Evolution of sex-specific wing shape at the widerwing locus in four species of Nasonia

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Abstract

How do morphological differences between species evolve at the genetic level? This study investigates the genetic basis of recent divergence in male wing size between species of the model parasitoid wasp Nasonia. The forewings of flightless N. vitripennis males are 2.3 times smaller than males of their flighted sister species N. giraulti. We describe a major genetic contributor to this difference: the sex-specific widerwing (wdw) locus, which we have backcrossed from N. giraulti into N. vitripennis and mapped to an 0.9 megabase region of chromosome 1. This introgression of wdw from large-winged N. giraulti into small-winged N. vitripennis increases male but not female forewing width by 30% through wing region-specific size changes. Indirect evidence suggests that cell number changes across the wing explain the majority of the wdw wing size difference while changes in cell size are important in the center of the wing. Introgressing the same locus from the other species in the genus, N. longicornis and N. oneida, into N. vitripennis produces intermediate and large male wing sizes. To our knowledge, this is the first study to introgress a morphological quantitative trait locus (QTL) from multiple species into a common genetic background. Epistatic interactions between wdw and other QTL are also identified by introgressing wdw from N. vitripennis into N. giraulti. The main findings are 1) the changes at wdw have sex- and region-specific effects and could therefore be regulatory, 2) the wdw locus appears to be a co-regulator of cell size and cell number, and 3) the wdw locus has evolved different wing width effects in three species.

Keywords

wing size; QTL; introgression; cell size; cell number; sexual dimorphism

INTRODUCTION

A primary goal of biology is to understand the mechanisms behind the diversity of phenotypes among species. Genetic investigation of morphological differences between species has emerged only recently (Orr 2001). Because morphological differences often
involve the modification of basic developmental processes, careful study of these traits can also reveal developmental mechanisms of patterning, growth and size control (Stern 2000). For example, a major component of the size difference between wild and domesticated tomato fruit has been found to act by changing cell number (Frary et al. 2000). This gene, \textit{fw2.2}, was positionally cloned and subsequently implicated as a regulator of the cell cycle in plants (Cong and Tanksley 2006).

Recently, controversy has been building over what types of genetic alteration can cause the sex- and organ-specific changes that are seen in many cases of morphological evolution (e.g., Hoekstra and Coyne 2007, Lynch and Wagner 2008, Stern and Orgogozo 2008). Basically, some have argued that noncoding \textit{cis}-regulatory mutations are a primary source of morphological novelty because regulatory change is thought to be more likely to have spatially or temporally restricted effects than protein sequence change (Stern 2000, Carroll 2005). Others argue that this conclusion is premature: a number of other types of mutations that affect gene regulation have been identified and may be important in morphological evolution (Hoekstra and Coyne 2007, Lynch and Wagner 2008). The controversy is perhaps most acute because relatively few morphological differences between species have been mapped to a fine enough genetic level to ascertain the genetic cause (Hoekstra and Coyne 2007, Stern and Orgogozo 2008).

Sexually dimorphic traits are often the most rapidly evolving traits between species, making them prime candidates for dissection of the genetic basis of development (Kopp and True 2002). For example, investigation of a recently evolved sex-specific abdominal pigmentation difference between \textit{Drosophila} species led to the identification of the \textit{bric-a-brac} gene as a major contributor (Kopp et al. 2000). The pigmentation difference caused by the \textit{bric-a-brac} locus involves changes in \textit{cis}-regulatory binding sites for the sex signaling gene \textit{Doublesex} and the abdominal patterning gene \textit{ABD-B} (Williams et al. 2008). Investigation of recently evolved sexual differentiation can therefore lead to understanding how gene networks acquire sex and tissue specificity in the early stages of their evolution.

Morphological differences between species often involve changes at multiple genetic loci, as revealed by F2 quantitative trait locus (QTL) mapping (e.g., Alpert et al. 1995, Orr 2001, Gadau et al. 2002). A powerful approach to reducing this complexity is introgression of individual QTL through backcrossing into a common genetic background. The introgression approach creates an effectively Mendelian locus which can be analyzed in detail, both phenotypically and genetically. Between-species introgression lines have been widely used to isolate QTL behind important morphological and physiological differences in cultivated plants and their wild relatives (Lippman et al. 2007). The tomato \textit{fw2.2} QTL mentioned above was found in a between-species introgression line, which made it straightforward for the causative gene to be positionally cloned (Frary et al. 2000). In animals, introgression lines have been used in relatively few cases to isolate morphological differences between species, e.g., sex comb morphology in \textit{Drosophila} (Graze et al. 2007), wing size in \textit{Nasonia} (Weston et al. 1999) and male fertility traits in mice (L’Hôte et al. 2007).

The insect wing is essentially a two-dimensional sheet of cells and is therefore a relatively simple model in which to study the cellular mechanisms behind morphological evolution.
Specifically, changes in the size and shape of the wing can be readily attributed to changes in size, shape and number of cells (Dobzhansky 1929). Studies of wing size and shape variation in *Drosophila melanogaster* have revealed, among other things, that cell size and cell number may be under compensatory regulation (McCabe et al 1997). A number of genes which, when mutated, affect wing shape and cell size have been identified. These are often members of conserved growth regulation pathways such as cell cycle, insulin signaling and apoptosis (Hafen and Stocker 2003). Studies of quantitative wing shape variation within *D. melanogaster* have implicated several of these genes as potential contributors to intraspecific variation (Dworkin and Gibson 2006, Weber et al 2008). Wing shape across the *Drosophila* genus is remarkably conserved (Houle et al. 2003) and may be under stabilizing selection or canalization (Gilchrist et al. 2000).

To make full use of the wing as a model of the cellular and genetic basis of morphological evolution, it will be necessary to also study larger differences in wing size and shape. Here, we investigate the genetics of recent sex-specific wing size evolution in *Nasonia*, a genus of parasitic wasps containing four species which have dramatic differences in male wing size (Fig. 1, Darling and Werren 1990, Raychoudhury et al. 2009). *Nasonia* is an emerging genetic model of the evolution of species differences: the species are interfertile after removal of *Wolbachia* bacteria, allowing introgression of QTL for wing size and other traits between species (Breeuwer and Werren 1995, Weston et al. 1999, Clark et al. 2010, Desjardins et al. 2010). In addition, three species’ genomes have now been sequenced and a range of genomic and genetic resources have been produced (Werren et al. 2010). It should also be emphasized that *Nasonia* males are haploid, which simplifies the genetic analysis of complex traits (Weston et al. 1999, Werren and Loehlin 2009 in press).

The most striking morphological difference between *Nasonia* species is the 2.3-fold male-specific wing size difference between small-winged *N. vitripennis* and large-winged *N. giraulti* (Darling and Werren 1990, Weston et al 1999). QTL underlying this difference have been mapped (Gadau et al. 2002), as have QTL for wing length differences between *N. vitripennis* and medium-winged *N. longicornis* (Rütten et al 2004). These analyses have revealed both large effect and epistatic contributors, albeit with a limited level of resolution. Using an introgression approach, one large effect wing-size locus (*ws1*) that increases male wing size by 63% has been previously isolated from *N. giraulti* by introgression into *N. vitripennis* (Weston et al 1999). We have subsequently been able to use *ws1* introgression lines to determine that cell size and cell number changes contribute about equally to its wing size change and to identify the genetic basis of *ws1* by positional cloning (Loehlin et al., 2010).

Here, we report the discovery of a second large-effect wing size QTL in *Nasonia*, called *widerwing* (*wdw*). We identify its genomic location, sex specificity, and morphological effects in multiple introgression lines. Introgression of this QTL from *N. giraulti* into an inbred *N. vitripennis* background increases the wing width in males but not females. An 880 kilobase region containing *wdw* is identified from the overlap of three introgression lines, leading the way to future positional cloning. The effects of *wdw* on cell size and cell number in different regions of the wing are inferred using the pattern of seta on the wing surface. Introgression lines from two other *Nasonia* species reveal that the *wdw* QTL has diverged in
multiple lineages. Epistatic interaction of \textit{wdw} with other QTL is demonstrated using a reciprocal introgression of \textit{N. vitripennis} \textit{wdw} into \textit{N. giraulti}. These approaches allow us to characterize the \textit{wdw} QTL to a level of detail not possible in standard F2 QTL analyses.

**METHODS**

**Widerwing introgressions**

To identify the genetic basis of male-specific wing size, we conducted a set of experiments to backcross male-specific wing QTL from \textit{N. giraulti} into \textit{N. vitripennis}. Here we describe the three introgression strains (B3, D1 and SB2C) that contain the \textit{wdw} QTL. Other wing-size QTL from this screen will be described elsewhere. The B3 and D1 strains were isolated from an experiment where \textit{N. giraulti} males from wildtype strain RV2R were first mated to \textit{N. vitripennis} females from visible marker strain \textit{bl13,st5219}. F1 females were then backcrossed to \textit{bl13,st5219} males and F2 sublines were founded. Subsequent backcrossing of hybrid females was to males of the wildtype \textit{N. vitripennis} LabII strain, removing the unlinked visible markers in the process. Each generation of the backcross, hybrid virgin females were given two \textit{Sarcophaga bullata} hosts to produce haploid male offspring for 48h. The females were then mated to LabII males and given hosts to produce female offspring. We examined the wing size of male offspring of the virgin females and selected on females who were segregating for apparently Mendelian male wing-size loci. Virgin females for the next generation of the backcross were collected from the offspring of these selected females (as in Weston et al. 1999). After 8–12 generations of backcrossing, strains carrying single wing size QTL were made homozygous. The SB2C strain was isolated similarly, except that the initial cross was between different strains: \textit{N. vitripennis} strain \textit{st5219,or123} females and \textit{N. giraulti} strain R16A males (RV2R and R16A are both descendents of the same inbred lab strain, RV2, but with different mitochondria; Breeuwer and Werren 1995). Subsequently, B3, D1 and SB2C were found to contain the same QTL, termed \textit{widerwing} (\textit{wdw}). The B3 strain was later cured of its \textit{Wolbachia} infection and backcrossed into the \textit{N. vitripennis} strain used for genome sequencing (AsymCX) for 14 generations, to produce a homogenous genetic background. The resulting strain (B3bc14) was used for subsequent analyses and is referred to in this paper as \textit{wdw}_s \textit{V} (i.e., \textit{wdw} from \textit{N. giraulti} in \textit{N. vitripennis} background).

The \textit{N. longicornis} allele of \textit{wdw} was similarly isolated, starting with males of the genome sequenced IV7u strain and backcrossing into \textit{N. vitripennis} (AsymCX) for 8 generations. A linked molecular marker discovered in the mapping experiments (S1-i534) was used to select for heterozygous females. Two strains were derived independently after the F1 generation, \textit{wdw}_V-A and \textit{wdw}_V-B, and made homozygous. One \textit{N. oneida} \textit{wdw} introgression into \textit{N. vitripennis}, \textit{wdw}_o-V-B, was isolated from males of an antibiotically treated \textit{N. oneida} female (NoNYBr1136 strain) using the same procedure. Similarly, \textit{wdw} was backcrossed from \textit{N. vitripennis} (AsymCX) into \textit{N. giraulti} (R16A) in 9 generations, producing the \textit{wdw}_G-A introgression strain.
Visible Marker Mapping

Initial mapping suggested distant linkage between wdw and the Linkage Group II (= chromosome 1, Rütten et al 2004) marker reddish5 (rdh5). Visible mapping was therefore conducted between wdw*G, rdh5 and a third chromosome 1 marker, distantennapedia (dant) (Werren and Perrot-Minnot 1999). dant males were mated to B3,rdh5 females and then F1 females were hosted as virgins to generate a visible mapping population of haploid F2 males.

Genotyping

Polymerase chain reaction (PCR) genotyping assays were developed using the Nasonia vitripennis 1.0 genome assembly and N. giraulti and N. longicornis Sanger sequencing reads, which were aligned with NCBI BLAST and Sequencher (Gene Codes). We designed primers to amplify species-specific length polymorphisms (indels) identified on genome assembly SCAFFOLD1. Sequences for these wdw-region primers are: S1-i451-F CTGTGTGGTGACCCCTGATTCC, S1-i451-R AACCAGAGTCTAAAGCCAG, Nv: 563bp, Ng: 501bp. S1-i481-F2 TTTTGCGGGGAAATCCTCG, S1-i481-R GGCTGACTTATGGGCGATTCTC, Nv: 781bp, Ng: 719bp. S1-i534-F TCCAACCATTGTTTGATGATACC, S1-i534-R GCAGATTCTCCGCGATTCT, Nv: 781bp, Ng: 719bp. S1-i540-F GTGGCGTTTGGCGGTTAC, S1-i540-R GTTTTTCAAATACGCACACTTCC, Nv: 455bp, Ng: 535bp. Genomic locations are given in Appendix 1. Each of these assays was found to discriminate N. vitripennis alleles from N. giraulti, N. oneida and N. longicornis.

Genomic DNA was extracted from single wasps using the Squish protocol (Gloor and Engels 1992). All PCR reactions were carried out using 14.3uL ddH2O, 2uL 10x PCR buffer, 0.6uL 10mM MgCl, 0.4uL 10mM dNTPs, 0.8uL 10mM forward primer, 0.8uL 10mM reverse primer, 0.1uL 5u/uL Taq Polymerase (Invitrogen), and 1uL genomic DNA (males) or 0.2uL genomic DNA (females). Reaction cycle for all primer pairs in iCycler (BioRad) thermalcyclers was: 94C for 2 min, 36 repeats of (94C for 30s − 55C for 30s − 72C for 60s), and 72C for 10 min.

Wing measurements

For wing measurements, individual females were provided two fly hosts for 48 hours in a vial at 25C, and measurements were made on the offspring. This design reduces allometric effects of crowding on body size because fewer offspring are produced than can consume the host (Weston et al. 1999). Wildtype strains used were AsymC (N. vitripennis), R16A (N. giraulti), IV7u (N. longicornis), and NoNYBr1136 (N. oneida). Male wing samples were collected from the offspring of virgin females, while female wing samples were collected from the offspring of mated females. The one exception to this was wdw*G. Male samples of this strain were collected from the offspring of cultures containing five mated females and five hosts, as single-female settings only produced diapause larvae. Wings from 5 offspring per vial and 4–10 vials per genotype were collected and mounted (Table 1); occasionally, damaged mounts meant that fewer than 5 wings were measured per vial. Adult fore- and hindwings were dissected at the hinge adjoining the thorax and dry mounted on microscope slides under coverslips. Heads were mounted on double-sided tape to provide a comparative
measure of body size. Wings were photographed on a Zeiss AxioImager Z1 compound scope at 10X as mosaic images. For wing seta counts, wings were re-imaged at 20X through multiple focal planes (z-stacks). Heads were imaged at 4x under darkfield and green autofluorescence to highlight the eyes.

Measurements were performed on the wing images using AxioVision 4.6 software (Zeiss). In this study, wing length is defined as the maximum distance between the notch at the proximal edge of the costal cell and the distal part of the wing (Fig. 2). Wing width is the perpendicular distance between two lines parallel to the length axis that intersect the most anterior and posterior points of the wing. These length and width measurements also defined the proximal-distal (P-D) axis and the anterior-posterior (A-P) axis, respectively (Fig. 2). Wing area was measured by drawing an outline around the wing, starting at the notch. Head width (interocular width), a measure of body size, was measured as the length of a line segment with endpoints at the inner edges of the compound eyes that is tangent to the anterior edges of the paired ocelli.

Linear measurements of forewing sections were calculated from the position of landmarks placed along the P-D axis and A-P axis. Forewing landmark positions for this series of measurements are shown in Fig. 2 (arrowheads). To determine forewing landmarks along the A-P (width) axis, a line parallel to the A-P axis was drawn tangent to the distal part of the stigma. Landmarks were then placed at the intersections between the line and the various veins and folds (Fig. 2).

Regional seta size, number and interseta shape measurements

Prominent, hairlike setae are produced by cells on the wing blade, and have been used in other studies (particularly in Drosophila) to infer cell size and cell number contributions to wing size (e.g. Zwaan et al. 2000). Local differences in cell size can be inferred based on changes in area per seta, while cell number differences can be inferred based on changes in seta number. We have found a 3:1 ratio of cells per seta in both N. vitripennis and another introgressed wing-size QTL (ws1) despite large differences in cell size and number between the two (Werren et al. unpublished). However, we have not experimentally determined the relationship between seta number and cell number for the wdw QTL. Therefore, we refer to changes in seta number, area per seta and interseta shape specifically rather than the inferred mechanisms of cell number, area and shape change.

Locations of all setae on the dorsal forewing surface were determined for a subset of wings (one male wing per vial). Coordinates of all dorsal setae were recorded from 20x z-stacked wing images, excluding setae at the wing margin and on the darkened vein. For interseta shape measurements, each seta’s nearest neighbors in the anterior, posterior, proximal and distal directions were identified using a Perl script. Interseta shape was calculated as the ratio of anterior-posterior to proximal-distal nearest neighbor distances. Wings were then divided into 12 regions using the landmarks shown in Fig. 2. Region area was calculated from landmark coordinates or manually outlined (regions 5 and 9) to exclude the stigma and include the wing margin curvature. Each seta was assigned to a region and the regional seta number and average interseta shape were computed. Area per seta for each region was calculated by dividing the region area by the number of setae.
Statistics

Pairwise comparisons between strains were conducted using Tukey’s Honestly Significant Difference (HSD), based on ANOVAs with vial as a nesting factor, with the sample sizes shown in Table 1 unless noted otherwise. Because several morphological variables were measured per wing, we used the conservative Bonferroni correction for multiple tests. P-values shown were corrected by multiplying by the number of tests conducted in each analysis (Basic wing measurements (Table 1), 7 variables; Linear wing sections (Table 2), 8 variables; Regional seta patterns (Fig. 4), 52 variables).

We tested for compositional epistasis between \( wdw \) and the genetic (species) background using an additive and a multiplicative model of wing length and width. In the additive null model, if there is no epistasis, there should be no difference between \((wdw_gV - wdw_vV)\) and \((wdw_gG - wdw_vG)\). In the multiplicative null model, there should be no difference between \((wdw_gV / wdw_vV)\) and \((wdw_gG / wdw_vG)\). T-tests (planned comparisons based on nested ANOVAs) were calculated for the additive and multiplicative models using variances for the difference and ratio of two independent random variables, respectively.

RESULTS

Isolation of widerwing introgression lines

Major-effect wing size QTL were backcrossed from large-winged \( N. \) giraulti into small-winged \( N. \) vitripennis. Three Mendelian segregating wing width QTL were isolated and made into homozygous introgression strains (B3, D1, and SB2C). All F2 male offspring of crosses between the three strains had the same wider wing phenotype, indicating that the three strains contained the same QTL. This locus was named widerwing (\( wdw \)) for its distinct width effect. This \( wdw_gV \) introgression phenotype can be readily distinguished by eye from the “tapered” shape wings of \( N. \) vitripennis \( wdw_vV \) males. In particular, \( wdw_gV \) gives the forewing a specific “triangular” shape and greatly broadens the spacing between the stigmal vein and the median fold, whose curvature is also changed (Fig. 2). A descendant of the B3 strain, called B3bc14, was chosen for detailed analysis of wing features because it is in the same background used to produce \( wdw \) introgressions from the other species. Forewing length, width and area are not significantly different between males of the B3, D1, SB2C, or B3bc14 strains (Tukey’s HSD, \( p < 0.05; n = [40,20,20, and 40] \) males from \( b = [8,4,4, and 9] \) vials).

Mapping the widerwing introgressions

A future goal is to identify the \( wdw \) gene by positional cloning. As a first step, we identified the location of \( N. \) giraulti DNA in the \( wdw_gV \) lines using the newly sequenced \( Nasonia \) genomes. We first mapped the \( wdw_gV \) introgression phenotype using visible markers. The \( wdw \) phenotype shows linkage to the Linkage Group II (Chromosome 1, Rütten et al. 2004) markers \( rdh5 \) and \( dant \) (chi-square tests, \( p < 0.01, n=696 \)). Map distances are 61cM (Kosambi corrected) between \( wdw \) and \( rdh5 \), 78cM between \( wdw \) and \( dant \), and 21cM between \( rdh5 \) and \( dant \).
We used this visible map to estimate the location of the wdwV introgressions in the sequenced N. vitripennis and N. giraulti genomes. A molecular marker in the inferred wdw region (NVC1_13 in SCAFFOLD1, Niehuis et al. 2008) was identified based on its map position relative to a homolog of the Drosophila gene spineless-aristapedia, which was proposed to be a candidate for the dant mutation (Werren and Perrot-Minnot 1999). The NVC1_13 marker shows giraulti genotypes for two of the three wdwV introgressions (Appendix 1). Additional markers were then designed along SCAFFOLD1 and used to define the introgressed giraulti region within the three wdwV lines (Fig. 3 and Appendix 1). Confirming that this was the correct region, the wdw phenotype cosegregated perfectly with the i481 and i540 markers in 180 F2 male offspring of crosses between the D1 introgression and wildtype N. vitripennis. The shared overlapping giraulti regions of the three wdwV lines define the possible location of wdw to a region of 880 kilobases (Fig. 3).

**Wing shape effects of the wdwV introgression**

Widerwing is a major contributor to the 2.3-fold male wing size difference between N. vitripennis and N. giraulti (Fig. 1 and Table 1). The wdwV introgression recovers 36% of the difference in forewing width and 28% of the difference in length between the species. Relative to wildtype N. vitripennis (wdwV), the wdwV introgression increases male forewing length by 8%, forewing width by 29%, and forewing area by 32% (Tukey’s HSD, p < 0.05) (Table 1). Male hindwing size is also increased, though not as severely; length and width increase by 10% and area increases by 20%. In contrast, female forewing and hindwing length, width and area are completely unaffected by the introgression (p > 0.05). It is noteworthy that such a large effect locus is entirely sex-specific (Fig. 1).

To determine whether the observed male wing shape changes derive from uniform changes or localized changes in size and shape, we divided forewings into sections using landmarks along the length (A-P) and width (P-D) axes (Fig. 2A). Section distances were significantly greater across most of the wdwV forewing when compared with wdwV (Table 2). Large localized changes are also seen. The central section increases in width by a striking 74%, corresponding to the displacement of the median fold (Fig. 2), while the other sections along the A-P axis increase by 16–25% (p < 0.05). Along the P-D axis, the two distal sections are 15–16% longer. The proximal section is also significantly increased, but only by 4%. To summarize, the wdwV introgression increases wing size across the forewing, but the magnitudes of these increases are region-specific.

**Changes to cell size, number, and shape inferred from wing seta pattern**

The locally different changes in wing shape seen in the wdwV introgression could be caused by changes in cell number, size or shape. Setae (cell hairs) cover most of the forewing and can be used as a proxy for cells. The forewing was divided into regions based on sections of the A-P and P-D axis (Fig. 2) and the pattern of seta number, area per seta and shape was determined in a subset of wings.

wdwV increases seta number across most of the wing and area per seta at a local level (Fig. 4). Seta number is significantly greater across the central and distal sections of the wdwV forewing (Fig. 4B and data not shown) (p < 0.05, n=8). This corresponds to a significant
43% increase in seta number across the wing (Fig. 4A), suggesting an overall increase in cell number. The most dramatic local changes occur in the central-stigmal region (region 6, Fig. 4B). In this region, area increases by 161%, seta number by 99% and area per seta by 32%. Significant area per seta increases are found in this region but not overall, suggesting a local increase in cell size. Interseta shape is significantly wider overall, but by a relatively minor 1.7%. We can therefore infer that \( \text{wdw}_g V \) causes increases in cell number in most of the wing and increases in cell size restricted to the center of the wing.

**Widerwing from \( N. \text{longicornis} \)**

We subsequently backcrossed the region containing \( \text{wdw} \) from other \( Nasonia \) species. \( N. \text{longicornis} \) and \( N. \text{giraulti} \) share a common ancestor with \( N. \text{vitripennis} \) but are more closely related to one another (Campbell et al. 1993, Raychoudhury et al. 2009). In addition, \( N. \text{longicornis} \) male wings are intermediate in size between \( N. \text{vitripennis} \) and \( N. \text{giraulti} \) (Fig. 1 and Table 1). We therefore speculated that the \( \text{wdw} \) phenotype might be shared between \( N. \text{longicornis} \) and \( N. \text{giraulti} \). The \( N. \text{longicornis} \) allele of \( \text{wdw} \) was backcrossed into \( N. \text{vitripennis} \) for 8 generations. Molecular markers found in the \( \text{wdw}_g V \) introgressions were used to select on the same genomic region during the backcrosses. Two strains were isolated, \( \text{wdw}_l V-A \) and \( \text{wdw}_l V-B \). Both strains share \( \text{longicornis} \) genotypes at markers in the \( \text{wdw} \) region and most flanking regions (Appendix 1). Male wings of these two strains were not significantly different for forewing or hindwing length or width (\( p > 0.05 \), \( n=20 \), \( b = 5 \)), so the measurements were pooled.

The \( N. \text{longicornis} \) \( \text{wdw} \) locus (\( \text{wdw}_l V \)) has an intermediate effect when introgressed into \( N. \text{vitripennis} \) (Fig. 1 and Table 1). Specifically, \( \text{wdw}_l V \) male forewings are 11% wider and have 11% larger area than \( \text{wdw}_V \) (\( p < 0.05 \)). Forewing length and hindwing length, width and area are not significantly different (\( p > 0.05 \)). These changes are not as large as those seen in \( \text{wdw}_g V \); \( \text{wdw}_l V \) has only 24% of the increase in forewing width and 38% of the increase in forewing area seen in \( \text{wdw}_g V \). The \( \text{wdw}_l V \) introgression recovers 23% of the species difference in male forewing width between \( N. \text{longicornis} \) and \( N. \text{vitripennis} \), which is less than the 36% recovery seen in \( \text{wdw}_g V \).

The \( \text{wdw}_l V \) introgression also shows small but significant reductions in female wing size (4–6% smaller than \( \text{wdw}_l V, p < 0.05 \), Table 1) and head width (4%). Head width has been shown to covary with wing size (Weston et al. 1999). Including head width as a covariate with wing length and width (not shown) reduced the mean forewing length and width differences between \( \text{wdw}_l V \) and \( \text{wdw}_V \) females to 2.8%, although these adjusted means were still significantly different. Male and female \( \text{wdw}_l V \) wings are both smaller than \( \text{wdw}_g V \). However, the relative wing width difference is much greater in the males (area is 11% larger than \( \text{wdw}_V \) and 16% smaller than \( \text{wdw}_g V \)). Further reduction of \( \text{longicornis} \) flanking regions around \( \text{wdw}_l \) will be needed to resolve whether these effects are due to \( \text{wdw}_l \) or linked genes.

**Changes in wing sections and seta pattern in \( \text{wdw}_l V \)**

At both a broad and a local level, the \( N. \text{longicornis} \) introgression of \( \text{wdw} \) has wing effects intermediate between the \( N. \text{giraulti} \) and \( N. \text{vitripennis} \) loci. The central forewing is also the
largest changed section in wdwV, though the magnitude (28%) is less than is seen in wdwgV (Table 2, p < 0.05). In contrast, the distal section of the A-P axis, which was longer in wdwgV than in wdwV, is not significantly longer in wdwV. This expansion of distal section length in wdwgV but not wdwV is noteworthy, since it differs from the pattern of intermediate size seen in other wing sections and may therefore be a discrete effect.

The seta pattern in wdwV is consistent with its intermediate wing size (Fig. 4). In the center of the wing (region 6; Fig. 4B), region area is increased by 50%, seta number is increased by 21% and area per seta is 26% larger (p < 0.05, n=8). While the area per seta increase in the center of the wing is similar to wdwgV, the seta number expansion in wdwV appears to be more spatially restricted than with wdwgV as no seta number difference across the wing was detected (Fig. 4A). Therefore, a more localized change in cell size and number may be responsible for the intermediate wing width increase in wdwV.

Widerwing from N. oneida

N. oneida, a newly discovered species that is sister to N. giraulti, has large male wings, similar to but slightly smaller than N. giraulti (Raychoudhury et al. 2010). Backcross of the wdw region from N. oneida into N. vitripennis yielded a strain (wdw_oV-B) with male forewings visibly similar to wdwgV (Fig. 1). Supporting visual observations, forewing length, width, area and hindwing length are not significantly different (p > 0.05) (Table 1). However, hindwing width is 4% smaller relative to wdwgV males (p < 0.05). These effects are also male specific: females of the wdw_oV-B introgression do not differ from wdwgV in forewing or hindwing length or width (p > 0.05). With the exception of male hindwing width, the wdw introgression from N. oneida has essentially the same phenotype as the introgression from its sister species N. giraulti.

N. vitripennis widerwing in the N. giraulti background

To determine if wdw has epistatic interactions with other QTL, we performed the reciprocal introgression of the N. vitripennis allele of wdw into large-winged N. giraulti, again using a linked molecular marker. Consistent with this wdwG strain containing the wdw QTL, forewing width is 9% smaller and hindwing width is 8% smaller than N. giraulti (wdwG) (p < 0.05) (Table 1). Forewing length and hindwing length are not significantly different between strains (p > 0.05). The most obvious similarity between the reciprocal wdwG and wdwG introgressions is that the giraulti alleles have wider wings in both backgrounds (Fig. 5). The wdwG introgression recovers 17% of the species difference in wing width, about half of the recovery caused by the wdwG introgression.

If wdw had no epistatic interactions with other QTL that differ between the species, the effects of wdw genotype and genetic background should add (or possibly multiply) together (Phillips 2008). However, the interaction between wdw and species background is not consistent with either an additive or multiplicative model of forewing length, width or area (t-tests, p < 0.05). This can be seen visually in Fig. 5, where the distance between the smaller wings (vitripennis background) is greater than the distance between the larger wings (giraulti background). Therefore, wdw interacts epistatically with other QTL that differ between vitripennis and giraulti. Such compositionally epistatic QTL are likely to be in the
same pathway as wdw and will be interesting candidates for future study. The introgression experiments do not rule out that mitochondrial type contributes to the wing phenotype, although it would be surprising that this would be sex specific. Furthermore, this would be an epistatic interaction, albeit between nuclear and mitochondrial genotypes. Epistatic interactions could also involve maternal genotype influences upon male phenotype. However, our genetic analysis clearly shows that wdw is inherited in a mendelian fashion from heterozygous mothers. Therefore, any maternal effects of wdw or epistatic interactors are likely to be subtle, if they occur at all.

DISCUSSION

Widerwing, a large effect wing size QTL, identified and mapped by introgression

The four Nasonia species differ substantially in male wing size. We took advantage of the interspecies genetic tools in this system to introgress and characterize alleles of a large effect QTL, wdw. The introgression approach allowed us to map the genomic location of the locus and to determine the morphological effects of microevolution at this QTL in four separate species. Differences between multiple species at a single QTL have been examined before at the level of F2 analysis (e.g., Alpert et al. 1995). To our knowledge, though, this is the first report of a morphological QTL introgressed from multiple species into a common genetic background.

Further highlighting the value of the introgression approach, this substantial wing width effect was not identified in the original F2 wing QTL maps (Gadau et al 2002, Rütten et al. 2004). This is particularly remarkable because wdw introgressions recover 36% of the N. vitripennis - N. giraulti species difference in wing width in the N. vitripennis background and 17% in the N. giraulti background. A likely explanation for the absence of wdw from the F2 QTL map is the effects of a hybrid inviability locus which has been identified 20–30cM away from the wdw region (Niehuis et al. 2008). Such hybrid incompatibility loci limit the effectiveness of F2 mapping between species by creating ‘pseudo-linkage’ (Weston et al. 1999). However, incompatibilities can be beneficial in the introgression approach, as they select for recombinants between the incompatibility and the QTL and can therefore increase the effective recombination rate during the backcross.

Sex and region specificity of wdw suggests regulatory evolution

In addition to mapping the locus, a finding of this study is that the degree of male-specific wing size change at the wdw locus parallels the overall species differences in male wing size (i.e. N. giraulti large, N. longicornis intermediate, and N. vitripennis small). Sexually dimorphic traits in other taxa tend to be rapidly evolving and are therefore useful for investigating the evolution of development between closely related species (Kopp and True 2002). Changes in cis-regulatory binding sites for members of the sex-determination system have been implicated in recently evolved sexual dimorphism in Drosophila (Williams et al. 2008) and could be involved here, as well.

The sex specificity and regional effects of wdw introgressions are evidence that the underlying genetic changes have low pleiotropy. Reduced pleiotropic effects are considered
to be indicative of cis-regulatory rather than coding sequence changes (Carroll 2005). Under this hypothesis, the observed low pleiotropy, sex-specific, and region-specific differences between wdw alleles indicate cis-regulatory mutations at the locus. The generality of the cis-regulatory hypothesis is controversial, however. Existing evidence for cis-regulatory causes of morphological evolution is limited and cis-regulation is not the only possible molecular change through which pleiotropy can be reduced (Hoekstra and Coyne 2007, Lynch and Wagner 2008). Currently we do not know whether cis-regulatory or coding changes are involved at wdw, and further fine scale mapping and functional analysis are needed to resolve this question.

**Multiple genetic changes at the wdw locus occurred across species**

The wdw loci from several Nasonia species have varying effects on wing length and width in the common N. vitripennis background. What is surprising about this result is that the same locus is responsible for multiple cases of wing size evolution within the relatively short divergence time of the Nasonia genus (≤1 MYA, Campbell et al. 1993). This raises the question of whether the phenotypic differences between wdw\textsubscript{V}, wdw\textsubscript{L}V and wdw\textsubscript{G}V are due to multiple changes in a single gene, or at different but tightly linked genes. At the present megabase resolution of mapping, we cannot distinguish between these possibilities. A case of multiple substitutions at the same morphological locus (cis-regulatory elements of the shavenbaby gene), each with isolated regional effects, has been reported for larval trichome patterns between Drosophila species (McGregor et al. 2007). We similarly observed a combination of discrete and continuous lineage specific wing region effects in the wdw introgressions from three species. The wdw locus could therefore represent another case of multiple substitutions affecting the same gene. Involvement of a single locus in evolution of sex specific wing size regulation in three different species would imply that only a few targets are available for such evolutionary changes.

**wdw interacts with other diverged QTL**

Reciprocal introgression showed that the effects of wdw\textsubscript{G} and wdw\textsubscript{V} differ based on the genetic (species) background. Such epistatic effects are evidence of genetic interaction, and in this case epistasis reveals that genes that interact with wdw have also diverged between N. vitripennis and N. giraulti. Identifying the location and genetic basis of these QTL has the potential to uncover multiple components of the sex specific wing-size gene network in the Nasonia.

**Cell size, cell number and organ size evolution**

How the size of organs such as the wing is established has long interested biologists (Dobzhansky 1929, Leevers and McNeill 2005, etc.). The size of an organ is determined ultimately by the size and number of its cells. In recent years, a number of genes involved in regulation of cell size and number have been identified by mutation analysis. These include members of the Insulin Receptor and Tor signaling pathways (cell size and number regulation) and the cell cycle and Hippo signaling pathways (cell proliferation and apoptosis) (Johnston and Gallant 2002, Leevers and McNeill 2005). These pathways may not be directly involved in the regulation of organ size, however, because other signals
appear to compensate organ size by increasing cell size when cell number is experimentally reduced and vice versa (Crickmore and Mann 2008). Organ size may instead be established independently of cell size and number by instructive patterning signals such as secreted morphogens or cell adhesion genes, which have been proposed to potentiate physical size sensing between cells (Leavers and McNeill 2005, Crickmore and Mann 2008). Although much is known about how these pathways operate in Drosophila and mice, which pathways are important for establishing differences in organ size remains unexplored. We are aware of only one gene that has been identified as causing the evolution of organ size: *fw2.2*, a previously undescribed cell cycle regulator in plants (Cong and Tanksley 2006). Identifying the mechanisms behind more cases of cell size and number evolution should lead to further understanding of how organ size is determined.

We found that a combination of broad seta number and localized seta area changes contribute to interspecific wing-size differences at the *wdw* locus. In an investigation of clinal variation in *Drosophila* wing size, Zwaan et al. (2000) found that cell number (as estimated by setae) was the predominant mechanism behind variation in wing area as well, and concluded that cell size and cell number compensate one another in that system. With *wdw*, we see no evidence of compensation between cell size and number. This result suggests either that *wdw* operates at the level of patterning or else that the organ size compensation mechanism may not be as important as has been argued.

The *wdw* QTL has two effects: a region-specific difference in area per seta (cell size) and a broader change to seta number (cell number). It is possible that multiple genes in the *wdw* region separately encode the cell size and cell number effects. Assuming that this is not the case, how could changes to one gene increase cell size in one region while simultaneously increasing cell number across the wing? One hypothesis is a change in morphogen pattern. Specifically, wing cell lineages in certain regions might respond differently to changes in a single *wdw* signal. Second, changes in sensitivity to the concentration gradient of a morphogen could give a similar result. Third, the regional area per seta change could be an indirect effect of increasing wing width, such as by diluting an inhibitory effect of the wing vein on cell size.

**Future directions**

This study raises a number of questions about the structure of the *wdw* locus, whether the underlying changes are *cis*-regulatory, and how *wdw* regulates cell size and cell number. These can only be answered by positional cloning the *wdw* QTL and by identifying the genes downstream of *wdw*. Positional cloning and gene expression studies are practical in *Nasonia*, given the advantages of male haploidy and a suite of genome resources (Werren et al. 2010). Interspecies introgression is a powerful genetic tool. Our finding that introgression is practical between four different *Nasonia* species opens the door to future studies of a wide range of complex traits.

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Fig. 1.
Male forewing size differs across the *Nasonia* genus and in introgressions of the *wdw* locus from each species, but female forewings of *wdw* introgressions are not affected. Cladogram depicts relationships between the *Nasonia* species (Raychoudhury et al. 2009). Scale bar: 200um.
Fig. 2. Landmarks, axes and wing regions used in this study. Black lines represent the length (P-D) and width (A-P) axes. Arrowheads show forewing landmarks used for calculating distances along the A-P axis. Regions used for seta counts are depicted with numbers and are defined by landmarks (white circles) placed at the intersection of either the wing margin, folds (thick white curves) or translations of the A-P and P-D axis (thin white lines).
Fig. 3.
Map of the wdw region. Genotype at length polymorphism (indel) markers is shown for the three \( wdw_g V \) introgressions. Dotted line: Consensus location of the \( wdw \) QTL based on shared overlapping markers in the region. Black bars: \( N. \) vitripennis (background) genotype. Open bars: \( N. \) giraulti (introgression) genotype. Shading: Genotype between two markers is unknown. Marker locations in SCAFFOLD1 and map data for other introgressions are given in Appendix 1.
Fig. 4.
Seta number, area and shape contributions to forewing regions. a: Change averaged across all twelve seta-containing regions. b: The largest changed region (region 6) shows local area-per-seta effects. Interseta shape values greater than one indicate that seta are farther apart along the A-P (width) axis. Relative mean ± standard deviation is shown. Letters denote contrast groups for multiple comparisons (Tukey’s HSD, n=b=8).
Fig. 5.
Compositional epistasis between \textit{wdw} and genetic background. If \textit{wdw} does not interact with other QTL in the species background and wing length (for example) is additive, $\Delta \text{length}[V]$ should equal $\Delta \text{length}[G]$. 
Table 1

Basic measurements of \textit{wdw} introgression and wildtype strains. Length, width, area and sample size of forewings and hindwings is shown. Absolute values (length and width in \textmu m; area in \textmu m$^3$) are shown for \textit{wdw}_V males. Measurements relative to \textit{wdw}_V males are shown for both sexes of all other genotypes. Mean ± standard deviation is shown. N: sample size for males, nested in vials (b) N.d., measurement not determined.

| genotype | Forewing length | Forewing Width | Forewing Area | Hindwing length | Hindwing width | Hindwing area | head width | N (b) |
|----------|-----------------|---------------|--------------|----------------|---------------|--------------|-----------|-------|
| Males (absolute) | \textit{wdw}_V | 1065 ± 28 | 326 ± 11 | 242000 ± 13000 | 716 ± 26 | 176 ± 6 | 86000 ± 5000 | 402 ± 9 | 40(8) |
| Males (relative) | \textit{wdw}_V | 1.00 ± 0.03 | 1.00 ± 0.03 | 1.00 ± 0.05 | 1.00 ± 0.04 | 1.00 ± 0.04 | 1.00 ± 0.05 | 1.00 ± 0.02 | 40(8) |
| \textit{wdw}_V | 1.08 ± 0.03 | 1.29 ± 0.06 | 1.32 ± 0.09 | 1.10 ± 0.04 | 1.10 ± 0.05 | 1.20 ± 0.08 | 1.01 ± 0.03 | 40(9) |
| \textit{wdw}_V | 1.06 ± 0.04 | 1.28 ± 0.08 | 1.27 ± 0.12 | 1.07 ± 0.06 | 1.03 ± 0.06 | 1.12 ± 0.11 | 0.98 ± 0.04 | 40(9) |
| \textit{wdw}_V | 1.02 ± 0.03 | 1.11 ± 0.04 | 1.11 ± 0.07 | 1.04 ± 0.04 | 1.05 ± 0.05 | 1.08 ± 0.08 | 1.01 ± 0.03 | 40(10) |
| \textit{wdw}_G | 1.28 ± 0.04 | 1.81 ± 0.05 | 2.16 ± 0.13 | 1.39 ± 0.04 | 1.48 ± 0.06 | 2.15 ± 0.15 | 1.01 ± 0.03 | 40(8) |
| \textit{wdw}_G | 1.27 ± 0.03 | 1.66 ± 0.05 | 2.00 ± 0.11 | 1.39 ± 0.04 | 1.36 ± 0.05 | n.d. | 1.00 ± 0.03 | 20(4) |

Females (relative) | \textit{wdw}_V | 1.88 ± 0.03 | 2.80 ± 0.06 | 4.84 ± 0.18 | 2.01 ± 0.04 | 2.41 ± 0.08 | 5.08 ± 0.23 | 1.23 ± 0.04 | 40(8) |
| \textit{wdw}_V | 1.88 ± 0.03 | 2.78 ± 0.05 | n.d. | 2.01 ± 0.04 | 2.42 ± 0.06 | n.d. | 1.24 ± 0.02 | 20(5) |
| \textit{wdw}_V | 1.81 ± 0.06 | 2.72 ± 0.10 | n.d. | 1.93 ± 0.08 | 2.34 ± 0.12 | n.d. | 1.19 ± 0.05 | 20(5) |
| \textit{wdw}_V | 1.78 ± 0.07 | 2.63 ± 0.11 | n.d. | 1.91 ± 0.09 | 2.27 ± 0.11 | n.d. | 1.18 ± 0.04 | 20(5) |
| \textit{wdw}_G | 1.62 ± 0.03 | 2.44 ± 0.05 | 3.64 ± 0.16 | 1.73 ± 0.03 | 2.16 ± 0.06 | 3.91 ± 0.18 | 1.21 ± 0.03 | 40(8) |
Table 2

Linear measurements of male forewing sections. Sections are defined in Fig. 2. Section width or length was calculated as the component of interlandmark distance along the anterior-posterior (A-P) or proximal-distal (P-D) axis, respectively. Absolute and relative means ± standard deviations are shown for \( w_{dv}, V \) while relative means ± relative standard deviations are shown for \( w_{dg}, V \) and \( w_{dl}, V \). Letters denote contrast group for multiple comparisons (Tukey’s HSD, \( b=8 \)).

| Region          | \( w_{dv}, V \) (um) | \( w_{dv}, V \) relative to \( w_{dv}, V \) |
|-----------------|-----------------------|--------------------------------------------|
| Forewing anterior | 95 ± 5                | 1.00 ± 0.05 A                            |
|                 |                       | 1.25 ± 0.06 B                            |
|                 |                       | 1.13 ± 0.05 C                            |
| Central         | 60 ± 4                | 1.00 ± 0.07 A                            |
|                 |                       | 1.74 ± 0.13 B                            |
|                 |                       | 1.28 ± 0.13 C                            |
| Median          | 97 ± 6                | 1.00 ± 0.06 A                            |
|                 |                       | 1.20 ± 0.06 B                            |
|                 |                       | 1.03 ± 0.07 A                            |
| Posterior       | 63 ± 4                | 1.00 ± 0.06 A                            |
|                 |                       | 1.16 ± 0.06 B                            |
|                 |                       | 1.13 ± 0.08 B                            |
| Forewing proximal| 463 ± 14              | 1.00 ± 0.03 A                           |
|                 |                       | 1.04 ± 0.04 B                            |
|                 |                       | 1.02 ± 0.04 AB                           |
| Marginal        | 189 ± 12              | 1.00 ± 0.06 A                            |
|                 |                       | 1.03 ± 0.05 A                            |
|                 |                       | 0.99 ± 0.04 A                            |
| Stigmal         | 133 ± 8               | 1.00 ± 0.06 A                            |
|                 |                       | 1.16 ± 0.07 B                            |
|                 |                       | 1.06 ± 0.06 C                            |
| Distal          | 278 ± 10              | 1.00 ± 0.04 A                            |
|                 |                       | 1.15 ± 0.05 B                            |
|                 |                       | 1.02 ± 0.03 A                            |
Appendix 1

Molecular marker map of wdw introgressions. Marker positions are for *N. vitripennis* genome assembly v1.0, SCAFFOLD1. Primer sequences for markers are in the Methods section. N.d., not determined.

| Marker Position (bp) | S1-4451 4519674 | S1-4481 4817310 | S1-4534 5348235 | S1-4540 5400567 | NvC1-13 5488802 | S1-4580 5803622 |
|----------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Strain               |                 |                 |                 |                 |                 |                 |
| wdwV B3BC14          | vitripennis     | giraulti        | giraulti        | giraulti        | giraulti        | giraulti        |
| wdwV B3              | vitripennis     | giraulti        | giraulti        | giraulti        | giraulti        | giraulti        |
| wdwV D1              | giraulti        | giraulti        | giraulti        | giraulti        | giraulti        | giraulti        |
| wdwV SB2C            | giraulti        | giraulti        | giraulti        | vitripennis     | vitripennis     | vitripennis     |
| wdwV-B               | oneida          | oneida          | oneida          | oneida          | n.d.            | oneida          |
| wdwV-A               | longicornis     | longicornis     | longicornis     | longicornis     | n.d.            | longicornis     |
| wdwV-B               | longicornis     | longicornis     | longicornis     | longicornis     | n.d.            | vitripennis     |
| wdwV-G               | vitripennis     | vitripennis     | vitripennis     | vitripennis     | n.d.            | giraulti        |