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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WING-PATTERNING NETWORK IN THE WINGLESS CASTES

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 Nohthiger, 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 (vgQ), 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).

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′–C′) 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), scollop (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. morrisi (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 (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′-GCAAACCAACGTGAACGCAAG-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 disc (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
lability can be 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; Shibailat 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**


