or non-homologous genes, gene expression patterns, or gene functions. DSD reflects a pattern, which is known to occur in a broad range of traits and organisms, such as the vulva in nematodes (Eizinger and

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Sommer, '97; Louvet-Vallee et al., 2003; Kiontke et al., 2007), horns in dung beetles (Moczek and Nagy, 2005; Moczek et al., 2006; Moczek and Rose, 2009), segmentation in insects (Lynch et al., 2006), and sex determination in turtles (Torres Maldonado et al., 2002; Shoemaker et al., 2007; Valenzuela, 2010) and flies (Marin and Baker, '98; Schutt and Nothiger, 2000). Despite its ubiquity as well as its name that unfortunately suggests that DSD is synonymous with neutral drift, we actually have little understanding of the evolutionary and developmental dynamics and processes underlying DSD. To address this problem, we focus on wing polyphenism in ants, which is the ability of a single genome to produce winged and wingless castes in response to environmental cues, such as photoperiod, temperature, and nutrition (Abouheif and Wray, 2002). Wing polyphenism in ants is a classic example of DSD; it is a universal and homologous trait across all ant species (Abouheif and Wray, 2002; Brady et al., 2006), yet the gene network that underlies wing polyphenism is evolutionarily labile (Abouheif and Wray, 2002; Bowsher et al., 2007; Nahmad et al., 2008; Shbailat et al., 2010). Previous studies have shown that expression of this "wing-patterning" network is largely conserved in the wing discs of winged castes (queens and males) of ants relative to *Drosophila* and other holometabolous insects (Cohen, '93; Carroll et al., '94, 2005; Keys et al., '99; Weatherbee et al., '99; Abouheif and Wray, 2002; Tomoyasu et al., 2005, 2009; Bowsher et al., 2007; Shbailat et al., 2010). In contrast, the network is evolutionarily labile in wingless castes, that is, it is interrupted at different points in the wingless castes of different ant species (Abouheif and Wray, 2002; Bowsher et al., 2007; Shbailat et al., 2010). The current pattern of DSD underlying wing polyphenism in ants suggests that the expression of all genes in the network in wingless castes is labile because the interruption of all genes whose expression we have sampled in wingless castes is labile between species, castes, and even within castes (Abouheif and Wray, 2002; Bowsher et al., 2007; Shbailat et al., 2010). Therefore, an important question arising from this current pattern is whether all genes in the network are labile or are there genes that are non-labile?

Recent theoretical and empirical studies in ants (Nahmad et al., 2008; Shbailat et al., 2010) have focused on the gene *brinker* (*brk*) as a potential key node that mediates the suppression of wings in the wingless castes of ants. *brk* is part of the Decapentaplegic (Dpp) signaling pathway (Campbell and Tomlinson, '99; Jazwinska et al., '99; Minami et al., '99; Fig. 1) and plays an important role in mediating growth, patterning, and apoptosis in the wing discs of *Drosophila*. Dpp and Brk form inverse gradients that have opposing effects on growth and modulate cell proliferation rate throughout *Drosophila* wing disc (Schwank et al., 2008). Up-regulation of *brk* (Martin et al., 2004; Schwank et al., 2008) or down-regulation of the Dpp signaling pathway (Zecca et al., '95; Campbell and Tomlinson, '99; Jazwinska et al., '99; Martin-Castellanos and Edgar, 2002; Martin et al., 2004; Schwank et al., 2008) reduces growth and induces apoptosis in the *Drosophila* wing disc (Moreno et al., 2002), and down-regulates the expression of the Dpp signaling target genes: *spalt* (*sal*), *optomotor blind* (omb), *vestigial* Quadrants (vg^{Q}), and *daughters against dpp* (*dad*; Campbell and Tomlinson, '99; Jazwinska et al., '99; Minami et al., '99; Martin et al., 2004; Affolter and Basler, 2007; Fig. 1). *brk* (and not *dpp*) is thought to be a key node because when both *brk* and *dpp* signaling are ubiquitously expressed, wing discs and adult wings become significantly reduced in size (Martin et al., 2004; Schwank et al., 2008).

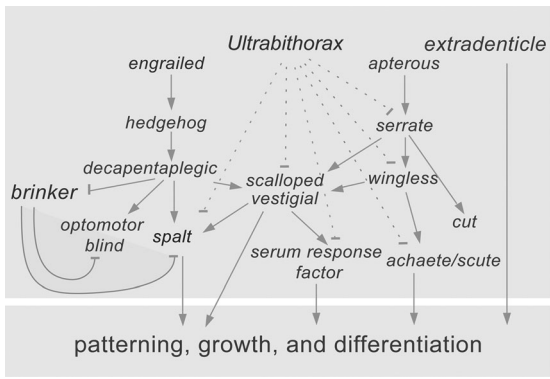

Figure 1. A general representation of the wing-patterning network in *Drosophila* during last larval instar. A set of transcription factors and signaling molecules regulates the patterning and growth of the anterior–posterior (A–P) and dorsal–ventral (D–V) compartments (Cohen, '93; Carroll et al., 2005). In the A–P compartments, *en* acts as a selector gene that specifies the posterior compartment. En activates the expression of *hedgehog* (*hh*). Hh then acts as a short-range signaling molecule that activates the expression of *decapentaplegic* (*dpp*). Dpp signaling activates the expression of the downstream target genes primarily by repressing the expression of *brk*. In the D–V compartments, *apterous* (*ap*) acts as a selector gene that specifies the dorsal compartment. Ap activates *serrate* (*ser*). Ser ligand binds to the Notch receptor, which subsequently activates Notch signaling. Notch signaling in turn activates *cut* (*ct*), *vestigial* (*vg*) at the boundaries of the wing pouch, and *wingless* (*wg*). Wingless signaling then activates the expression of other downstream target genes.

In the wing discs (Fig. 2G′) of winged castes (Fig. 2G) of the ant species *Pheidole morrisi*, Shbailat et al. (2010) showed that, expression of *brk* is conserved relative to its expression in *Drosophila* wing discs. In the wingless castes of *P. morrisi*, however, *brk* expression was predicted to be up-regulated or ubiquitously expressed in vestigial wing discs or primordia to slow growth and induce apoptosis (Nahmad et al., 2008). The wingless caste of *P. morrisi* is composed of "soldier" (Fig. 2H) and "minor worker" (Fig. 2I) sub-castes, where soldier larvae possess only one

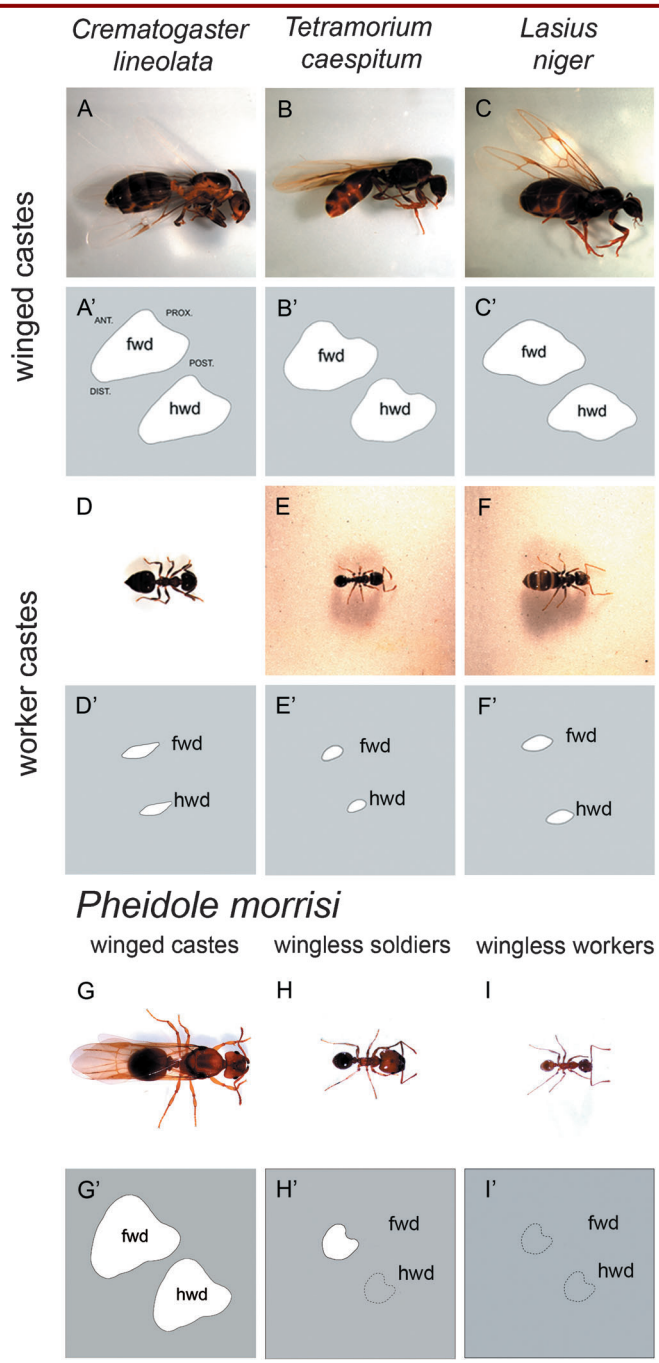

Figure 2. The size and morphology of wing discs in winged castes and vestigial wing discs in wingless workers of Myrmicine and Formicine ant species during the last larval instar. (A–C) winged queens and (D–F) wingless workers of *Crematogaster lineolata*, *Tetramorium caespitum*, and *Lasius niger*. (A –F) Schematic diagrams of the fore- and hindwing discs (fwd, hwd) showing (A –C) wing discs of winged castes and (D – F) vestigial wing discs of wingless workers in the above ant species. (G) Winged queen, (H) wingless soldier, and (I) wingless minor worker of *Pheidole morrisi*. Schematic diagrams showing (G) fore- and hindwing discs of winged castes, (H) vestigial forewing disc and expected position of vestigial hindwing primordium of soldiers (dotted line), and (I) expected positions of vestigial fore- and hindwing primordia of minor workers (dotted line) in *P. morrisi*. ANT represents anterior, POST represents posterior, PROX represents proximal, and DIST represents distal. [Color figure can be seen in the online version of this article, available at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1552-5015]

pair of relatively large vestigial forewing discs but no visible hindwing discs (Fig. 2H′), and minor worker larvae lack visible vestigial wing discs entirely (Fig. 2I′). Shbailat et al. (2010) showed that, in the vestigial forewing disc in soldiers, *brk* expression is absent throughout its development. This absence of *brk* is likely to be actively and independently maintained throughout development because other genes in the network, such as *en*, *wingless* (*wg*), *extradenticle* (*exd*), *Ultrabithorax* (*Ubx*), *scalloped* (*sd*), *dpp*, and *sal* are expressed (Abouheif and Wray, 2002; Shbailat et al., 2010). Furthermore, the absence of *brk* expression in this disc is correlated to its rapid growth in the last larval instar (Wheeler and Nijhout, '81), and the abnormal growth of the anterior and posterior compartments (Shbailat et al., 2010). These correlations suggest that *brk* may play a role in controlling the growth of this disc, and more generally, suggest that the differential expression of *brk* between winged and wingless castes plays an important role in wing polyphenism in *P. morrisi* (Shbailat et al., 2010).

The main goal of our study was to determine whether the absence of *brk* expression is a unique (evolutionarily labile) interruption point of the vestigial soldier forewing discs in *P. morrisi* or whether it is a conserved (non-labile) interruption point in the vestigial wing discs of the wingless castes of other ant species. In addition, we chose to examine *en* expression because: (1) it is a gene that is upstream of *brk*; (2) it lies at the top of the network that patterns the anterior–posterior of the *Drosophila* wing disc (Held, 2002; Fig. 1); and (3) it is known from previous studies to be a labile interruption point between different ant species (Abouheif and Wray, 2002; Bowsher et al., 2007; Shbailat et al., 2010). Therefore, we wanted to determine how the lability or non-lability of *brk* expression occurs relative to *en*, an upstream gene known to be labile. Because *brk* expression has only been characterized in the vestigial forewing discs of *P. morrisi* soldiers, we examined the expression of *brk* and *en* in the other vestigial wing primordia and sub-castes of *P. morissi* (i.e., vestigial hindwing primordia of *P. morrisi* soldiers and vestigial wing primordia of *P. morrisi* minor workers; Fig. 2H′ and I′), as well as in vestigial wing discs in the worker castes of three additional ant species: *Crematogaster lineolata*, *Tetramorium caespitum*, and *Lasius niger* (Fig. 2D–F′). We chose these ant species (Fig. 2A–F′) because they represent a balanced phylogenetic sample of species from the Myrmicinae and Formicinae (Brady et al., 2006; Moreau et al., 2006), the two largest sub-families of ants (Brady et al., 2006; Moreau et al., 2006), and their vestigial wing disc size and shape (Fig. 2D′–F′) are representatives of the size range observed in Myrmicines and Formicines (Wheeler and Nijhout, '81; Fig. 2H′ and I′).

MATERIALS AND METHODS

Ant Collection and Staging

We collected *P. morrisi*, *T. caespitum*, and *C. lineolata* colonies in Long Island, New York, USA, and *L. niger* in Montreal, Quebec, Canada. We kept colonies of these species in (33 cm × 22 cm × 11 cm) plastic boxes with glass tubes filled with water constrained by cotton wool, and we fed them on a combination of mealworms, crickets, and the Bhatkar-Whitcomb diet (Bhatkar and Whitcomb, '70). We maintained all colonies under 27°C, 70% humidity, and 12:12 hr of day:night cycle. We identified the last larval instar for both winged and wingless castes of *P. morrisi*, *T. caespitum*, *C. lineolata*, and *L. niger* according to the following criteria published by Wheeler and Nijhout ('81): (1) the color of the gut was brown; (2) the wings and legs reached their maximum sizes but not evaginated; and (3) the prepupal cuticle was not formed.

Gene Cloning and Sequencing

We extracted genomic DNA using the CTAB DNA preparation (Levitan and Grosberg, '93). We amplified fragments of *brk* from *C. lineolata*, *T. caespitum*, and *L. niger*. The amplified fragments flanked part of the coding region that starts near the 5′ end of the gene and continues toward the 3′ end. We designed and used the following primers based on *brk* sequence from *P. morrisi* (Shbailat et al., 2010): forward 5′-GCAAAACCAACGTGCAACTGCAAG-3′ and 5′-CTGCAAGGAAATACGGTATTCATCG-3′, and reverse 5′-GAGGAGGTACATCGTAGGAGTCGTC-3′. We used the following PCR conditions: 94°C for 3 min (1 cycle), 94°C for 45 sec, 55°C for 45 sec, and 72°C for 2 min (40 cycles), and 72°C for 5 min (1 cycle). By aligning *brk* DNA sequence of *P. morrisi* (Shbailat et al., 2010) with *brk* DNA sequences from all the above species, we confirmed that *brk* sequences from all of these species lack introns.

Sequence Comparisons

We aligned Brk sequences from *P. morrisi*, *C. lineolata*, *T. caespitum*, and *L. niger* to their respective ortholog from *D. melanogaster* (GenBank accession number: NP_511069) using Clustal W. In the examined ant species, we searched for the conserved first and second Brk DNA binding domains (BrkDBDs) using the NCBI Conserved Domain Database (Cordier et al., 2006). GenBank accession number for *brk* from *P. morrisi* is HM045781 (Shbailat et al., 2010), *C. lineolata* is JF682358, *L. niger* is JF682359, and *T. caespitum* is JF682360.

Gene Expression Analysis

We fixed final instar larvae for 2 hr at room temperature, then we washed and stored them in methanol at −20°C as described in Patel ('94). We performed antibody staining for En protein using the anti-En antibody (Mab 4D9, Patel et al., '89, a gift from Prof. Nipam Patel) following the procedure in Patel ('94). We incubated the larvae overnight at 4°C with anti-En antibody at 1/3 dilution, and then with anti-mouse antibody conjugated with horseradish peroxidase at 1/300 dilution. We performed *brk* in situ hybridization according to the protocol in Tautz and Pfeifle ('89) with some modifications. For penetration, we incubated dissected larvae of *P. morrisi* and *C. lineolata* with sodium dodecyl

sulfate (SDS). Because SDS was insufficient to penetrate wing discs in winged queens and males in *T. caespitum* and *L. niger*, we incubated dissected larvae with proteinase K. For probe hybridization, we hybridized larvae of the four examined species overnight at 58°C with *brk* Digoxigenin labeled probe (Roche Diagnostic Canada, Laval, Quebec, Canada). For detection of labeled probe, we incubated the larvae overnight at 4°C with anti-Digoxigenin antibody conjugated with alkaline phosphatase (Roche Diagnostic Canada) at 1/2,000 dilution. In the examined ant species, to confirm that the absence of En and *brk* expression is real, and not an artifact, we stained larvae of winged castes within the same tube as worker larvae as a control for the expression pattern in the wing discs. Moreover, we over developed the staining reaction for larvae of both winged castes and workers to ensure that there is no weak signal present in the vestigial discs. Finally, we stained for *brk* expression in winged castes and wingless soldiers of *P. morrisi* using both sense and antisense mRNA (Fig. S1).

RESULTS

Cloning and Analysis of *brk* Orthologs From Ants

In *Drosophila melanogaster*, *brk* contains a single Brk DNA binding domain (DBD), which plays a role in repressing the expression of its target genes (Winter and Campbell, 2004; Cordier et al., 2006). We cloned fragments of *brk* which correspond to part of the coding region of the gene that starts near the 5′ end and continues toward the 3′ end. We cloned: 2,080 bp from *C. lineolata*, 2,097 bp from *T. caespitum*, and 2,043 bp from *L. niger*. A protein sequence analysis showed that these cloned fragments as well as that from *P. morrisi* (Shbailat et al., 2010) contain two Brk DNA binding domains: BrkDBD1, which is located near the N-terminal end of the protein, and BrkDBD2 (Cordier et al., 2006). In the examined ant species, BrkDBD2 follows BrkDBD1 by 415–431 amino acids, and the cloned fragments include part of BrkDBD1 and all BrkDBD2. Furthermore, a protein sequence alignment shows high similarity between BrkDBD1 in different ant species and that in *Drosophila* (Fig. 3A), as well as between BrkDBD1 and BrkDBD2 in ants and between these two domains and the single DBD in *Drosophila* (Fig. 3B). Collectively, these results indicate that the fragments we cloned from ants are *brk* orthologs.

The Expression of En and *brk* Is Conserved Between Winged Castes of Four Ant Species and *Drosophila*

In the winged castes of *C. lineolata*, *T. caespitum*, *L. niger*, and *P. morrisi*, En is expressed in the posterior compartment of wing discs (Fig. 4A–C and G), whereas *brk* expression is restricted to the lateral regions of the anterior and posterior of the wing hinge, as well as the upper peripheral region of the wing pouch (Fig. 4D–F and H). Furthermore, *brk* expression in the posterior compartment is more expanded relative to the anterior one (Fig. 4D–F and H). The expression of both En and *brk* is similar to their expression in ants (Abouheif and Wray, 2002; Bowsher et al., 2007; Shbailat et al., 2010) and *Drosophila* (Garcia-Bellido and Santamaria, '72; Campbell and Tomlinson, '99; Jazwinska et al., '99; Minami et al., '99) indicating that the expression of both genes is conserved.

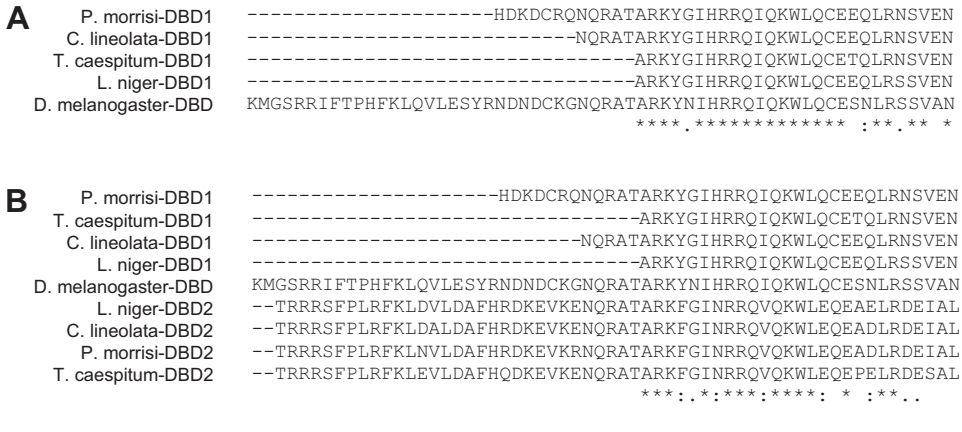

Figure 3. Comparative sequence analysis of Brk between different ants and its respective ortholog in *Drosophila melanogaster*. (**A**) Amino acid sequence alignment for part of BrkDBD1 from the following ant species: *P. morrisi*, *C. lineolata*, *T. caespitum*, and *L. niger* with BrkDBD from *D. melanogaster*. (**B**) Amino acid sequence alignment of BrkDBD1 and BrkDBD2 from *P. morrisi*, *C. lineolata*, *T. caespitum*, and *L. niger* with BrkDBD from *D. melanogaster*. In the examined ant species, BrkDBD1, which is located near the N-terminal regions of the protein, is followed by BrkDBD2.

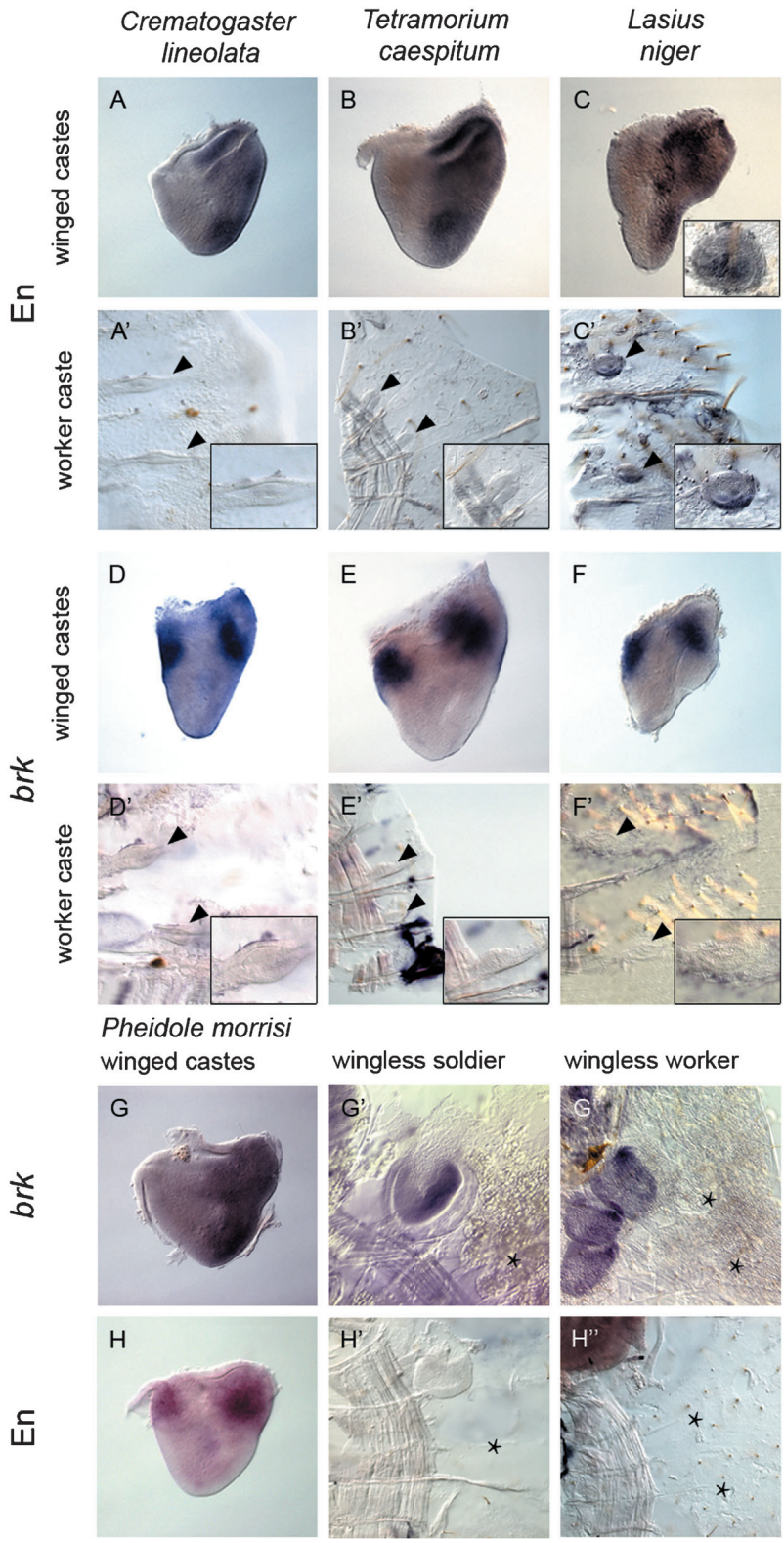

Figure 4. Continued.

In Wingless Castes of Four Ant Species, En Expression Is Absent in Some Species But Is Conserved in Others, While *brk* Expression Is Absent in All of These Species

We found that in the vestigial wing discs of *C. lineolata* and *T. caespitum* workers, En expression is absent relative to its expression in the wing discs of winged castes (compare Fig. 4A′ and B′ with A and B). However, in the vestigial wing discs of *L. niger* workers, En is expressed in the bottom half, which represents the posterior compartment of the vestigial wing discs (Fig. 4C′). This pattern of En expression in the vestigial wing discs of workers is similar to its expression in early wing discs of winged castes, where it is also expressed in the bottom half of the wing discs (Inset in Fig. 4C). In *P. morrisi*, En is expressed in the posterior compartment of the vestigial forewing disc of soldiers, but is absent in the expected position of vestigial hindwing primordium of soldiers and vestigial fore- and hindwing primordia of minor workers (Abouheif and Wray, 2002; Shbailat et al., 2010; Fig. 4G′ and G″). Therefore, the expression of En is labile within and between wingless castes of different ant species in that it is absent in some species but is conserved in others. Regardless of the presence or absence of En expression (Fig. 4A′–C′ and G′ and G″), *brk* expression is absent in the vestigial wing discs in all examined ant species (Fig. 4D′–F′ and H′; Shbailat et al., 2010), as well as in the expected position of the vestigial hindwing primordium of soldiers and vestigial wing primordia of minor workers of *P. morrisi* (Fig. 4H′ and H″). Therefore, in all four species, the interruption of *brk* expression in the vestigial wing discs or primordia of wingless castes is non-labile.

DISCUSSION

Our results show that: (1) in all four ant species, the expression of En and *brk* in the wing discs of winged castes is conserved relative to that in *Drosophila*; and (2) in the wingless castes of these species, *brk* expression is absent in all species, whereas En expression is present in the vestigial wing discs of some species but not in others. These results indicate that the absence of *brk* expression may be a conserved non-labile interruption point in the network of vestigial wing discs in the wingless castes of species that belong to the two largest and derived sub-families of ants: the Myrmicinae and Formicinae. The conservation of *brk* expression in winged castes of these species indicates that the role of *brk* as a growth repressor may be conserved. This is a reasonable assumption given that RNAi knockdown of genes in the network controlling wing development in the beetle *Tribolium castaneum*, which is a close relative of ants, shows that their function is largely conserved (Tomoyasu et al., 2005, 2009). Furthermore, all genes in the wing network sampled, including *brk*, show a similar pattern of expression relative to *Drosophila* (Abouheif and Wray, 2002; Bowsher et al., 2007; Shbailat et al., 2010). In wingless castes, however, the absence of *brk* expression in these species may also play a role in growth control. This assumption is built on the finding that in *P. morrisi* soldier larvae the absence of *brk* expression is correlated to the rapid growth of this disc during the last larval instar as well as disruption of normal growth of the anterior and posterior compartments within the vestigial disc (Wheeler and Nijhout, '81; Shbailat et al., 2010). To understand the possible role and origin of *brk* absence, future studies should test the function of *brk* in these ant species. Moreover, Myrmicinae and Formicinae are derived subfamilies of ants, and therefore, species of ants that belong to the basal sub-families should be sampled to uncover whether the absence of *brk* is general and evolved near the origin of wing polyphenism in ants or whether it is only a feature of derived ant species.

Our results also have important implications for understanding the dynamics of DSD underlying wing polyphenism in ants. All of the genes in the network whose expression has been previously sampled have been shown to be evolutionarily labile between wingless worker castes of different ant species (Abouheif and Wray, 2002; Bowsher et al., 2007; Shbailat et al., 2010). This lability can be explained by directional natural selection, where each interruption point plays a functionally important role in suppressing wing development (Abouheif and Wray, 2002; Abouheif, 2004; Nahmad et al., 2008; Shbailat et al., 2010). In this case, the lability of interruption points is caused by directional selection acting directly on many different genes in the network in different species, or indirectly through directional selection on a correlated phenotype (Abouheif and Wray, 2002; Abouheif, 2004; Nahmad et al., 2008; Shbailat et al., 2010). Alternatively, this

Figure 4. En and *brk* expression in the wing discs of winged castes and vestigial wing discs of wingless castes in different ant species during the end of last larval instar. (**A–F**) Wing discs in winged castes of *C. lineolata*, *T. caespitum*, and *L. niger* showing (A–C) En protein expression and (D–F) *brk* mRNA expression. (**A′–F′**) Vestigial wing discs in the wingless workers of the above ant species representing (A′–C′) En protein expression and (D′–F′) *brk* mRNA expression. Arrowheads indicate vestigial fore- and hindwing discs of workers. Insets in (A′–F′) show the expression of En and *brk* in the vestigial forewing discs of wingless workers, while inset in (C) shows the expression of En in early wing disc in winged castes of *L. niger*. (**G–G″**) En expression and (**H–H″**) *brk* expression in *P. morrisi* in (G and H) wing disc of winged castes, (G′ and H′) vestigial forewing disc and expected position of vestigial hindwing primordium of soldier (asterisk), and (G″–H″) expected positions of vestigial fore- and hindwing primordia of minor worker (asterisks). All images were taken at the same magnification and are all to scale. All insets were also taken at the same magnification and are all to scale. The wing discs of winged castes are oriented such that hinge is to the top and posterior is to the right.

lability can been explained by neutral drift, where interruption points do not play a functional role in repressing wing development (Abouheif and Wray, 2002; Abouheif, 2004; Nahmad et al., 2008; Shbailat et al., 2010), and the wing imaginal disc is simply eliminated through apoptosis in the last stages of development (Sameshima et al., 2004; Shbailat et al., 2010). The differential lability between *brk* expression (non-labile) and an upstream gene *en* (labile) raises the possibility that the non-labile interruption points in the network may be under stabilizing selection, whereas the labile ones are either under directional selection or neutrally drifting (Nahmad et al., 2008). This possibility is supported by: (1) the general absence of *brk* expression in the wing primordia and vestigial wing discs in wingless castes of four Myrmicine and Formicine ant species,

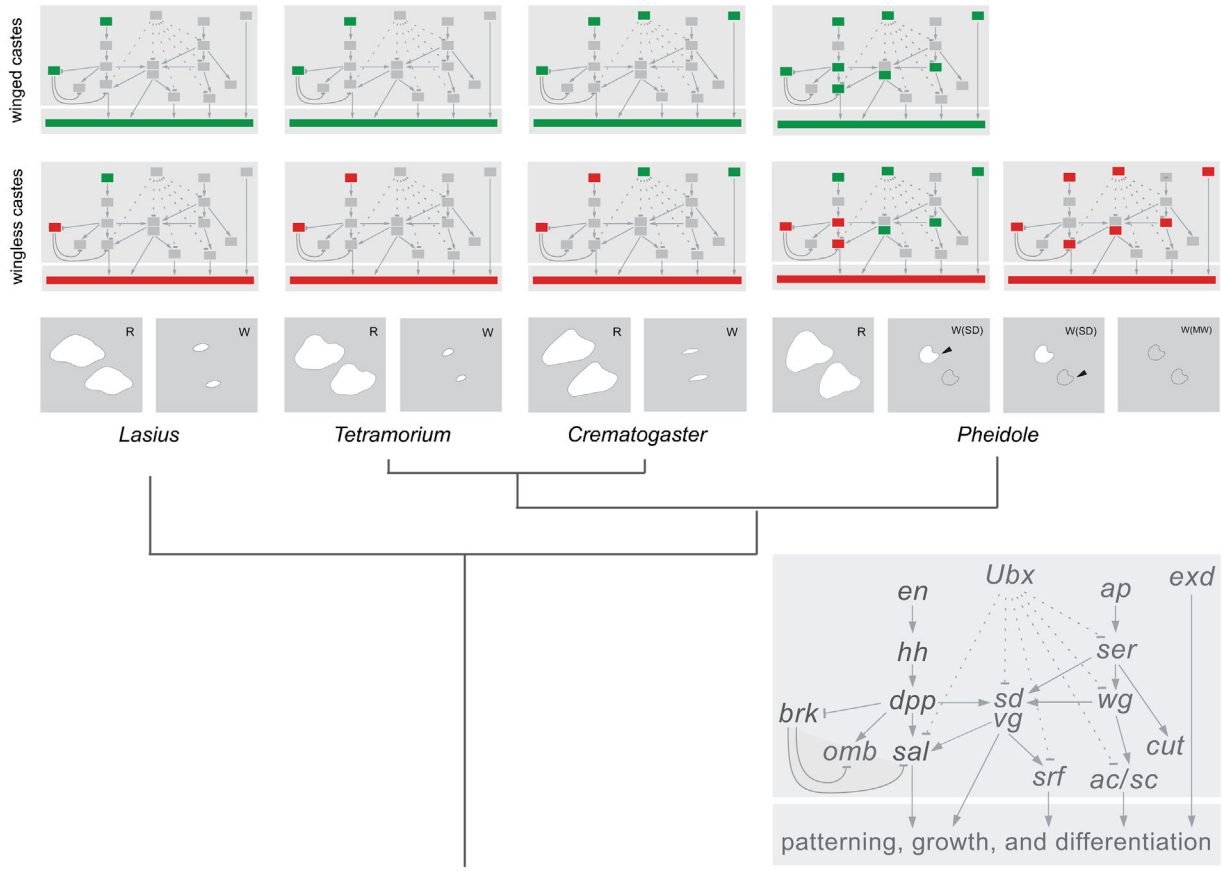

Figure 5. Differential lability of genes in the network underlying wing polyphenism in ants. The bottom row above the phylogeny shows schematic diagrams of wing discs in winged castes (R), and vestigial wing discs in wingless workers (W) of the following ant species: *C. lineolata*, *T. caespitum*, and *L. niger*. For *P. morrisi*, schematic diagrams are shown for vestigial wing discs and primordia for both wingless soldiers [W(SD)] and wingless minor workers [W(MW)], where arrowheads mark the forewing disc and hindwing primordium of soldier, and dotted vestigial wing discs represent the expected position of the hindwing primordium of soldier as well as the expected positions of the fore- and hindwing primordia of minor worker. The middle row shows diagrams of the wing network in the wingless castes of ants. For *P. morrisi*, left panel represents the network in the forewing discs of soldiers, while right panel represents the network in the expected position of hindwing primordia of soldiers as well as the expected positions of fore- and hindwing primordia of minor workers. The top row shows diagrams of the wing network in winged castes of ants. Within each of the panels, green boxes mark conservation of gene expression relative to *Drosophila* and red boxes mark interruption of gene expression. Below the phylogeny is the gene network that regulates growth and patterning of *Drosophila* wing disc throughout development (see Fig. 1). The expression of *en*, *Ubx*, and *exd* in the winged castes and wingless workers of *C. lineolata*, and the expression of *en*, *Ubx*, *exd*, *sal*, *sd*, and *wg* in the winged castes and wingless soldiers and minor workers of *P. morrisi* are based on Abouheif and Wray (2002). The expression of *dpp* and *brk* in the winged castes and wingless soldiers of *P. morrisi* is based on Shbailat et al. (2010). Phylogenetic relationships are based on Brady et al. (2006) and Moreau et al. (2006).

especially in the case of *P. morrisi* soldiers and *L. niger* workers, where En expression is present while *brk* expression is absent (Fig. 5); and (2) the stark contrast between the evolutionary non-lability of *brk* and the lability of all of the other interruption points, including *en*, known to be labile in the gene network (Abouheif and Wray, 2002; Bowsher et al., 2007; Shbailat et al., 2010; Fig. 5). This mix of evolutionary labile and non-labile genes in the network in wingless castes of ants may be a general feature of networks underlying polyphenic traits.

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LITERATURE CITED

Abouheif E. 1997. Developmental genetics and homology: a hierarchical approach. Trends Ecol Evol 12:405–408.

Abouheif E. 2004. A framework for studying the evolution of gene networks underlying polyphenism: insights from winged and wingless ant castes. In: Hall BK, Pearson RD, Muller GB, editors. Environment, development, and evolution: toward a synthesis. MA: Massachusetts Institute of Technology. p 125–137.

Abouheif E, Wray GA. 2002. Evolution of the gene network underlying wing polyphenism in ants. Science 297:249–252.

Affolter M, Basler K. 2007. The Decapentaplegic morphogen gradient: from pattern formation to growth regulation. Nat Rev Genet 8:663–674.

Bhatkar A, Whitcomb WH. 1970. Artificial diet for rearing various species of ants. Fla Entomol 53:229–232.

Bowsher JH, Wray GA, Abouheif E. 2007. Growth and patterning are evolutionarily dissociated in the vestigial wing discs of workers of the red imported fire ant, *Solenopsis Invicta*. J Exp Zool (Mol Dev Evol) 308B:769–776.

Brady SG, Schultz TR, Fisher BL, Ward PS. 2006. Evaluating alternative hypotheses for the early evolution and diversification of ants. Proc Natl Acad Sci USA 103:18172–18177.

Campbell G, Tomlinson A. 1999. Transducing the Dpp morphogen gradient in the wing of *Drosophila*: regulation of Dpp targets by *brinker*. Cell 96:553–562.

Carroll SB, Gates J, Keys DN, et al. 1994. Pattern-formation and eyespot determination in butterfly wings. Science 265:109–114.

Carroll SB, Grenier JK, Weatherbee SD. 2005. From DNA to diversity: molecular genetics and the evolution of animal design. Malden, MA: Blackwell Science.

Cohen SM. 1993. Imaginal disc development. In: Bate M, Martinez-Arias A, editors. The development of *Drosophila melanogaster*. NY: Cold Spring Harbor Laboratory Press. p 747–843.

Cordier F, Hartmann B, Rogowski M, Affolter M, Grzesiek S. 2006. DNA recognition by the Brinker repressor—An extreme case of coupling between binding and folding. J Mol Biol 361:659–672.

Eizinger A, Sommer RJ. 1997. The homeotic gene *lin-39* and the evolution of nematode epidermal cell fates. Science 278:452–455.

Garcia-Bellido A, Santamaria P. 1972. Developmental analysis of the wing disc in the mutant engrailed of Drosophila melanogaster. Genetics 72:87–104.

Held LI. 2002. Imaginal discs: the genetic and cellular logic of pattern formation. Cambridge: Cambridge University Press.

Jazwinska A, Kirov N, Wieschaus E, Roth S, Rushlow C. 1999. The *Drosophila* gene *brinker* reveals a novel mechanism of Dpp target gene regulation. Cell 96:563–573.

Keys DN, Lewis DL, Selegue JE, et al. 1999. Recruitment of a *hedgehog* regulatory circuit in butterfly eyespot evolution. Science 283:532–534.

Kiontke K, Barriere A, Kolotuev I, et al. 2007. Trends, stasis, and drift in the evolution of nematode vulva development. Curr Biol 17:1925–1937.

Levitan DR, Grosberg RK. 1993. The analysis of paternity and maternity in the marine hydrozoan *Hydractinia-symbiolongicarpus* using randomly amplified polymorphic DNA (RAPD) markers. Mol Ecol 2: 315–326.

Louvet-Vallee S, Kolotuev I, Podbilewicz B, Felix MA. 2003. Control of vulval competence and centering in the nematode *Oscheius* sp. 1 CEW1. Genetics 163:133–146.

Lynch JA, Brent AE, Leaf DS, Pultz MA, Desplan C. 2006. Localized maternal *orthodenticle* patterns anterior and posterior in the long germ wasp *Nasonia*. Nature 439:728–732.

Marin I, Baker BS. 1998. The evolutionary dynamics of sex determination. Science 281:1990–1994.

Martin-Castellanos C, Edgar BA. 2002. A characterization of the effects of Dpp signaling on cell growth and proliferation in the *Drosophila* wing. Development 129:1003–1013.

Martin FA, Perez-Garijo A, Moreno E, Morata G. 2004. The *brinker* gradient controls wing growth in *Drosophila*. Development 131:4921–4930.

Minami M, Kinoshita M, Kamoshida Y, Tanimoto H, Tabata T. 1999. Brinker is a target of Dpp in *Drosophila* that negatively regulates Dpp-dependent genes. Nature 398:242–246.

Moczek AP, Nagy LM. 2005. Diverse developmental mechanisms contribute to different levels of diversity in horned beetles. Evol Dev 7:175–185.

Moczek AP, Rose D, Sewell W, Kesselring BR. 2006. Conservation, innovation, and the evolution of horned beetle diversity. Dev Genes Evol 216:655–665.

Moczek AP, Rose DJ. 2009. Differential recruitment of limb patterning genes during development and diversification of beetle horns. Proc Natl Acad Sci USA 106:8992–8997.

Moreau CS, Bell CD, Vila R, Archibald SB, Pierce NE. 2006. Phylogeny of the ants: diversification in the age of angiosperms. Science 312:101–104.

Moreno E, Basler K, Morata G. 2002. Cells compete for Decapentaplegic survival factor to prevent apoptosis in *Drosophila* wing development. Nature 416:755–759.

Nahmad M, Glass L, Abouheif E. 2008. The dynamics of developmental system drift in the gene network underlying wing polyphenism in ants: a mathematical model. Evol Dev 10:360–374.

Patel NH. 1994. Imaging neuronal subsets and other cell-types in whole-mount *Drosophila* embryos and larvae using antibody probes. In: Goldstein LSB, Fyrberg E, editors. Methods in cell biology. San Diego: Academic Press, Inc. p 445–487.

Patel NH, Martinblanco E, Coleman KG, et al. 1989. Expression of engrailed proteins in arthropods, annelids, and chordates. Cell 58:955–968.

Sameshima SY, Miura T, Matsumoto T. 2004. Wing disc development during caste differentiation in the ant *Pheidole megacephala* (Hymenoptera: Formicidae). Evol Dev 6:336–341.

Schwank G, Restrepo S, Basler K. 2008. Growth regulation by Dpp: an essential role for Brinker and a non-essential role for graded signaling levels. Development 135:4003–4013.

Schutt C, Nothiger R. 2000. Structure, function and evolution of sex-determining systems in Dipteran insects. Development 127:667–677.

Shbailat SJ, Khila A, Abouheif E. 2010. Correlations between spatiotemporal changes in gene expression and apoptosis underlie wing polyphenism in the ant *Pheidole morrisi*. Evol Dev 12:579–590.

Shoemaker C, Ramsey M, Queen J, Crews D. 2007. Expression of Sox9, Mis, and Dmrt1 in the gonad of a species with temperature-dependent sex determination. Dev Dyn 236:1055–1063.

Tautz D, Pfeifle C. 1989. A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. Chromosoma 98:81–85.

Tomoyasu Y, Wheeler SR, Denell RE. 2005. *Ultrabithorax* is required for membraneous wing identity in the beetle *Tribolium castaneum*. Nature 433:643–647.

Tomoyasu Y, Arakane Y, Kramer kJ, Denell RE. 2009. Repeated co-options of exoskeletion formation during wing-to-elytron evolution in beetles. Curr Biol 19:2057–2065.

Torres Maldonado LC, Landa Piedra A, Moreno Mendoza N, et al. 2002. Expression profiles of Dax1, Dmrt1, and Sox9 during temperature sex determination in gonads of the sea turtle *Lepidochelys olivacea*. Gen Comp Endocrinol 129:20–26.

True JR, Haag ES. 2001. Developmental system drift and flexibility in evolutionary trajectories. Evol Dev 3:109–119.

Valenzuela N. 2010. Multivariate expression analysis of the gene network underlying sexual development in turtle embryos with temperature-dependent and genotypic sex determination. Sex Dev 4:39–49.

Weatherbee SD, Nijhout HF, Grunert LW, et al. 1999. *Ultrabithorax* function in butterfly wings and the evolution of insect wing patterns. Curr Biol 9:109–115.

Weiss KM, Fullerton SM. 2000. Phenogenetic drift and the evolution of genotype-phenotype relationships. Theor Popul Biol 57:187–195.

Wheeler DE, Nijhout HF. 1981. Imaginal wing disks in larvae of the soldier caste of *Pheidole-bicarinata-vinelandica* forel (Hymenoptera, Formicidae). Int J Insect Morphol 10:131–139.

Winter SE, Campbell G. 2004. Repression of Dpp targets in the *Drosophila* wing by Brinker. Development 131:6071–6081.

Wray GA, Abouheif E. 1998. When is homology not homology? Curr Opin Genet Dev 8:675–680.

Zecca M, Basler K, Struhl G. 1995. Sequential organizing activities of *engrailed*, *hedgehog* and *decapentaplegic* in the *Drosophila* wing. Development 121:2265–2278.