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The *Tribolium spineless* ortholog specifies both larval and adult antennal identity

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Abstract

The morphology of insect antennae varies widely among species, but our understanding of antennal development comes almost solely from studies of one species - the fruit fly, *Drosophila melanogaster*. Moreover, this knowledge applies mostly to adult structures, since *Drosophila* lacks external larval appendages. In contrast to *Drosophila*, the red flour beetle, *Tribolium castaneum*, has both larval and adult antennae, which are very different from one another in morphology. Thus, *Tribolium* provides an ideal system to compare modes of antennal development both within and between species. Here we report that the *Tribolium* ortholog of *spineless* (*Tc-ss*) is required in both the larval and adult antennae. Knockdown of *Tc-ss* by RNAi during either larval or imaginal development causes transformation of the distal portion of the antennae to legs. Thus, the function of *ss* is conserved between *Drosophila* and *Tribolium* with respect to adult antennal specification, and also between *Tribolium* larval and adult antennal development. The similarity of the *Tc-ss* RNAi phenotype to that of a classically described *Tribolium* mutation, *antennapedia* (*ap*) (of no relationship to the *Drosophila* Hox gene of the same name), led us to characterize the original *ap* mutation and two newly identified *ap* alleles. Our mapping and phenotypic data suggest *Tc-ss* is the best candidate for the *ap* locus. These results represent a first step in characterizing larval and adult antennal patterning in *Tribolium*, which should provide important insights into the evolution of insect antennal development.

Keywords

Tribolium; antenna; spineless; appendage; insect

Introduction

Virtually all our knowledge about the specification of insect antennal identity comes from analysis of *Drosophila* imaginal development. These studies suggest that the key factor in determining antenna versus leg identity is the extent to which *homothorax* (*hth*) and *Distal-less* (*Dll*) expression overlap within the developing imaginal disc (Dong et al., 2000). Co-expression of these genes triggers a cascade of gene expression that ultimately leads to antennal identity (Dong et al. 2002; Emerald et al. 2003). An important target of these genes is *spineless* (*ss*) (Dong et al. 2002; Emmons et al. 2007), a member of the bHLH-PAS transcription factor family (Duncan et al. 1998). Loss of *ss* function causes transformation of the distal antennal segments (A3 and arista) to leg, indicating that *ss* plays a key role in the specification of antennal identity (Duncan et al. 1998; Struhl 1982). Like many genes involved in antennal development, *ss* is also expressed in the legs (Duncan et al. 1998) where it is required for normal development of the tarsal segments (Struhl 1982). However, expression

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in the antennae is more extensive and persistent than that in the legs, with a high level of expression apparently being important for antennal identity (Duncan et al. 1998). In addition to these appendage roles, *ss* has an additional function in bristle development as shown by the reduced bristle size of *ss* mutants.

Although *ss* is strongly expressed in the *Drosophila* embryonic antennal segment (Duncan et al. 1998; Emmons et al. 2007), it is not clear whether it actually plays a role in establishing embryonic antennal identity. The antennae of *Drosophila* larvae are represented only by sense organs, which in *ss* mutants are somewhat abnormal and/or sclerotized, but not obviously transformed (Duncan et al. 1998).

In contrast to *Drosophila*, the red flour beetle, *Tribolium castaneum*, has both larval and adult antennae. Antennal morphology is drastically different at these two life stages (compare Fig. 1d to 2a), suggesting that the antennal tissue undergoes substantial re-patterning during metamorphosis. This raises the question of whether the same genes specify and pattern antennae during embryonic and imaginal development. To begin to address this issue, we have examined the function of the *Tribolium ss* ortholog. We show here that RNAi-induced knockdown of *Tc-ss* during either embryonic or adult development causes transformation of antennae to legs, suggesting that at least some aspects of patterning are conserved between larval and adult antennae. We also revisit the classical mutation *antennapedia* (*ap*) - of no relationship to the Hox gene Antennapedia - which causes transformation of the antennae to legs (Englert and Bell 1963). We describe two new alleles of *ap* and provide phenotypic and mapping evidence that suggests *ap* corresponds to the *Tc-ss* locus.

Materials and Methods

Sequence Analysis

BLAST analyses against the *Tribolium* genome and predicted genes (Tribolium Genome Sequencing Consortium 2008) were performed at the NCBI (<http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=7070>) and Baylor College of Medicine (<http://www.hgsc.bcm.tmc.edu/blast.hgsc?organism=13>) web sites. BLAST analyses against *Drosophila melanogaster* sequences were performed at FlyBase (<http://flybase.bio.indiana.edu/blast/>).

Gene Cloning

Predicted coding sequences (NCBI RefSeq XP_967876, GLEAN_11104 and GLEAN_11105) identified by BLAST searches with *Drosophila* Spineless were used to design primers (*ss3* (5'-ggaaattcaccgtctccaccg-3') and *ss4* (5'-acatactcccgtctctccattt-3')) to amplify a 1.875 kb fragment of the predicted *Tc-ss* cDNA. The resulting cDNA fragment was cloned and sequenced (GenBank accession EU912437).

In situ hybridization

The 1.875 kb fragment of *Tc-ss* described above was used as template for riboprobe synthesis by standard methods. In situ hybridization was performed essentially as described by Nagaso et al. (2001)

RNAi

A 755 bp fragment of *Tc-ss* (bases 785-1539 of Genbank EU912437) was used as template for dsRNA synthesis. Injections of dsRNA into embryos (2 ug/ul), larvae (0.5-3.25 ug/ul) and pupae (1.225 ug/ul) were performed as previously described (Brown et al. 1999; Bucher et al. 2002; Tomoyasu and Denell 2004).

Documentation

Cuticle preparations were mounted either in Hoyer's medium or as described by Shippy et al. (2008). Adult heads were mounted in ethanol on a depression slide and covered with a cover slip. Images of several focal planes were captured using a Nikon DXM1200F digital camera and 3-D reconstructions were made using Auto-Montage software (Syncrosopy). Scanning electron micrographs were obtained using a Hitachi S-3500N scanning electron microscope (Hitachi Science Systems) at the Kansas Agricultural Experiment Station Scanning Electron Microscope Laboratory, Kansas State University Entomology Department.

Beetle Genetics

Beetles were reared by standard methods on whole wheat flour supplemented with 5% brewers yeast. *ap*² and *ap*³ were identified as spontaneous, recessive mutations in laboratory stocks of *Df(HOMC)/mxp^{Sibd}* and Ga-2, respectively. Complementation tests using either *ap*¹ (to test *ap*²) or *ap*² (to test *ap*³) indicated that these mutations were all alleles of the same locus.

Mapping

To identify a piggyBac insertion linked to *ap*, transgenic lines carrying GFP-marked transposons were crossed to the multiple marker strain (mms), which is homozygous for *ap* (Beeman and Brown 1999). GFP-positive progeny were then backcrossed to mms. The progeny were scored for GFP and *ap* to assess potential linkage. The insertion site junction of the line showing linkage (PU-105) was amplified by vectorette PCR as described by Lorenzen et al. (2007), cloned and sequenced (GenBank Accession EU912436). This sequence was then BLASTed against the *Tribolium* genome to determine the location of the PU-105 insertion. To estimate the cM positions of *Tc-ss* and PU-105, marker sequences from LG10 of the genetic linkage map (Lorenzen et al. 2005) were BLASTed against the *Tribolium* genome to identify those markers closest to the sequences of interest. Relevant marker sequences and their GenBank accession numbers are 17.K01s (CZ012512), 23.A01t (CZ012536) and 2D10 (CB334987 and CB334988).

Results and Discussion

Tribolium has a single *spineless* ortholog

We used *Drosophila* Spineless as the query sequence in a tBLASTn search of the *Tribolium* genome (Tribolium Genome Sequencing Consortium 2008) and identified a locus on LG10 predicted to encode a protein with extensive identity to Spineless (data not shown). A reciprocal BLAST against *Drosophila* recognized Spineless as the top hit for this sequence, suggesting one-to-one orthology. Therefore, we have named the *Tribolium* gene *Tc-spineless* (*Tc-ss*).

Tc-ss is expressed in the embryonic antennae

Tc-ss expression is first observed in the antennae at the extended germ band stage (Fig. 1a). During germband retraction, this expression domain becomes more extensive, eventually encompassing the entire distal portion of the antenna (Fig. 1b). Expression also appears in the maxillary and labial appendages (Fig. 1b), and later in small patches in the legs (Fig. 1c). Late in embryogenesis, *Tc-ss* is also expressed in a punctate pattern that probably represents cells of the peripheral nervous system (Fig. 1c).

Tc-ss is required for larval and adult antennal identity

To assess the function of *Tc-ss*, we first injected *Tc-ss* dsRNA into *Tribolium* embryos. The resulting larvae display transformations of the distal antennal segments to legs (Fig. 1e), consistent with the expression domain of *Tc-ss*. The basal antennal segment (scape) is unaffected (compare to wild-type in 1d), but the pedicel, flagellum and seta are replaced by a

femur, tibiotarsus and pretarsal claw. Parental RNAi with *Tc-ss* produced progeny with the same phenotype (data not shown).

To determine the function of *Tc-ss* during adult development, we injected *Tc-ss* dsRNA into last instar larvae. The resulting adults show abnormal development of several structures. First, the antennae are transformed toward leg identity (Fig. 2d). The most basal antennal segments (the scape and pedicel) are unaffected. The funicle and club segments (which together make up the flagellum) are replaced by a tibia-like segment, a partial tarsus, and a terminal claw. Second, the thoracic legs show fusions and/or deletions of the tarsal segments (Fig. 2e). Third, the sensory bristles of the body wall are greatly reduced in length (Fig. 2f) compared to wild type (Fig. 2c).

These RNAi results indicate that the role of *Tc-ss* in adult development is very similar to the role of *ss* in *Drosophila*. One difference, however, is that the antennal transformations in *Tribolium* include more proximal leg segments. In *Drosophila ss* null mutants, the arista is transformed into tarsal segments, while A3 seems to lose any identifying characteristics (Duncan et al. 1998). In contrast, *Tc-ss* RNAi produces a fused segment with definite leg identity (probably tibia) in addition to the terminal tarsal region. As in *Drosophila ss* nulls, the tarsal regions of the transformed antennae as well as the thoracic legs are reduced.

The transformation of larval antenna to leg produced by *Tc-ss RNAi* is even more extensive than that of the adult antenna, in that it includes a femur in addition to a tibiotarsus and claw. (Consistent with this difference, the larval pedicel is affected while the adult pedicel remains intact.) Despite the apparent difference between the two developmental stages with respect to the proximal extent of *Tc-ss* function, our results suggest that at least some aspects of patterning are shared between embryonic and imaginal antennal development. It will be interesting to determine whether genes acting upstream and downstream of *ss* in *Drosophila* adult antennal specification also play similar roles in *Tribolium* adult and/or embryonic antennal development.

The *antennapedia* locus maps near *Tc-spineless*

The transformation of antenna to leg produced by *Tc-ss* RNAi is reminiscent of the *Tribolium antennapedia* (*ap*) mutant phenotype described by Englert and Bell (1963). This mutation, which has no connection to the Hox gene *Antennapedia*, produces transformation of both larval and adult antennae to legs. Like *Tc-ss*, *ap* maps to LG10 (formerly LG8). To determine a more precise map position for *ap*, we identified a piggyBac insertion (PU-105) that showed linkage to *ap*. Out of 96 mapping progeny, we identified 19 recombinants between PU-105 and *ap*, indicating that these loci are separated by roughly 20 cM. The insertion site of PU-105 maps approximately 165 kb away from the distal-most marker (47.1 cM) on an SSCP-based genetic linkage map (Lorenzen et al. 2005). The recombination rate in this region of the genome is unknown, but we estimate that PU-105 maps somewhere between 45-49 cM on LG10 (Fig. 3). The proximity of PU-105 to the end of the linkage group means that *ap* almost certainly maps proximal to PU-105. Thus, allowing some room for error, we estimate that *ap* maps in the range of 24-30 cM on LG10 (Fig. 3).

To evaluate candidate genes for the *ap* locus, we determined map positions for the *Tribolium* orthologs of genes important for antennal identity in *Drosophila*. In addition to *spineless*, these include *tango* (Emmons et al. 1999), *hth* (Casares and Mann 1998; Pai et al. 1998), *extradenticle* (Gonzalez-Crespo and Morata 1995; Rauskolb et al. 1995), *Dll* (Cohen and Jurgens 1989; Dong et al. 2000; Sato 1984; Sunkel and Whittle 1987), *distal antenna* (*dan*) and *distal antenna-related* (*danr*) (Emerald et al. 2003; Suzanne et al. 2003). *Tc-Dll* has previously been mapped to LG7 (Beeman and Brown 1999) and thus can be removed from consideration. We found that *Tc-homothorax* and the single *Tribolium* ortholog of *dan/danr*

also map to LG7, while *Tc-tango* maps to the X chromosome, eliminating these genes as candidates for *ap*. *Tc-extradenticle*, like *ap*, maps to LG10. However, its position near a marker at 15.8 cM is quite far from the estimated position of *ap*. Interestingly, *Tc-ss* maps to LG10 between markers at 24.0 cM and 25.3 cM (Fig. 3), putting it within range of the *ap* locus. Although finer mapping is needed, the similar map positions of *Tc-ss* and *ap* make *Tc-ss* the most likely candidate gene for the *ap* locus.

antennapedia mutant phenotypes are similar to the effects of *Tc-ss* RNAi

In addition to the original *ap* allele (Englert and Bell 1963), which we will hereafter call *ap*¹, we have isolated two new recessive alleles of this locus (see Materials and Methods). To further investigate whether *ap* mutations might affect the *Tc-ss* locus, we carefully examined the phenotype produced by each *ap* allele. In the descriptions below, we have specifically noted whether structures affected by *Tc-ss* RNAi are altered in the *ap* mutants.

*ap*¹ causes partial transformation of antenna toward leg at both the larval (Fig. 1f) and adult (Fig. 2g) stages. The most obvious leg-like feature in *ap*¹ larval antennae is a distal pretarsal claw. In adults, the expressivity of the phenotype is somewhat variable, but typically a distal tarsal claw and a fused region with some degree of leg identity are present. The funicle segments are either partially fused or altogether absent. The two basal segments always retain antennal identity. The legs (Fig. 2h) appear normal, as do the bristles of the body wall (Fig. 2i).

*ap*² causes a more complete transformation to leg than *ap*¹ in both larvae (Fig. 1g) and adults (Fig. 2j). The larval pedicel, flagellum and seta are replaced by a fused segment (apparently tibiotarsus and femur) and pretarsus. In adults, the funicle and club segments are transformed into tibia and tarsal segments. Similar to *ap*¹, the legs (Fig. 2k) and bristles show no defects (Fig. 2l).

*ap*³ homozygous larvae display a severe antenna to leg transformation (Fig. 1h), similar to that seen for *ap*². One difference in *ap*³ is the presence of an obvious boundary between the tibiotarsus and femur of the transformed antenna, a feature that is also seen in *ss* RNAi larvae. The antennal transformations of *ap*³ adults are quite variable. Mildly affected individuals (Fig. 2m) display normal funicles but fused club segments. Interestingly, one or more tarsal segments and a terminal claw are sometimes present distal to the three club segments. Other *ap*³ antennae (Fig. 2p) resemble those of *ap*² homozygotes. In contrast to the other alleles of *ap*, *ap*³ homozygotes sometimes show leg defects, namely fusion or deletion of the adult tarsal segments (Fig. 2q), as well as a reduction in bristle length (Fig. 2r). These additional phenotypes are very similar to those produced by *Tc-ss* RNAi.

To try to confirm the relationship between *ap* and *ss*, we sequenced the coding region (CDS) of *ss* from *ap*¹, *ap*² and *ap*³ homozygotes. However, we found no changes predicted to affect the encoded protein. This result is not particularly surprising, given that none of the *ap* alleles appear to be nulls (see below).

Despite the lack of a definitive mutant lesion, several lines of evidence suggest that *Tc-ss* is the gene affected by *ap* mutations. First, both genes map to a similar position on LG10. Second, the phenotypes of *ap* mutations, particularly the pleiotropic effects of *ap*³, are very similar to those produced by *Tc-ss* RNAi. Third, the existence of different classes of *ap* alleles, some affecting only the antennae and others affecting the legs and bristles as well, is reminiscent of *Drosophila ss*. While null *ss* alleles affect the antennae, legs and bristles (similar to the most severe effect of *ap*³), many alleles affect only the antennae (as seen for *ap*¹ and *ap*²). In *Drosophila*, these different classes of alleles seem to be the result of the complex regulatory structure of the *ss* locus. Separate enhancer regions driving expression in antennae, legs and bristles have been identified (Emmons et al. 2007). The antennal enhancers are located some

distance upstream of the *ss* transcription unit, while the leg and bristle enhancers are within or just upstream of the transcription unit. Therefore, antenna-specific *ss* alleles can be explained by deletions or rearrangements that affect only the antennal enhancers (Emmons et al. 2007).

If, indeed, *ap* mutations are *Tc-ss* alleles, we would predict that the *ap*¹ and *ap*² mutations affect only antennal expression. It is interesting that both of these alleles affect antennal identity in larvae as well as adults. In *Drosophila*, there are at least two embryonic antennal segment enhancers for *ss*. Both of these map near imaginal antenna enhancers, but at least one of them is clearly a separate element. Unfortunately the evolutionary distance between *Drosophila* and *Tribolium* (~300 million years) is too great to permit conservation-based recognition of cis-regulatory elements, but if the regulatory structure of *Tc-ss* is similar to *Drosophila ss*, we might expect *ap*¹ and *ap*² to be deletions or rearrangements with breakpoints upstream of *Tc-ss*. Such lesions might affect expression of *Tc-ss* in both embryonic and adult antennae, but not adult legs or sensory bristles.

In contrast to *ap*¹ and *ap*², the *ap*³ lesion probably affects all aspects of *Tc-ss* expression. However, this allele is unlikely to be a null, since not all *ap*³ homozygotes show the severe phenotype. It is possible that a second site mutation is responsible for the variation observed in *ap*³ homozygotes. However, the Ga-2 background of *ap*³ is highly inbred and believed to be homozygous at most loci (Lorenzen et al. 2005). It is more likely that the variable phenotype of *ap*³ is caused by differences in gene expression levels among individuals.

The results described here highlight the advantages of using *Tribolium* as a model organism to study the patterning of larval and adult antennae. The two-pronged approach of using both forward genetics and candidate genes from the *Drosophila* adult antennal paradigm, promises to provide important insights into *Tribolium* development, as well as the evolution of insect antennae.

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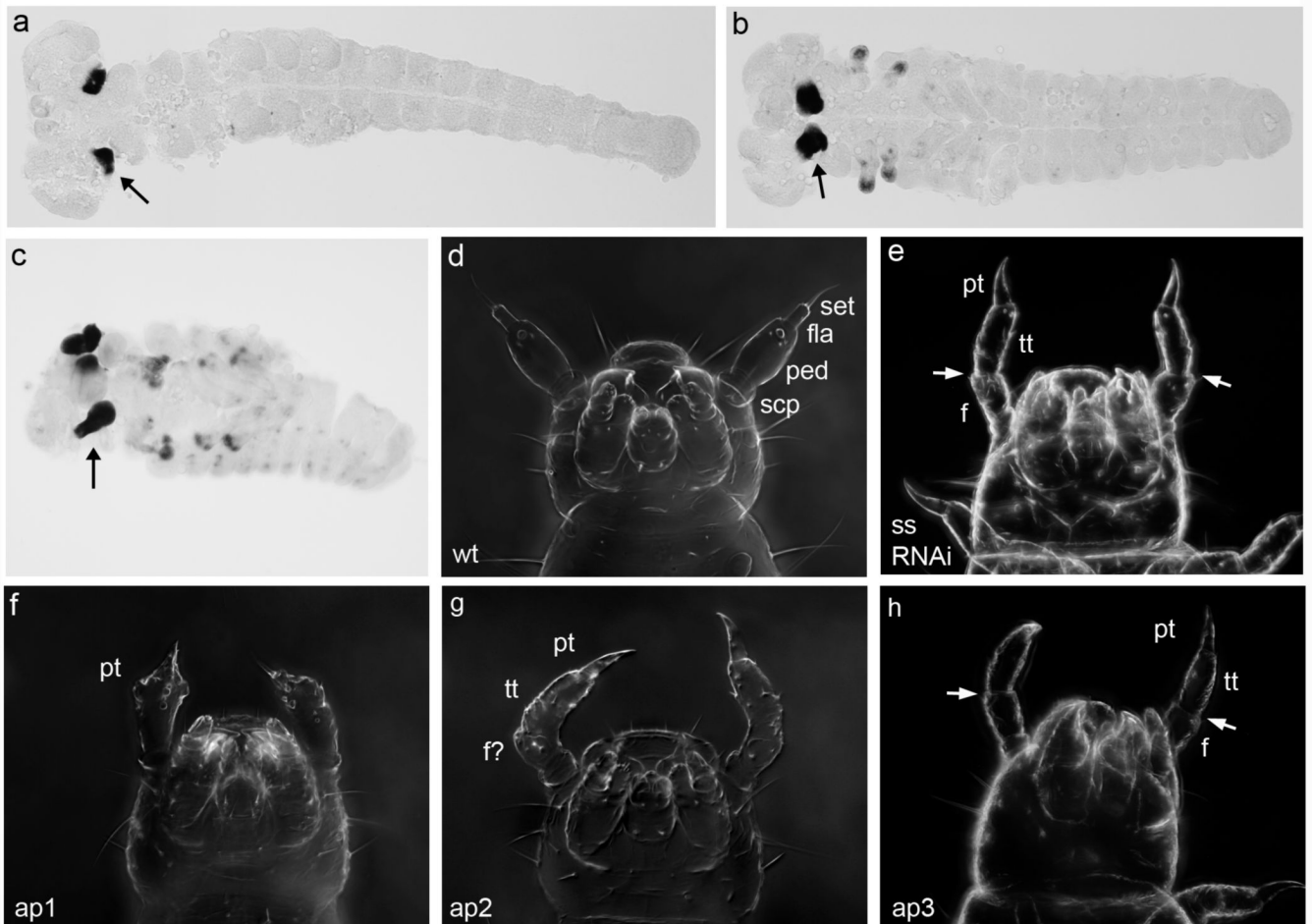


Fig. 1.

Embryonic expression and larval phenotypes. **a-c** *Tc-ss* expression in *Tribolium* embryos. An arrow points to the antennal expression in each panel. **a** *Tc-ss* expression in the antennae of an extended germ band stage embryo. **b** As the germ band retracts, *Tc-ss* is expressed in the maxillary and labial appendages, as well as the antennae. **c** A dorsally closing embryo continues to show strong *Tc-ss* expression the antennae as well as patches of expression in the other appendages. Additional punctate staining probably corresponds to peripheral nervous system cells. **d-h** Cuticle preparations of first instar larvae. **d** Wild-type larval antennae have a basal scape (scp), a pedicel (ped), a short cylindrical flagellar segment (fla) and a terminal seta (set). The antennae of *Tc-ss* RNAi (**e**), *ap1* (**f**), *ap2* (**g**) and *ap3* (**h**) larvae are transformed toward leg. Leg segments are identified where relevant: pretarsus (pt), tibiotarsus (tt) and femur (f). Arrows in **e** and **h** point to the clear boundary between femur and tibiotarsus.

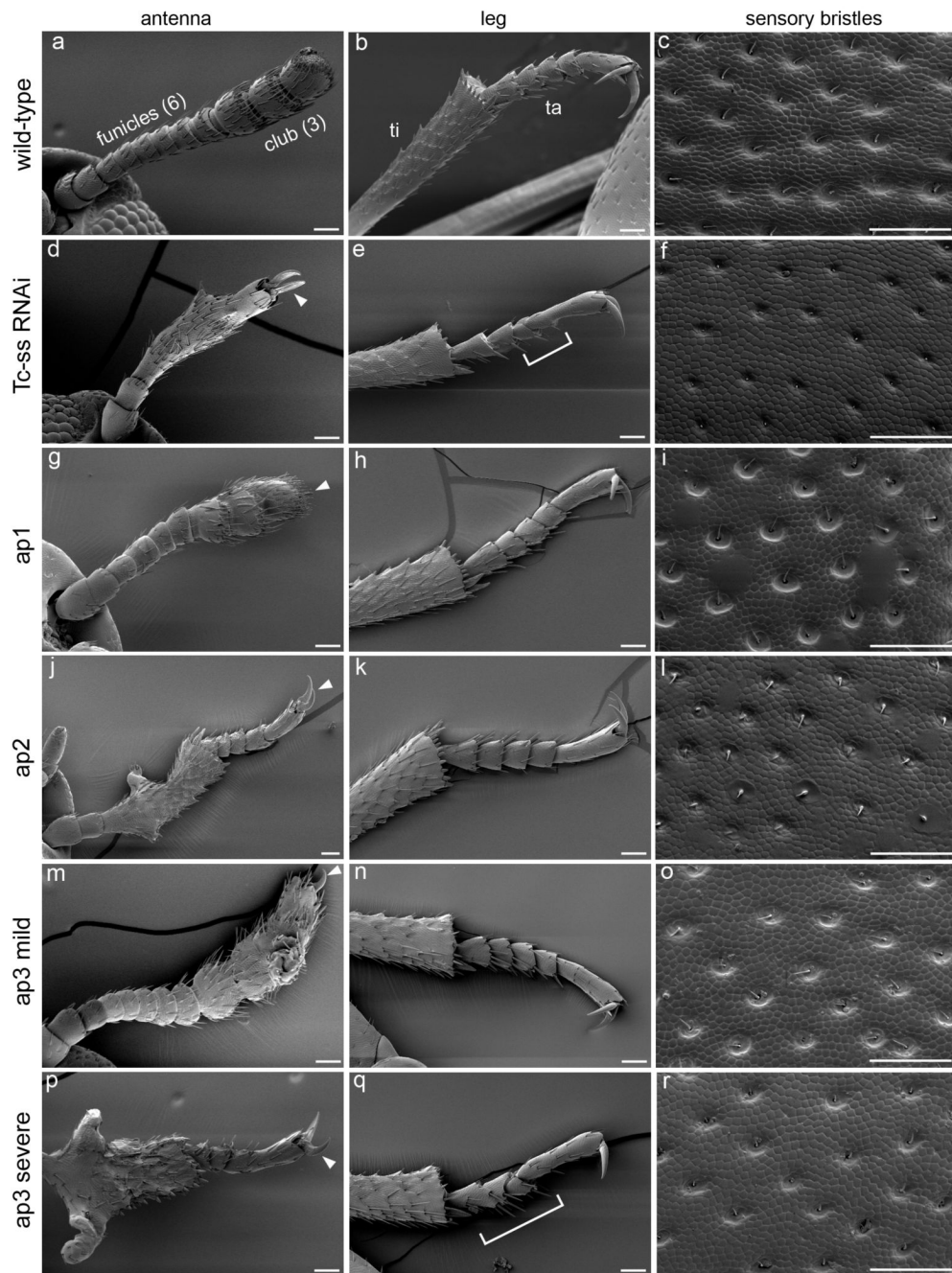


Fig. 2. Scanning electron micrographs of adult structures. Scale bars represent 50 μ m. **a** The wild-type antenna has two basal segments, six funicle segments and three club segments. **b** The distal portion of a wild-type T2 leg consists of the tibia (ti), five tarsal segments (ta) and a terminal claw. **c** Sensory bristles on a wild-type pronotum. These structures are also shown for *Tc-ss* RNAi (**d-f**), *ap*¹ (**g-i**), *ap*² (**j-l**), mildly affected *ap*³ (**m-o**), and severely affected *ap*³ (**p-r**) adults. Brackets in **e** and **q** denote regions of tarsal fusion. Arrowheads point to terminal claws, which are indicative of leg identity.

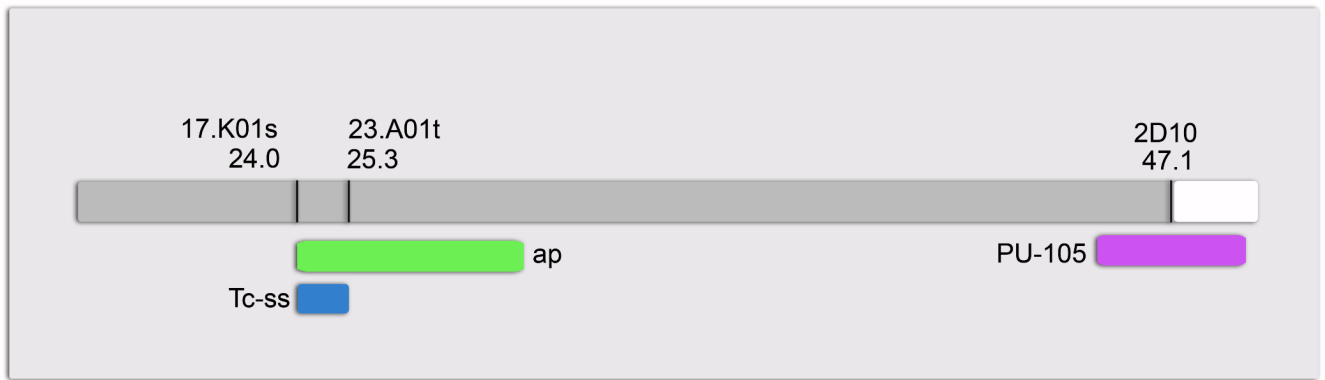


Fig. 3. Schematic diagram of a portion of LG10. Genetic markers shown are from the molecular map produced by Lorenzen et al. (2005). Approximate positions of *ap* (green), *Tc-ss* (blue), and PU-105 (purple) are shown below the chromosome (gray). The undefined region of the chromosome beyond the distal-most genetic marker is shown in white.