INTRODUCTION

Understanding the evolutionary and developmental basis of novel morphological, physiological and behavioral traits is a critical problem in genetics. Such traits can represent fundamental differences between closely related species, and dissecting their genetic and developmental emergence can help to uncover the evolutionary processes that occur during species divergence. In combination with elucidating the genetic basis of phenotypic differences between species, it is additionally essential to characterize the variation that segregates within natural populations, and articulate the relationship between inter- and intraspecific trait variation. One of the oldest debates in evolutionary biology is over the forces that maintain standing phenotypic variation within species (Provine, 1971; Lewontin, 1974). One set of models posit that variation is largely a result of intermediate-frequency polymorphisms that are actively maintained in populations by balancing selection (Barton and Keightley, 2002; Turelli and Barton, 2004). These models are consistent with Darwin’s idea that variation between species is simply an extension of preexisting variation segregating within populations (Darwin, 1859; Lewontin, 1974). An alternative series of models propose that the bulk of variation is due to continuously arising, individually rare deleterious mutations (Johnson and Barton, 2005). If true, species diversification occurs by the rapid fixation of highly infrequent spontaneous advantageous mutations, such that there are qualitative differences between variants leading to species differences and variants that segregate within species. One effective way to determine the evolutionary processes acting on complex trait variation is to experimentally identify and characterize the underlying molecular genetic basis of multiple quantitative trait loci (QTLs), identify the genes involved and ultimately detect the molecular signatures of specific selective forces at functional loci (Mitchell-Olds et al., 2007).

Male secondary sexual traits—courtship displays, body coloration, ornaments, armaments/weapon and so on—provide abundant examples of evolutionary novelty, with many instances of rapidly evolving traits discriminating closely related species (Eberhard, 1985; Andersson, 1994). Evolution of these traits is ultimately because of competition for reproduction, driven by sexual selection on variation in male traits via processes such as female choice, male-to-male competition and sexual conflict (Parker, 1979; Andersson, 1994; Arnqvist, 1998; Hosken and Stockley, 2004; Emlen et al., 2005; Emlen, 2008). By studying the genetic control of male-specific secondary sexual traits within and between species, we can learn about the processes and selective forces leading to diversification.

In Drosophila one such male-limited secondary sexual trait is the sex comb, a cluster of specialized bristles present on the forelegs of some species within the melanogaster and obscura groups (Kopp and True, 2002; Kopp, 2011). Among closely related species within these clades, sex combs vary radically in the number of ‘teeth’ on each comb, the morphology of the teeth, the orientation of the comb relative to the leg axis and the position and number of comb arrays on the forelegs (Kopp and True, 2002; Barmina and Kopp, 2007; Kopp, 2011).
The function of the sex comb has been examined in a number of studies that have physically and genetically ablated the entire comb, or experimentally reduced the number of teeth on each comb, to assess the behavior of manipulated males and their ability to copulate. Early studies showed reduced copulation/insemination success in Drosophila mauritiana, D. persimilis, D. pseudoobscura and D. simulans males that had forelegs severed to remove the region carrying the sex comb (Speth, 1952; Coyne, 1985), and in D. melanogaster and D. simulans males where comb teeth were physically removed with forceps (Cook, 1977). A reduction in the mating success of combless D. melanogaster males was also observed by Ng and Kopp (2008) using genetic ablation of the sex comb. Recently, an elegant study employed precise laser ablation of comb teeth in both D. melanogaster and D. bipectinata, finding that the removal of all comb teeth in either species effectively eliminates copulation in both no choice and competitive trials (Hurtado-Gonzales et al., 2015). The failure of males to copulate in these experiments appears to be because of the inability of the male to grasp the female’s abdomen/genitalia as a result of the absence of the sex comb teeth (Hurtado-Gonzales et al., 2015).

If removal of all comb teeth is deleterious to males, the question arises of whether the precise number of teeth that a male possesses is related to function, and is under selection. Surveys of several populations and strains of D. melanogaster show that males generally have 7–14 teeth per comb (Ahuja and Singh, 2008; Sharma et al., 2011; Snook et al., 2013). Ahuja and Singh (2008) employed divergent artificial selection and generated high and low sex comb tooth populations. Competing pairs of males with differing tooth numbers (means of 5.45 and 3.31 teeth) from a low-selected strain revealed that those with more teeth were significantly more likely to achieve a successful copulation, suggesting sexual selection against very low sex comb tooth number (Ahuja and Singh, 2008). Nevertheless, an experiment that maintained D. melanogaster and D. pseudoobscura populations for many generations under regimes allowing various levels of sexual selection failed to lead to any correlated selection for differences in sex comb tooth number (Snook et al., 2013). Studies to assess the level of sexual selection acting on the sex comb in natural, wild-caught populations (by counting teeth on the sex combs of males captured while copulating or not) have shown similarly mixed outcomes. Markow et al. (1996) found that D. simulans with relatively fewer teeth have greater copulation success. Conversely, Polak et al. (2004) found that D. bipectinata had greater mating success when the number of teeth on one of the three comb tooth rows was higher. No evidence for an effect of the number of teeth on mating was evident for D. pseudoobscura (Markow et al., 1996). Thus, although the comb as a whole appears to be critical for male mating, the relationship between mating success and the precise number of comb teeth is not yet clear.

The sex comb has become a valuable developmental genetics model for the rapid evolution of trait variation because of its exceptional morphological diversity among related species (Kopp, 2011). The diversity of comb morphology throughout the melanogaster and obscura groups is strongly related to the expression of the HOX transcription factor Sex combs reduced (Scr). Scr is upregulated in the sex comb precursor cells, often showing sexually dimorphic expression in species with sex combs (higher Scr expression in males), with no such increased expression or dimorphism in species lacking combs (Barmina and Kopp, 2007). Scr serves to activate doublesex (dsx) in the tarsal region during the late larval stage, and dsx expression subsequently initiates sex-specific development of the comb, and defines its morphology (Tanaka et al., 2011). The role of dsx in the morphogenesis of the sex comb appears to be at least partly carried out through a repression of dachshund (dac) as Dac is absent from the sex comb during pupal development (Atallah et al., 2009, 2014). An unanswered question is whether these high-level developmental regulatory genes that are responsible for development of the sex comb also segregate for allelic variation that confers intraspecific variation in sex comb morphology.

In this study we sought to map loci contributing to variation in sex comb tooth number variation in the model D. melanogaster system. Our goal was to ask whether genomic regions contributing to intraspecific trait variation were consistent with those for interspecific variation mapped in prior studies. In addition, we ask whether genes involved in the developmental specification of the comb are also involved in intraspecific variation in comb tooth number. Using genotypes derived from the Drosophila Synthetic Population Resource (DSPR) (King et al., 2012b), a large set of recombinant inbred lines (RILs) derived from a multiparental advanced-generation intercross, we were able to map QTLs with greater resolution than in previous genome-wide studies (True et al., 1997; Macdonald and Goldstein, 1999; Nuzhdin and Reiwitsch, 2000; Kopp et al., 2003; Tatsuta and Takano-Shimizu, 2006). The three QTLs we map all make small but significant contributions to trait heritability, and our data suggest that these loci may each harbor a series of alleles affecting phenotype. The overlap we see between our QTLs and those mapped in interspecific crosses can be explained by chance alone, and no mapped QTL overlaps with a priori developmental candidate genes (for example, dac, dsc, Scr). We functionally test at least one gene under each mapped QTL using tissue-specific RNA interference (RNAi), validating effects at the genes disco-r, poly U binding factor 68K (pUf68), scribbled (scrib) and Tweedle8 (Tweed8). These genes are strong candidates to harbor functional polymorphisms that causally affect sex comb tooth number variation within D. melanogaster, and provide a set of plausible candidate loci for future functional and behavioral testing.

MATERIALS AND METHODS
QTL mapping population and phenotyping
We mapped QTLs contributing to sex comb tooth number variation using genotypes derived from the DSPR (FlyRILlong; King et al., 2012b). The DSPR consists of two sets of RILs (pA and pB), each descended from an advanced-generation intercross of eight founder lines, seven specific to a panel (A1–A7 or B1–B7) and one common to both panels (A8B). Each recombinant population was maintained as a pair of replicate subpopulations (pA1, pA2, pB1, pB2) at large population size for 50 generations to expand the genetic map. RILs were subsequently established from each subpopulation via full-sib mating, and genotyped by restriction site-associated DNA sequencing (Baird et al., 2008). Using full resequencing data for the 15 founder lines and hidden Markov model assigned to each region in each RIL probabilities that the genomic segment is derived from each of the eight founders. For 95% of all positions over all RILs the most likely founder has a probability of >0.95, allowing accurate inference of the mosaic founder haplotype structure of each RIL. King et al. (2012b) provide full details of the development and properties of the DSPR RILs.

For the current experiment we generated and assayed the male F1 progeny of crosses between independent pairs of pA and pB RILs, in each case crossing pA RIL females to pB RIL males. Given the unidirectional nature of these crosses can be explained by chance alone, and no mapped QTL overlaps with a priori developmental candidate genes (for example, dac, dsc, Scr). We functionally test at least one gene under each mapped QTL using tissue-specific RNA interference (RNAi), validating effects at the genes disco-r, poly U binding factor 68K (pUf68), scribbled (scrib) and Tweedle8 (Tweed8). These genes are strong candidates to harbor functional polymorphisms that causally affect sex comb tooth number variation within D. melanogaster, and provide a set of plausible candidate loci for future functional and behavioral testing.
phenotyping the F1 progeny of RIL-by-RIL crosses versus phenotyping RILs directly.

RIL flies were allowed to lay eggs in narrow, polystyrene Drosophila vials containing cornmeal–yeast–molasses media. Flies were allowed to lay for up to 2 days, and adults were periodically cleared from vials to maintain roughly equal egg density across experimental vials. After 8–10 days, we harvested 10 virgin female flies from each PA RIL and 10 male flies from each PB RIL under CO2 anesthesia. These flies—the parents of the experimental genotypes—were allowed to recover for at least 1 day before mating, and flies were again allowed up to 2 days to lay eggs before being removed from vials. After 12–14 days, we collected >10 F1 experimental males from each cross vial and stored at –20°C until phenotyping. One foreleg from each test male was removed (we made no attempt to record whether we scored the left or right leg), mounted in mineral oil on a glass slide and tooth number was manually scored at ×40 total magnification.

We counted the number of teeth present on one sex comb for each of 10 males from each experimental cross vial. This modest within-genotype replication is because of our principal interest in estimating the mean phenotype associated with each founder haplotype in the mapping population, rather than in providing highly accurate estimates of genotype means. We scored males from 713 genotypes across 6 experimental batches, collecting data from 57 to 168 genotypes per batch. For the majority of genotypes experimental males were derived from a single experimental batch/vial. However, 56 genotypes were generated and tested as described above in more than one batch, and the correlation between means calculated separately for each batch is high (Pearson’s r = 0.70, P < 10−4, Supplementary File S1), giving us confidence in our phenotypes. Raw phenotype scores are presented in Supplementary File S2.

Sex comb tooth number heritability

Following the method described in Marriage et al. (2014), the broad-sense heritability of the trait was estimated separately for each subpopulation by calculating the genetic and phenotypic variance components from a linear model of the form: Yi,k = μ + bY + gijk + εijk, where Yi,k is the kth observation of the ith genotype on the jth batch, μ is the grand mean, bi is the random effect of batch, gijk is the random effect of RIL cross genotype nested within batch and εijk is the error term. The components were calculated in R (http://www.R-project.org) using the lm and VarCorr functions in the nlme package (Pinheiro et al., 2011). Note that as individuals of the same genotype were typically raised in the same vial, our heritability estimates will inevitably include some environmental effects. Heritability of the pA × PB cross genotype means used for mapping was estimated as the general variance component divided by the total variance of genotype means.

QTL mapping

The general approach to mapping QTL in the DSPR is described in detail in King et al. (2012a, b), the full analytical model is provided in King et al. (2014) and we implemented mapping routines within the DSPRqtl R package (github.com/egking/DSPRqtl: FlyRILs.org). Briefly, we regress mean male sex comb tooth number for each genotype on the 16 additive probabilities that correspond to the probabilities that paternal RIL is derived from each of the eight PA founders, and the probabilities that paternal RIL is derived from each of the eight PB founders. We additionally include subpopulation as a covariate as there is a difference between the two subpopulations in the average tooth number for each genotype on the 16 additive probabilities that correspond to the position of previously mapped sex comb QTLs on our genetic map (see Supplementary File S4). When QTL positions were provided as cytological locations in these studies, we converted to nucleotide positions (D. melanogaster reference genome release 5) using the map conversion tools on FlyBase (dos Santos et al., 2015). When the QTL positions were given as the intervals between gene-based markers, we simply used the known positions of these genes from FlyBase. Nucleotide positions were subsequently converted to genetic positions on the DSPR map by virtue of the high-density genotyping conducted on all DSPR RILs, and accurate estimation of genetic distances throughout the genome (King et al., 2012a, b).

We note that unlike the other four studies, Kopp et al. (2003) used a marker-based rather than an interval-based mapping methodology and provide only the positions of significant markers. Given the haplotype structure of the mapping population, 95% confidence intervals on QTL locations are likely to be substantially larger than reported.

Difficulties arose in two cases where Tatsuta and Takano-Shimizu (2006) mapped QTLs to intervals overlapping the proximal or distal boundaries of a large paracentric inversion on chromosome 3R that is fixed between D. melanogaster and the three species of the D. simulans clade (D. mauritiana, D. sechellia and D. simulans). The positions of this inversion were taken from Ranz et al. (2007), and for each of the D. simulans QTLs in question the equivalent region in D. melanogaster is represented by a pair of noncontiguous genomic segments.

Functional validation of plausible candidate genes

Assessment of the genes within mapped QTL intervals led us to functionally test effects on the sex comb of five genes using the bipartite Gal4-UAS RNAi system. To drive knockdown we used rn-Gal4 (Bloomington Drosophila Stock Center number 8142), an enhancer trap present just upstream of the rotund transcription unit, that expresses Gal4 at the position of the presumptive tarsus in the leg disc (St Pierre et al., 2002). In combination with the female splice isoform of the transformer (tra) gene under the control of the UAS promoter, the rn-Gal4 driver has been used to genetically ablate the sex comb in Drosophila males (Ng and Kopp, 2008). We also employed three dsv-Gal4 drivers, gifts from the Baker (Ashburn, VA, USA) and Goodwin labs (Oxford, UK). These are targeted knock-ins of Gal4 into the native doublesex gene and
express Gal4 in a pattern that generally recapitulates that of endogenous dsx expression (Rideout et al., 2010; Robinevet et al., 2010; Pan et al., 2011). Regulation of dsx expression is critical for the proper development of the sex comb (Tanaka et al., 2011).

Transgenic RNAi Project (TRIP) UAS-RNAi and co-isogenic control strains were obtained from the BDSC (Bloomington, IN, USA), specifically stock numbers 36303 (chromosome 3 landing site control strain), 35786 (UAS-GFP control), 35788 (UAS-Luciferase control), 41683 (UAS-disco-r-RNAi), 31960 (UAS-Dsp1-RNAi), 29591 and 343758 (UAS-pUf68-RNAi), 29552 (UAS-scrib-RNAi) and 61864 (UAS-TwIdS-RNAi). We also obtained UAS-RNAi stocks, and the appropriate control strains, from the Vienna Drosophila Resource Center (Vienna, Austria) (Dietzl et al., 2007) and 20144 (UAS-pUf68-RNAi), a ‘GD’ line harboring a P-element transgene, 109796 (UAS-pUf68-RNAi) and 105414 (UAS-scrib-RNAi), ‘KK’ lines harboring phiC31-based UAS transgenes, 60000 (w1118 host strain for GD library) and 60100 (KK landing site control strain). All Gal4 and UAS transgenes are inserted into autosomal locations (see Supplementary File S5 for additional details).

We generated RNAi knockdown/control male genotypes by crossing 10 virgin females from each UAS/control strain to 10 males from each Gal4 strain, establishing multiple vials per cross over multiple blocks. For each experimental male, the phenotype was scored as the average number of teeth on both sex combs (raw phenotype scores are presented in Supplementary File S6). The final sample size for each genotype varies because of marked differences in the number of males of the desired genotype emerging from each cross vial, likely a consequence of pre-adult lethality for some of the knockdown genotypes generated. Every experimental block included control genotypes to assess any block-to-block variation in phenotype.

**Fly rearing**

All stock maintenance and rearing of experimental individuals was carried out at 25 °C and 50% relative humidity with a 12 h light/12 h dark cycle.

**Statistical analysis**

All statistics were carried out using core, or custom written, routines in the R statistical programming language (http://www.R-project.org).

**RESULTS**

**Heritability**

Variation in male sex comb tooth number is substantial in the DSPR (Figure 1), with heterozygous pA × pB cross progeny genotype means ranging from 9.4 to 13.2 teeth per sex comb. This variation is similar to the range of 9.2–12.9 teeth observed across 32 lines of *D. melanogaster* derived from a worldwide set of collection sites (Ahuja and Singh, 2008), and similar to the 7–13 teeth (Sharma et al., 2011) and ~11 teeth (Snook et al., 2013) present in laboratory-adapted outbred populations. On average, genotype means in subpopulation 1 are lower than those in subpopulation 2 (Welch’s t = −9.1, P < 10−15). Given the subpopulations are derived from the same set of founders, but were maintained independently for 50 generations before the creation of RILs, differences most likely reflect variation in the founder composition of the subpopulations (see King et al., 2012b).

The broad-sense heritability for sex comb tooth number was estimated as 0.23 and 0.22 for subpopulations 1 and 2, respectively. Variance due to batch was <1% in each case. These heritability values are somewhat higher than, although not inconsistent with, narrow-sense heritability estimates of 0.07–0.21 from the response to artificial selection for increased and decreased comb tooth number in *D. melanogaster* (Ahuja and Singh, 2008). For QTL mapping we employ genotype means, averaging over all 10 sex comb measurements per genotype, and the broad-sense heritability of these mean phenotypes is 0.77 and 0.75 for subpopulations 1 and 2, respectively. The effect of environmental variation on sex comb tooth number has been reported to be much greater than that of genetic variation, with genetic and environmental coefficients of variation of 2.96–3.36 and 7.87–7.99, respectively (Ahuja and Singh, 2008). We see comparable values in this study (genetic coefficient of variation = 4.5, environmental coefficients of variation = 8.2), and hence by measuring multiple individuals we reduce the effect of environmental noise, and our mean sex comb phenotype has much higher heritability.

**Sex comb QTLs**

Using a similar approach to recent studies using the DSPR (Kislukhin et al., 2013; King et al., 2014) we mapped QTL contributing to variation in sex comb tooth number across 713 pA × pB cross progeny male genotypes. Three QTLs survived a permutation-derived 5% genome-wide threshold (Figure 2 and Table 1); Q1 is X-linked, and Q2 and Q3 are near the opposite ends of chromosome 3 on 3L and 3R, respectively. QTLs explain 4.4–7.4% of the genetic variation for sex comb tooth number and—assuming additivity—collectively explain 18.4% of the total genetic variation for the trait (Table 1). Given the fairly large sample size employed, we do not expect substantial upward bias in QTL effect estimates—the Beavis effect (Beavis, 1994; Xu, 2003)—nonetheless, the values we estimate should be considered as upper bounds on the variance explained by these loci.

Standard QTL mapping studies attempt to identify causative loci that segregate between a pair of parental genotypes (see Mackay, 2001). Thus, only a tiny fraction of the allelic variation in a population is interrogated, and such studies cannot evaluate the extent to which causative loci harbor a series of functional alleles with different effects on phenotype. With the DSPR we have the opportunity to evaluate the phenotypic effects of up to 15 alleles. Figure 3 shows the estimated founder haplotype effects at each of the mapped QTLs, and does not suggest a clear grouping of the founders into two groups, the pattern expected under simple biallelism. Although the patterns we observe could be because of allelic heterogeneity at single causative genes under each QTL, given the size of the implicated intervals (0.61–0.99 megabases; Table 1), it is not unlikely that multiple linked genes contribute to the effects estimated at each QTL. In addition, the variation in founder genotype frequency in the DSPR, where the frequency of each founder deviates from the expected 1/8 throughout the genome (King et al., 2012b), could make effects difficult to estimate and complicate their interpretation.

**Overlap of sex comb QTLs among studies**

Two previous studies have mapped QTL for sex comb tooth number in small panels of *D. melanogaster* RILS (Nuzhdin and Reiwitch, 2000; Kopp et al., 2003). The positions of these QTLs are depicted in the top panel of Figure 2 (blue bars, rows a and b). One of the two X-linked QTLs mapped in Nuzhdin and Reiwitch (2000) (Figure 2, row a) overlaps with Q1 mapped here, but otherwise none of the QTLs previously observed to contribute to sex comb tooth number variation within *D. melanogaster* overlap those we map in the DSPR. As 9.2% of the physical genome harbors previously mapped intraspecific QTL, finding one 1-Mb DSPR QTL that overlaps such a region is not unexpected by chance (Poisson probability, λ = 1, λ = 0.092, P = 0.09). We see the same result when considering that previously mapped intraspecific QTLs encompass 12.9% of the genetic map length (P = 0.011). True differences in the architecture of trait variation among the three *D. melanogaster* mapping populations could obviously explain this lack of overlap, and clearly studies initiated from 2 to 4 alleles (Nuzhdin and Reiwitch, 2000; Kopp et al., 2003) may not have captured functional alleles present in the DSPR. Differences in power among the studies could also plausibly explain the differences we see. In this context, previous simulations of the
power of the DSPR pA×pB cross design to identify QTLs contributing 5–10% of the variation is 68–95% (King et al., 2012a). Thus, if the genetic architecture of sex comb tooth number is dominated by small-to modest-effect QTLs, insufficient power may lead to studies routinely identifying different subsets of QTLs.

Two studies have previously mapped between-species variation for sex comb tooth number (Figure 2, top panel, red bars, rows c and d), using D. simulans–D. sechellia recombinants (Macdonald and Goldstein, 1999), and D. simulans–D. mauritiana recombinants (True et al., 1997). The largest-effect QTL mapped by True et al. (1997), positioned over the chromosome 3 cenromere (Figure 2), has been further resolved by Graze et al. (2007) who implicated a small handful of genes as plausibly contributing to the difference in sex comb tooth number between D. simulans and D. mauritiana. Only our Q3 overlaps an interspecific sex comb QTL, mapping at the same location as the small-effect QTL identified by True et al. (1997) (Figure 2). The apparent differences in the architecture of within-and-between-species variation we observe could be real or could be due to the technical differences in QTL mapping methods described above.

Collectively, across five previous studies, 53.3% (49.5%) of the physical (genetic) map has been implicated in the genetic control of sex comb tooth number variation, and by random chance we would expect that two of the QTLs we map would overlap these regions (Poisson probability, $k = 2$, $\lambda = 0.533/0.495$, $P = 0.08/0.07$), and this is what we observe. Interestingly, no previous studies have mapped a factor influencing sex comb tooth number variation to the left end of chromosome 3L where we identify QTL Q2 (Figure 2).

### Candidate sex comb tooth number genes

Several genes are known to affect the specification and development of the sex comb, such as Scr (Lewis et al., 1980a; b; Barmina and Kopp, 2007; Tanaka et al., 2011), dac (Atallah et al., 2014), the sex determination pathway genes dsx and transformer 2, tra2 (Baker and Ridge, 1980; Tanaka et al., 2011) and a series of genes named based on their effect on the sex comb (Additional sex combs, extra sex combs, multi sex combs, Posterior sex combs, Sex comb on midleg, Sex combs extra and super sex combs). Although some of these genes, such as dsx and Scr (Graze et al., 2007), have been implicated within QTL intervals identified in previous mapping studies, none of them are present within the three QTLs we map in the DSPR (Supplementary File S7).

In the absence of classic candidate genes within the modest number of genes in our QTL intervals (Table 1), we employed two strategies to attempt to define plausible candidates for subsequent testing. First, to identify any genes in FlyBase with a putative or functionally characterized role in sex comb development, we used the controlled vocabulary search terms ‘sex comb’, ‘sex comb tooth’ and ‘sex comb development’ to extract a total of 93 genes tagged with one or more of these terms (see Supplementary File S8). The genes Dsp1 (Dorsal switch protein 1) and pUf68 (poly U binding factor 68KD) were previously annotated as having some role in sex comb formation (Decoville et al., 2001; Quinn et al., 2004), and are present within QTL intervals Q1 and Q2, respectively. Second, we exploited data from a microarray study (Barmina et al., 2003) that identified two independent sets of genes of interest (see Supplementary Tables 1 and 3 from Barmina et al., 2005): (1) 16 genes differentially expressed

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**Table 1 Details of QTLs mapped for sex comb tooth number variation in the DSPR**

| Name | Chr | LOD score | Peak cM (3-LOD CI) | Peak Mb (3-LOD CI) | Number of genes | Percent of $H^2$ |
|------|-----|-----------|-------------------|-------------------|-----------------|-----------------|
| Q1   | X   | 6.9       | 53.0 (52.3–54.2)  | 16.05 (15.81–16.42) | 70              | 4.4             |
| Q2   | 3L  | 10.5      | 1.1 (0.6–1.9)     | 1.48 (1.22–1.90)   | 124             | 6.6             |
| Q3   | 3R  | 11.8      | 91.5 (90.1–92.9)  | 22.57 (22.09–23.08) | 114             | 7.4             |

Abbreviations: CI, confidence interval; Chr, chromosome; DSPR, Drosophila Synthetic Population Resource; LOD, logarithm (base 10) of odds; QTL, quantitative trait locus.

$^a$3-LOD CI indicates the 3-LOD support interval of the QTLs. Physical positions are given based on release 5 of the Drosophila reference genome.

$^b$The number of protein-coding genes in the 3-LOD support interval.

$^c$The percentage of broad-sense heritability of genotype means explained by the QTLs.
between male, sex comb-bearing T1 pupal legs (forelegs) and male, non-sex comb-bearing T2 pupal legs and (2) 143 genes differentially expressed between male T1 pupal legs and female, non-sex comb-bearing T1 pupal legs. *TwdlS* (*TweedleS*) was differentially expressed between male T1 and T2 legs, and is under Q3. Genes *disco-r* (*disco-related*) and *scrib* (*scribbled*) were differentially expressed between male T1 and female T1 legs, and are present within the Q1 and Q3 intervals, respectively.

We attempted to functionally validate the effect of these five genes on the number of sex comb tooth in *D. melanogaster* using the bipartite Gal4-UAS RNAi system. The two types of Gal4 drivers used — *rn-Gal4* and *dsx-Gal4* — are expected to knock down expression of target genes in slightly different spatiotemporal patterns, but in both cases include the presumptive sex comb precursor region of the foreleg.

Knockdown of *disco-r* (under Q1) shows no effect with the *rn-Gal4* driver (Figure 4), but we see a reduction in the number of sex comb teeth with two of the three *dsx-Gal4* drivers (Figure 5). Knockdown of *Dsp1* (also under Q1) shows no effect on the phenotype with any driver (Figures 4 and 5), potentially implying it has no role in the sex comb, although we cannot discount the possibility that *Dsp1* expression is simply not being reduced because of technical factors (see Booker et al., 2011).

For *pUf68* (under Q2) we see a significant change in phenotype for all *rn-Gal4* knockdowns tested (Figure 4), but none of the *dsx-Gal4* knockdowns (Figure 5). The situation is complicated by the observation that although three of the *pUf68 rn-Gal4* knockdowns yield lower sex comb tooth numbers than the control, in one case (construct ID 34785) we see significantly more teeth (Figure 4). We regenerated this latter *pUf68* RNAi genotype using the reciprocal cross (crossing males from the UAS strain to females from the Gal4 strain) that serves to swap the X chromosome present in the test males, but observed the same increase in comb tooth number relative to the control (Supplementary File S9). Given the irregular formation of the comb in *pUf68* RNAi animals, and the variable size and melanization of the teeth (Supplementary File S10), some of the variations across genotypes may simply represent experimental difficulty counting the number of teeth present.

For *scrib* (under Q3) there is a significant reduction in sex comb tooth number with *rn-Gal4* (Figure 4), and we also observe a reduction with one of the three *dsx-Gal4* drivers (Figure 5). For *TwdlS* (also under Q3) we see a dramatic reduction in sex comb tooth number across all drivers (Figures 4 and 5 and Supplementary File S10).

**DISCUSSION**

Darwin posited the conversion of variation within species into adaptive differences among species. If genes that contribute to polymorphism within a species are generally also those that influence a trait difference between species, there should be significant overlap between mapped intra- and interspecific QTL intervals. Several previous studies have observed QTL overlap in both within and between species (Nuzhdin and Reiwitch, 2000; Fishman et al., 2002; Lexer et al., 2005; Hall et al., 2006; Wittkopp et al., 2009; McNeil et al., 2011; Groot et al., 2013), supporting the idea that interspecific differences are not fundamentally distinct from variation within species in many instances. However, at least one study failed to identify any overlap in the positions of QTLs mapped within and between species (Gleason and Ritchie, 2004). QTL overlap does not provide information on whether those alleles responsible for species

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**Figure 3** Founder haplotype effects for mapped QTLs. Phenotype means (and s.e.) are presented for each founder at the peak position of each QTL. Data are only presented for founders present in at least 10 RILs at a probability of >0.95. AB8 was used to found both synthetic populations, and hence is depicted twice in the figure. Given that we phenotyped males resulting from crosses between population A females and population B males, for the X-linked Q1 only means for population A founders can be estimated.
demonstrating shared genetic control of trait variation within and between species.

Here, we genetically tested Darwin’s idea using variation in the number of teeth on the sex comb, a male-specific sexual structure in Drosophila. The gross morphology of the sex comb has rapidly diverged among closely related species (Kopp, 2011), there is variation among the four melanogaster complex species in the number of comb teeth (True et al., 1997) and there is some evidence that tooth number is under selection (see, for example, Ahuja and Singh, 2008). Using a multiparental, advanced intercross mapping population, and phenotyping well over 7000 animals, we mapped three QTLs contributing to sex comb tooth variation within D. melanogaster. We subsequently examined overlap between the positions of QTLs contributing to sex comb tooth number in the DSPR, and the positions of intra- and interspecific sex comb QTLs identified in previous work using the melanogaster group of Drosophilid species (True et al., 1997; Macdonald and Goldstein, 1999; Nuzhdin and Reivitch, 2000; Kopp et al., 2003; Tatsuta and Takano-Shimizu, 2006). The level of overlap between the QTLs that we identified and those identified in five previous mapping studies is not strong, and indeed is consistent with chance. Nonetheless, neither is overlap absent. Q1 overlaps with one of the two X-linked QTLs mapped by Nuzhdin and Reivitch (2000) in a cross between D. melanogaster strains (Figure 2), and Q3 overlaps with a QTL mapped to the end of chromosome 3R by True et al. (1997) in a between-species mapping panel (Figure 2). It is possible we have replicated effects at the same genes in these instances, but available data preclude any confident test of this assertion. The low resolution of early QTL mapping studies, which ensures large fractions of the genome are implicated in the control of trait variation, and the imperfect power to map small-effect QTLs in the DSPR, which means only a subset of the functional polymorphisms are resolved, makes assessing the similarity of intra- and interspecific genetic architecture for sex comb tooth number technically challenging.

One biological limitation of the within- and between-species comparison we present is the lack of an interspecific cross involving D. melanogaster. Such crosses are not possible because of hybrid inviability and sterility. A more appropriate test may be to build on the work of Tatsuta and Takano-Shimizu (2006) and generate high-resolution mapping data within D. simulans, allowing direct comparison with interspecific variation between D. simulans and either D. mauritiana or D. sechellia. Recent advances in high-throughput genotyping (see, for example, Andolfatto et al., 2011) allow very large mapping populations in non-model systems to be genetically analyzed (Huang and Erezylmaz, 2015). This offers the possibility of mapping sex comb number QTL in non-D. melanogaster recombinants with sufficient resolution that the similarity of trait architecture within and between species can be compared with confidence.

In contrast to previous mapping studies, we were able to use the DSPR to map modest-effect QTL to short, statistically defined regions, providing the opportunity to uncover likely candidate genes. None of the three QTLs we map implicate dac, dsh, Scr or tra2 (Supplementary File S7), the four genes that have been shown to be involved in the specification and development of the sex comb (Baker and Ridge, 1980; Lewis et al., 1980a, b; Barmina and Kopp, 2007; Tanaka et al., 2011; Atallah et al., 2014). Although not formally significant at a 5% permutation-derived threshold (Churchill and Doerge, 1994), we observed three additional peaks in the LOD (logarithm (base 10) of odds) score profile that are close to genome-wide significance (X, 0.5 cM; 2L, 54.0 cM; and 2R, 87.5 cM; Figure 2 and Supplementary File S3). These peaks could represent true, small-effect QTLs or simply be spurious. Regardless, 3-LOD support intervals about these peaks also do not implicate dac, dsh, Scr or tra2. These results imply that key players in sex comb developmental patterning either do not contribute to natural variation in sex comb tooth number in the DSPR or were undetectable in our study by virtue of having relatively small individual effects or by segregating for very rare functional alleles not captured in the DSPR.

Despite the absence of a priori candidate genes beneath mapped QTL intervals, we were able to leverage other data to define one or two plausible candidates for each QTL, and used RNAi directed to the region of the foreleg from which the sex comb is derived to show that four of these genes affect sex comb tooth number. disco-r is present within the interval implicated by X-linked QTL Q1, was shown to have lower expression in male compared with female pupal forelegs (Barmina et al., 2005) and has been suggested to be involved in leg development (Grubbs et al., 2013). pUf68 is implicated by QTL Q2 on
trait mutation leads to malformed sex combs (Quinn et al., 2005), and is required for splicing of the primary transcript, although it does not lead to false positives (Ma et al., 2006). However, such effects should be minimized in cases where multiple transgenes yield similar results.

In summary, in this study we have used a combination of high-resolution QTL mapping, published expression data and RNAi-based functional tests to implicate several short regions of the Drosophila genome, and a handful of loci, in the genetic control of male sex comb tooth number variation. Three of the candidate sex comb genes that we identify—disco-r, scrib and TwdlS—have not previously been shown to affect the morphology of the sex comb, and given their reported expression differences between sexes or segments in the Drosophila foreleg (Barmina et al., 2005), may be expected to segregate for functional regulatory polymorphisms that affect sex comb tooth number. Identification of the precise causative variants may be accomplished by association mapping in natural populations of flies (see, for example, Bastide et al., 2013), although the large environmental component to the variation of sex comb tooth number, the subtle effects of the QTLs we map and the possibility that QTLs are multiallelic imply that the kinds of sample size typically employed in the human disease association mapping literature will be required (that is, tens of thousands of individuals). Alternatively, it is now becoming feasible in D. melanogaster to employ CRISPR-Cas9 genome editing to compare different, putatively functional alleles in an otherwise standardized background and directly test the effects of specific genomic intervals, genes and nucleotide positions.

**DATA ARCHIVING**

All phenotype data collected as part of this study are available as Supplementary Information files. All data on the DSPR project is available at FlyRiLS.org.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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