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Quantitative developmental genetic analysis reveals that the ancestral dipteran wing vein prepattern is conserved in *Drosophila melanogaster*

Abstract Quantitative complementation tests provide a quick test of the hypothesis that a particular gene contributes to segregating phenotypic variation. A set of wild-type alleles is assayed for variation in their ability to complement the degree of dominance of the quantitative effect of a loss of function allele. Analysis of 15 loci known to be involved in wing patterning in *Drosophila melanogaster* suggests that the genes *decapentaplegic*, *thickveins*, *EGFR*, *argos* and *hedgehog*, each of which are involved in secreted growth factor signaling, may contribute to wing shape variation. The phenotype of one deficiency, *Df(2R)Px2*, which removes *blistered/Plexate*, is also highly sensitive to the wild-type genetic background and at intermediate expressivity reveals six ectopic veins. These form in the same locations as a projection of the ancestral pattern of dipteran wing veins onto the *D. melanogaster* wing. This atavistic phenotype indicates that the wing vein prepatterning mechanism can be conserved in highly derived species, and implies that homoplasic venation patterns may be produced by derepression of vein primordia.

Keywords Quantitative complementation test · Relative warp analysis · Atavistic · EGFR · TGF-β

Introduction

One of the major issues in the study of evolution and development is to determine the relationship between changes in regulatory gene expression that distinguish higher taxonomic levels, and variation at the species level. The basic conundrum is that if a genetic change that distinguishes e.g. a butterfly from a dipteran wing is in-

troduced into one of the species being compared, it is generally predicted to result in a maladaptive phenotype. Despite the presence of genetic variation that could potentially soften the deleterious effects of, and hence increase the probability of invasion of, a macromutation (Mackay and Fry 1996; Gibson et al. 1999), population geneticists generally downplay the contribution of saltationary genetic changes, particularly in animal evolution. Two models that might account for marked changes in the expression of regulatory genes are: (1) that the differences observed between orders result from the gradual accumulation of subtle differences at the species level; and (2) that significant evolutionary transitions involve genes considerably downstream in a genetic hierarchy, and that changes in regulatory genes occur at a later time, without dramatically affecting the phenotype. It is thus important to ask the question whether variation in regulatory genes affects morphology within modern day species.

While interval mapping has become the standard method for identification of regions of the genome that affect quantitative traits, its resolution is too low to locate candidate genes with confidence, and a new approach known as quantitative complementation testing (Long et al. 1996; Mackay and Fry 1996; Gurganus et al. 1999) has been proposed as a quick test for the possible involvement of known genes. Whereas a significant difference in mean phenotype of heterozygous (+/–) and wild-type (+/+) individuals across a range of genetic backgrounds indicates that a mutation affects a trait, the demonstration that a set of wild-type alleles differ functionally requires a test of the interaction between genotypic classes. That is to say, if there is significant variation in the difference between $(+_i/+_t)$ and $(+_i/-)$ for a set of $+_i$ alleles measured in siblings carrying a common tester allele $(+_t)$ or mutation (a *Deficiency* or strong loss of function allele), then the $+_i$ alleles may vary in their degree of dominance, which is a quantitative complementation test.

In practice, a set of isogenic lines carrying different wild-type alleles of a candidate gene are crossed to a

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common inbred line carrying the mutation over a marked tester ($-/+_t$) in replicate, the trait is measured in multiple individuals of each genotype, and analysis of variance is performed to test the significance of the genotype by line interaction term. Graphically, interaction effects are illustrated by crossing of line means in a plot of the mean phenotype of each line in the two backgrounds. If lines do not cross, the mutation has the same effect in each genetic background and hence the wild-type alleles do not vary in their mean effect. Here we use this technique to provide preliminary evidence that genes encoding morphogens with known roles in patterning and differentiation of placement of wing veins also contribute to subtle variation for components of wing shape.

Materials and methods

Fly crosses

All crosses were performed at 25°C with flies grown on standard cornmeal supplemented with live yeast paste. Stock numbers from the Bloomington stock center are indicated in Table 1. Six wild-type lines were chosen to cover a broad range of wing phenotypes, and included Oregon R, Russian 2b, two Ann Arbor inbred isofemale lines (AA3 and AA18), and two inbred isofemale lines from Kenya and South Africa (W6 and W29; Zimmerman et al. 2000). For all comparisons, the mutation-bearing stock was first crossed either to an inbred CyO/PmSp, CyO/Pm or In(2LR)EN/Gla stock, or to a TM6,Ubx/Sb stock, and then individual males of the genotype -/Pm, -/Gla, or -/Ubx were mated with individual virgin females of each wild-type line to obtain F2 siblings carrying either the mutant chromosome or the tester chromosome. Flies were reared at a density of 50–100 larvae/10 ml vial. Each cross was performed in duplicate, and five flies of each sex and genotype per cross were chosen at random for dissection and measurement of both wings.

Wing measurements

Hand-dissected wings were simply mounted between a glass microscope slide and cover slip, and TIFF images were immediately captured using a SPOT camera attached to a Nikon Eclipse microscope at low magnification. The images were then analyzed with M. Rasband's NIH/Scion Image software downloaded from http://www.scioncorp.com, on a Dell Dimension PC, by capturing the XY coordinates of landmarks at the junction of wing veins and/or the wing margin (Fig. 1A). A common file containing the coordinates of all 480 wings (2 replicates × 2 sexes × 2 genotypes × 6 lines × 5 flies × 2 sides) were analyzed using F.J. Rohlf's program TpsRelw Version 1.17 (downloaded from http://life.bio.sunysb.edu/morph) that performs a Procrustes transformation and computes relative warps. Each mutation was analyzed separately, and consequently the warps obtained are independent of those for every other mutation, and in general capture different aspects of shape variation.

Analysis of variance

The distribution of each of the six relative warp scores for each wing according to genotype (G), line (L) and sex (S) was studied by three way ANOVA with the following model:

$$Y = G + L + S + G{\times}L + G{\times}S + S{\times}L + G{\times}S{\times}L + R(G{\times}S{\times}L) + E$$

where all effects were considered fixed, and the error term includes within- and between-individual variance (which were generally of similar magnitude). S tended to be significant, but interaction terms involving S were not. The terms of interest for this study were thus the overall effect of G, and the $G{\times}L$ interaction term. Type III sums of squares were computed using ProcGLM in SAS.

Results and discussion

Quantitative effects on wing shape

Most genes affecting wing development have been characterized on the basis of the homozygous recessive ef-

Table 1 Significance of genotype by line interaction terms from ANOVAs of relative warps

Candidate gene	Allele	Stock	Tester	IVR-B		IVR-C		IVR-D	
				W1	W2	W1	W2	W1	W2
decapentaplegic	Df(2L)dpp^d33	Bellen	PmSp	*	**	***	*	.	.
thickvein	tkv^1	B-427	PmSp	**	.	***	.	.	.
wingless	Df(2L)J-H	B-1357	Pm	.	.	.	.	.	.
engrailed	Df(2R)en-A	B-190	PmSp	.	.	.	.	.	.
			Gla	.	.	.	.	.	.
EGF receptor	Df(2R)Pu-D17	B-2606	PmSp	.	***	.	.	***	**
			Pm	.	**	***	.	***	.
	Egfr^f2	B-2768	PmSp	**	.	***	.	.	.
			Pm	.	**	.	.	.	.
rhomboid	rho^ve-1	B-628	TM6	.	.	.	.	.	.
vein	vn^10567	B-P1749	TM6	.	.	.	.	.	.
spitz	spi^1	B-1859	PmSp	.	.	.	.	.	.
gurken	Df(2L)N22-14	B-2892	PmSp	.	.	*	.	.	.
	grk^2B	Schupbach	PmSp	.	.	.	.	.	.
argos	argos^Δ7	B-1004	TM6	.	.	.	**	**	.
ventral veinless	vvl^sep	B-822	TM6	.	.	.	.	.	.
messy	Df(3R)ry615	B-3007	TM6	.	.	.	***	.	.
	mesA^1	B-4279	TM6	.	.	.	.	.	.
elbow/wb	Df(2L)osp29	B-3078	PmSp	.	.	***	***	**	**
	elB^9	B-4743	PmSp	.	.	.	.	**	.
hedgehog	hh^1	B-450	TM6	.	.	***	.	**	***
	hh^2	B-3376	TM6	.	.	.	.	*	*

*$0.01 < P < 0.005$;
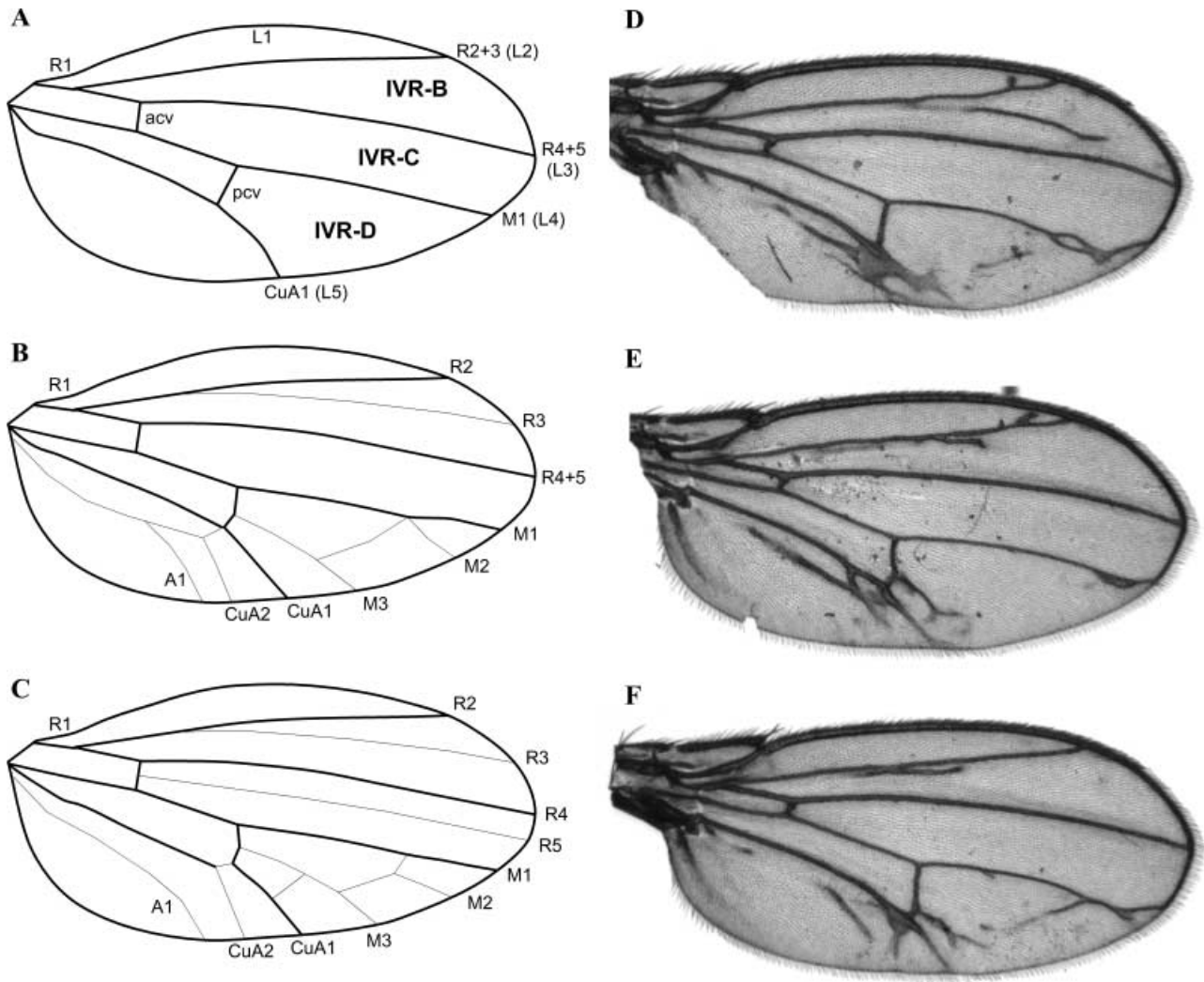**$0.005 < P < 0.0005$;
***$P < 0.0005$; . non-significant

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Fig. 1A–F Ectopic vein formation in *Df(2R)Px2*/AA18 males. **A** Outline of a typical *Drosophila melanogaster* wing, showing Comstock and Needham (1898–99) terminology (*L1*, *R2+3*, *R4+5*, *M1*, *CuA1*) and common developmental genetic usage in brackets (*L1*, *L2*, *L3*, *L4*, *L5*). The three intervein regions scored in this study are shown: landmark coordinates were captured at the junctions of veins, crossveins, and the wing margin (four points for IVR-B and IVR-D; five points for IVR-C; see Birdsall et al. 2000). **B** Projection of the location of ectopic wing veins in *Df(2R)Px2*/AA18 males onto the standard wing shape, based on extrapolation from 20 wings similar to those shown in **D**, **E**, and **F**. Other genetic backgrounds show a range of variation from complete repression of ectopic vein formation, to severe blistering, but veins that do form are consistent with this pattern. **C** Projection of the ancestral wing venation pattern onto the *D. melanogaster* wing, after interpretation of the *Protoplasa fitchii* pattern by Stark et al. (1999). The similarity with **B** is remarkable, differing only in the absence of R5, a connection between A1 and CuA2, and possibly the posterior crossvein between CuA1 and M3 (though a vestige of this may be seen in **E**)

fects of mutations on venation or overall wing shape. To test whether 14 such genes also have a quantitative dominant effect on shape in particular regions of the wing, the Procrustes-transformed landmark coordinates that define intervein regions B, C and D (see Fig. 1A) were subjected to relative warp analysis followed by ANOVA. Relative warps are a highly sensitive morphometric measure (Bookstein 1996) that parse local aspects of intervein region (IVR) shape, such as breadth near the margin or relative length of the crossvein. The measures are not significantly affected by size differences, and hence are almost invariant to the effects of sex and temperature on wing size (Birdsall et al. 2000). For wing shape, the first two relative warps for each IVR (W1 and W2 in Table 1) captured over 85% of the phenotypic variance. With the exception of one warp for each IVR of *rhomboid*, *messy* and *vein*, significant differences between mutant hemi- or heterozygotes ($+_i/-$) and wild-type ($+_i/+_t$) siblings were observed (data not shown). Thus each of the mutation-bearing chromosomes show a quantitative difference associated with the number of wild-type copies of the gene of interest. This result confirms the inference from QTL mapping studies that mutations in a large number of genes can potentially affect subtle aspects of wing shape (Weber et al. 1999; Zimmerman et al. 2000).

Support for the hypothesis that segregating variation at a particular locus affects a trait requires a much more stringent test, such as the quantitative complementation

test. Table 1 indicates the significance of *P*-values associated with the genotype by line interaction term in the ANOVA for each mutation tested against up to six different wild-type lines. Since six different traits (two warps for each of three intervein regions) were measured for each mutation, a significance level of 0.01 was chosen as a conservative indicator that wild-type alleles differ in their complementation of the mutant wing shape defect. This results in rejection of the null hypothesis of no effect for three loci for IVR-B, four loci for IVR-D, and seven loci for IVR-C. For the remaining loci, there is no evidence that wild-type variation has a quantitative effect on wing shape.

Neither *wingless* nor *engrailed* emerged as good candidate modifiers of wing shape, despite the overall effect of mutations at these loci on all three IVRs. Consequently, the *Sternopleural* allele of *wingless* on the *PmSp* marker chromosome is unlikely to be responsible for interaction effects detected with other second chromosome loci. Similarly, the loci encoding the putative EGFR ligands *vein, spitz* and *gurken* as well as the co-factor *rhomboid* can be excluded as good candidate modifiers of wing shape in our sample of six wild-type *D. melanogaster* lines.

The central and anterior portions of the wing, represented by IVR-C and IVR-B respectively, may be affected by variation in TGF-β activity, as both *dpp* and *tkv* show similar effects on both warps of these regions. The *EGF Receptor* also gave a positive result in these wing regions, as well as in the posterior IVR-D. Two different EGFR mutations were tested against two different tester chromosomes, and significant interaction terms were detected in all four cases. Since statistical power studies of quantitative complementation tests have not been performed, it is not clear whether the observed differences in significance levels are real, and hence whether there is allele-specificity to the interactions. Significant results for the repressor *argos* provide further support for the involvement of the EGF pathway in quantitative regulation of wing shape. In IVR-D, two different hypomorphic alleles of *hedgehog* and a mutation and Deficiency affecting *elbow* had strong interaction effects. In most of these cases, the significance of the interaction term is clearly attributable to one or two of the six lines, as visualized by the crossing of line means in the plots shown in Fig. 2B, C and D.

There are two major caveats to quantitative complementation tests that must be considered. Ideally the test should be performed after introgression of just the candidate mutation into a common wild-type tester background by repeated backcrossing so that as little as 5% of the genome is tested (Mackay and Fry 1996), rather than a whole chromosome as here. As a screening method, and dealing with homozygous lethal mutations, this is impractical. Our version of the quantitative complementation test must thus deal with the possibility that either the mutation-bearing chromosome or the marked tester chromosome (for example, *PmSp*, or TM6) also carries a mutation that affects the trait. The latter is con-
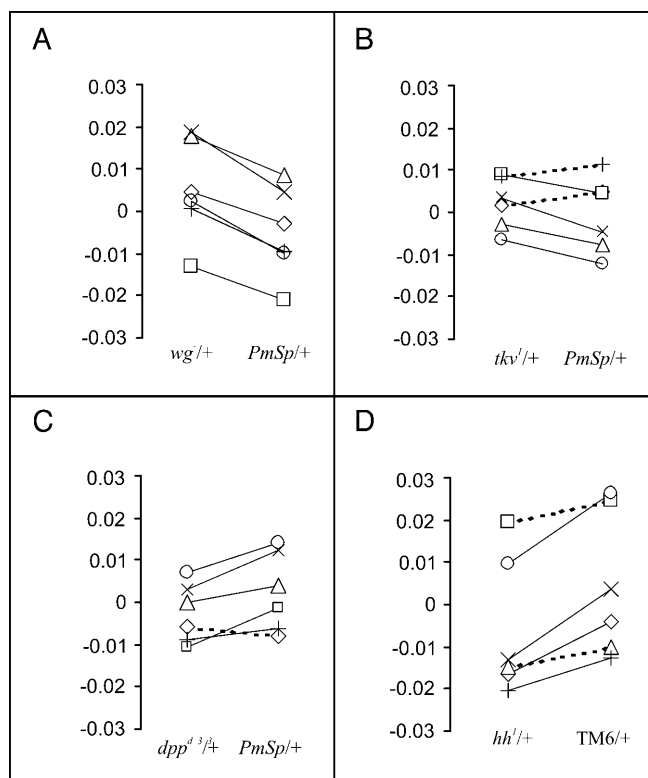

Fig. 2A–D Plots of line means from quantitative complementation tests. The *symbols* represent the mean relative warp values in mutant and tester genotypes from four experiments. In each case, a significant genotype effect is indicated by the *non-horizontal lines* joining means. **A** The lines for IVR-D, warp 2 in the *Df(2L)J-H* (*wg*⁻) cross are nearly parallel, indicating the absence of any genotype by line interaction effect (*P*=0.35). **B** Two lines (A18 and W29, *dotted*) show an increase in relative warp 1 for IVR-C over the tester relative to the *tkv¹* mutant, whereas each of the other lines show a decrease. Consequently, there is crossing of line means, which indicates a genotype by line interaction effect, which is significant from the ANOVA (*P*=0.0004). **C** Similarly, IVR-C warp 1 for *dpp^d33* shows a significant interaction effect (*P*<0.0001) due solely to A18. **D** For *hh¹* IVR-D warp 1, lines A3 and Oregon R produce the significant interaction (*P*=0.005)

trolled to some extent by utilizing the same tester chromosome for several mutations. While significant *G×L* interactions may be due to polymorphisms other than the identified mutation, negative results exclude the wild-type alleles opposite the mutation as a source of quantitative variance and are thus useful for screening candidate genes from further study, and for fine-structure mapping using overlapping deficiencies (Gurganus et al. 1999).

The second caveat concerns interpretation. The inference that a significant *G×L* interaction term indicates complementation of the degree of dominance of the mutation by wild-type variation opposite the lesion is parsimonious. The most obvious alternative is that interactions are produced by epistatic interactions between wild-type alleles anywhere in the genome, and the mutation. Fine structure QTL mapping suggests that epistatic interactions make little overall contribution to wing

shape variation relative to the additive genetic variance, but that they may nevertheless be prevalent, tending to cancel one another out (Weber et al. 1999). The dominance and epistasis models cannot be distinguished with the current experiments. Whether the significant interaction terms are due to dominance or epistatic interactions, our results are nevertheless consistent with an ability of the regulatory genes *dpp*, *tkv*, *EGFR*, *argos*, *elbow* and *hedgehog* to contribute to standing variation for wing shape. As with bristle number, which has been shown to be modified by wild-type variation in genes involved in neurogenesis (Mackay 1996), wing shape appears to be modified in part by genes identified by classical genetic methods.

Atavistic venation

It was not possible to score wing shape in most crosses involving *Df(2R)Px2*, due to the highly variable penetrance and expressivity of the appearance of ectopic veins and wing blisters covering up to two thirds of the wing blade. This deficiency removes cytological bands 60C6 to 60D9, uncovering the *SRF/blistered* locus, which encodes a transcriptional repressor of vein differentiation (Montagne et al. 1996) and has previously been shown to have venation and blistering phenotypes (Roch et al. 1998). The pseudoallelic locus *Plexate* is also removed by this deficiency. Wild-type genetic backgrounds clearly affect the phenotype of *Df(2R)Px2* hemizygotes, and blistering is much more severe in females than males (data not shown). One particular combination, AA18 / *Df(2R)Px2* produced a genetic balance in males (Fig. 1D–F) that allowed us to extrapolate the positions at which ectopic veins tend to form (Fig. 1B). We were able to stabilize this phenotype to some extent by backcrossing *Df(2R)Px2* into AA18 for three generations, with selection for ectopic veins but lack of blistering. Two replicates of this introgression gave similar responses as documented in Table 2, including the appearance of a fraction of females that show the same phenotype. In these lines, the frequency of short vein fragments also increased, though there was no consistent pattern to these and they are considerably less frequent than the six ectopic veins indicated.

The *Drosophila* wing is highly derived and differs from the plesiomorphic condition through the loss of at least a half dozen veins (Comstock and Needham 1898–99; Stark et al. 1999). Numerous authors have homologized the remaining veins as summarized in Fig. 1C, and it is often assumed that the longitudinal veins represent fusions of two adjacent ancestral veins after the loss of intervein tissue. In recent years, analysis of the location of ectopic vein tissue in mutants such as *net* and *plexus* has led to the alternative proposal that several veins are simply repressed, failing to form at boundaries of gene activity that still exist in *Drosophila* (Thompson 1974; Sturtevant et al. 1997). Our analysis of *Df(2R)Px2* provides direct support for this conclusion, as

Table 2 Percentage of *Df(2R)Px2*/AA18 wings showing ectopic veins. *A18* refers to F1 progeny of the cross of *Df(2R)Px2*/SM5 to the near isogenic line A18. *Intro 1* and *Intro 2* refer to replicate 3 generation introgressions of the deficiency into A18 with artificial selection for ectopic veins and against wing blistering

Vein[a]	Male			Female	
	A18	Intro 1	Intro 2	Intro 1	Intro 2
A1	95	69	75	74	76
CuA2	90	69	75	22	46
R3	63	43	37	15	38
M2	58	35	45	30	32
M3	58	31	52	11	22
distal cv	68	25	23	7	19
N	19	67	65	27	37

[a] See Fig. 1 legend for vein identities. *N* Number of wings scored

each of six ectopic veins that form lie in positions where they would be expected if the ancestral condition is simply projected onto the *Drosophila* wing (Stark et al. 1999; Fig. 1C), so the phenotype should be regarded as atavisitc. The ectopic veins include a distal crossvein in IVR-D, and five ectopic longitudinal veins. The only consistent exception is the lack of evidence for an extra vein primordium in the central region of the wing, although evidence for its presence can be seen in certain *plexus* mutants (Thompson et al. 1980).

It is not obvious why a disused prepatterning mechanism for vein formation would be conserved over one hundred million years (Powell and De Salle 1995), unless it plays an integral part in some other aspect of wing morphogenesis. Whatever the reason, its persistence and the observation that atavistic vein phenotypes can be produced by single mutations, implies that the evolution of homoplasic wing patterns may not be uncommon in dipterans. In addition to describing the mechanisms of phenotypic change, developmental studies should thus also contribute to a better understanding of the general tempo and mode of morphological evolution.

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