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A Double Segment Periodicity Underlies Segment Generation in Centipede Development

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Summary

The number of leg-bearing segments in centipedes varies extensively, between 15 and 191, and yet it is always odd [1, 2]. This suggests that segment generation in centipedes involves a stage with double segment periodicity and that evolutionary variation in segment number reflects the generation of these double segmental units. However, previous studies have revealed no trace of this [3–5]. Here we report the expression of two genes, an *odd-skipped* related gene (*odr1*) and a *caudal* homolog, that serve as markers for early steps of segment formation in the geophilomorph centipede, *Strigamia maritima*. Dynamic expression of *odr1* around the proctodaeum resolves into a series of concentric rings, revealing a pattern of double segment periodicity in overtly unsegmented tissue. Initially, the expression of the *caudal* homolog mirrors this double segment periodicity, but shortly before *engrailed* expression and overt segmentation, the intercalation of additional stripes generates a repeat with single segment periodicity. Our results provide the first clues about the causality of the unique and fascinating "all-odd" pattern of variation in centipede segment numbers and have implications for the evolution of the mechanisms of arthropod segmentation.

Results and Discussion

Geophilomorph centipedes hatch from the egg with the final adult number of segments. In *Strigamia maritima*, this number varies both within and between populations. The overall range is 45–53 leg-bearing (= trunk) segments [6]. These segments form during embryogenesis, in an anterior to posterior sequence. As they form, the segmented germ band extends anteriorly from an apparently undifferentiated disc of cells that remains at the posterior of the embryo [4, 5]. Thus, within a single embryo undergoing segmentation, one can trace the process of segmentation by following the differentiation and gene expression patterns along the body axis, with more posterior segments undergoing events that are earlier in the segmentation process.

Our previous study of segmentation in *Strigamia* [5] examined expression of the segmentation gene *engrailed*, the homolog of a *Drosophila* segment polarity gene that is a widely conserved marker for segment formation in arthropods. *engrailed* is first expressed in the forming germ band, after the emergence of presumptive segments from the posterior disc, and only a short time before the earliest morphological signs of segmentation.

We now report the expression of two genes that reveal much earlier steps in the segmentation process—a homolog of the *Drosophila* gene *caudal* and a member of the *odd-skipped* family that we term *odd-skipped related gene 1* (hereafter *odr1*). *caudal* genes encode homeodomain transcription factors, which in many species are early markers for the posterior part of the forming embryo [7, 8]. Genes of the *odd-skipped* family encode zinc finger transcription factors, which in *Drosophila* function during trunk and limb segmentation and during a number of later developmental events [9–11]. The *odd-skipped* related fragment that we have cloned is more similar to *Drosophila bowl* and *sob* than to the *odd-skipped* gene itself (see Supplemental Data).

The earliest reiterated pattern that we have observed in the formation of the trunk segments is the expression of *odr1*. Transcripts are initially detected in 2–3 concentric rings at the embryonic blastoderm stage, before germ band extension (Figure 1A). Later in development, throughout most of the period of segment formation, *odr1* transcripts can be detected in between 5 and 9 concentric rings of cells. These rings are centered on the proctodeum, which is strongly stained (Figures 1B and 1C). They extend from the embryonic into the extraembryonic territories (the border between the two being identified by differences in cell density [5]), with no obvious discontinuity between these domains. The pattern gives the appearance of a wave emanating from the proctodeum and decaying as it moves away. Within the perimeter of the internal ring, the pattern of expression is variable even between embryos of similar age (Figure 1D). We suspect that these patterns are samples from a cycle that begins with strong expression close to the proctodeum, expands to more diffuse expression within the perimeter of the internal ring, and resolves to expression in a broad ring that sharpens to generate a new *odr1* ring (Figure 1E). This entire pattern is generated in tissue that is not overtly segmented. In addition to the expression in unsegmented tissue, there is also staining in the forming limb buds, but this is only in segments that are well differentiated and clearly represents a distinct phase of gene expression. When *engrailed* and *odr1* are detected simultaneously in double in situ hybridization experiments, the posterior, most recently formed *engrailed* stripe is usually anterior to the outermost *odr1* ring (Figure 3A). In some preparations, faintly visible *odr1* rings reach as far as the most posterior *engrailed* stripe.

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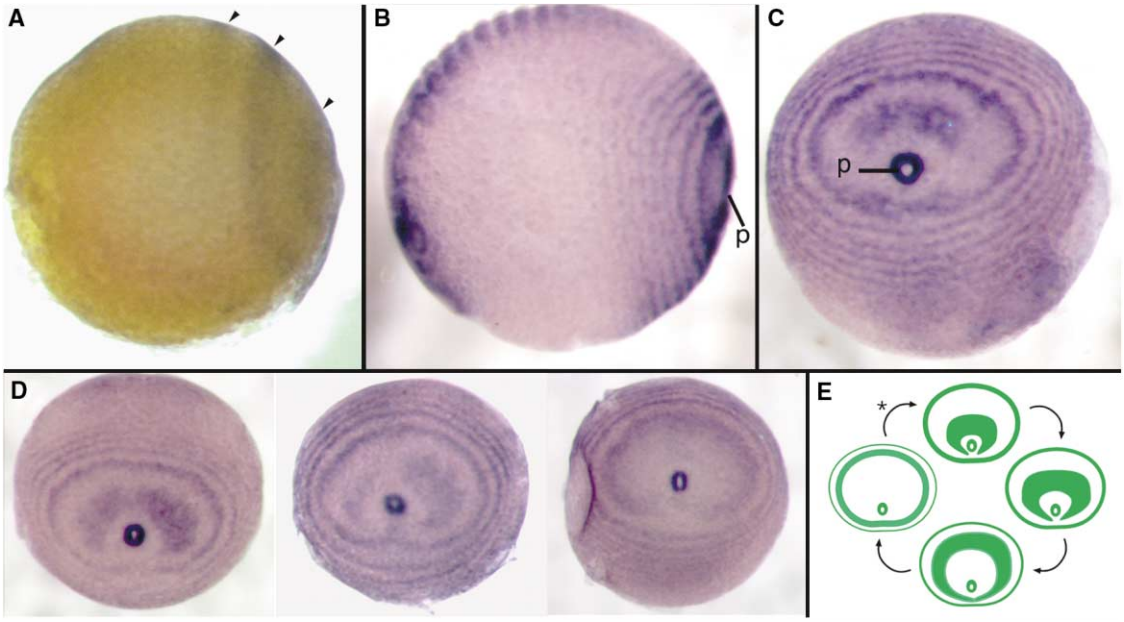

Figure 1. *Stmodr1* Expression in Embryos of *Strigamia maritima*

(A) The earliest detectable expression in 2–3 broad concentric rings (marked with arrowheads) before the appearance of the proctodeum or any sign of segmentation. Lateral view, anterior to the left.

(B) *Stmodr1* expression in an embryo with ~16 segments, lateral view, anterior to the left. The staining in the anterior of the embryo is late expression at the base of forming limb buds.

(C) Same embryo as (B) viewed from the posterior.

(D) Variable patterns of *Stmodr1* expression within the innermost defined ring in three embryos of similar age (12–14 segments) from the same clutch. (The crescent-shaped marking in the embryo to the right is not staining, but a dent in the embryo.)

(E) An interpretation of the sequence of events giving rise to the patterns seen in (D). At the point in the cycle marked by an asterisk, a new domain of expression initiates adjacent to the proctodeum. This domain expands and sharpens over the next two cycles to form a new ring of expression within the previously formed ring. (An outer ring is deleted from the diagram at the transition marked with an asterisk. In reality, each ring persists for several more cycles before fading.)

The *caudal* gene marks an intermediate phase of segment formation, effectively linking the patterns of *odr1* and *engrailed*. Before segmentation begins, *caudal* is expressed throughout the embryonic region of the blastoderm (i.e., the blastodisc) but not in the extra-embryonic territory. Levels of expression are somewhat higher in the posterior of the disc (not shown). As the germ band begins to extend from the anterior of the blastodisc, expression in this anterior territory resolves into stripes, fading completely from the cells between the stripes. More uniform expression is maintained in the posterior of the disc, which contracts as new segments form (Figures 2A–2D). A qualitatively similar pattern is maintained throughout the period of segment formation, except that the entire pattern moves closer to the proctodeum (Figures 4A–4C). By late stages, uniform *caudal* expression persists only around the proctodeum. The pattern of *caudal* stripes extends well into the overtly segmented region, fading as segment morphogenesis begins.

Careful examination of this *caudal* pattern, at any stage of segmentation, reveals a discontinuity in the form and spacing of stripes. The three or four most posterior, and thus most recently formed, *caudal* stripes are of approximately even breadth (left-right extent) and thickness (anteroposterior extent) and are almost evenly spaced. Anterior to these, the spacing between the stripes increases, and a faint secondary stripe appears intercalated between stripes of stronger *caudal* expression (Figure 2E). The distinction between primary and secondary stripes persists clearly for two repeats of this pattern, but further anterior, in older segments, the distinction between the primary and secondary stripes becomes less marked and the spacing more uniform.

Double staining with *engrailed* shows that the oldest *caudal* stripes fully overlap with stripes of *engrailed* expression (Figures 3B and 3C) at single segment intervals. Thus, if the secondary stripes are truly intercalated between primary stripes, the initial, posterior *caudal* stripes must be at two segment intervals. Detecting *odr1* and *caudal* together shows that, where both are well resolved, the periodicity of *odr1* rings is the same as that of the *caudal* stripes and that they largely or completely overlap (Figure 3D). Thus, the *odr1* pattern also resolves to a double segment periodicity. Within the central domain of dynamic *odr1* expression, *caudal* is ubiquitously expressed.

Although we did not notice it previously, the distinction between primary and secondary stripes can be detected in the expression of *engrailed* itself in newly formed segments. In the posterior 6–8 *engrailed* stripes, stronger and weaker expression alternates (Figures 3A and 3B). In a few specimens, there is a two-segment wide gap between the youngest complete stripes of *engrailed* expression and a very weak stripe just forming

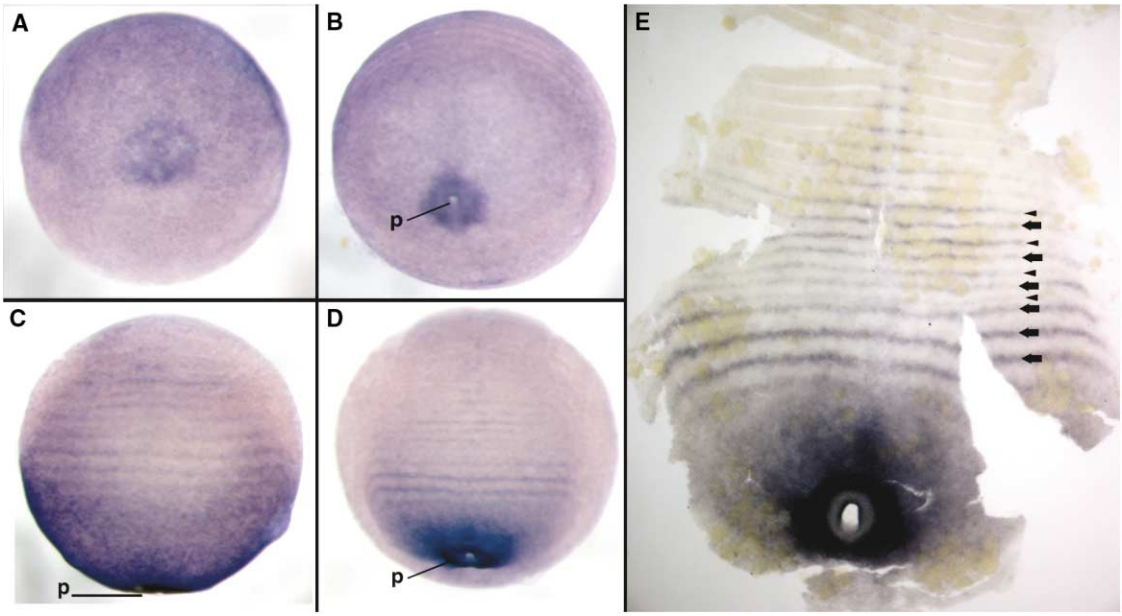

Figure 2. *Stmcad* Expression in Embryos of *Strigamia maritima*

(A) Early expression in an embryo with no morphologically distinct segments, viewed from the posterior. *Stmcad* is expressed throughout a hemispherical domain and starting to be upregulated in the forming proctodeum. A striped domain with 2–3 stripes is in the anterior (hidden in this view).

(B) *Stmcad* expression in an embryo with ~10 segments, viewed from the posterior.

(C) The same embryo as (B), viewed from above, anterior is toward the top.

(D) *Stmcad* expression in an embryo with ~30 segments. The terminal disc has contracted and the continuous expression domain is narrower.

(E) An embryo of a stage similar to (D), dissected from the yolk and mounted flat. Primary *Stmcad* stripes are marked with solid arrows and secondary, intercalated stripes are marked with small arrowheads. p, proctodeum.

between them. The *engrailed* stripes that appear in conjunction with the primary *caudal* stripes develop relatively earlier within the cycle of segment formation than those in conjunction with secondary *caudal* stripes, and they are slightly broader.

We interpret these patterns as evidence that the initial patterning of the blastodisc generates a double segment periodicity. In a slightly later step, this is subdivided to generate individual segments (Figure 4). From our static pictures of gene expression, we cannot formally rule out an alternative model, that the initial stripes of *odr1* and *caudal* are at single segment periodicity and that the pattern we see is generated by a combination of rapid changes in the levels of *caudal* expression as segments mature and rearrangements of cells that alter the spacing of segments as they emerge from the blastodisc. However, this requires a number of ad hoc assumptions about cell behavior and gene expression that make it, in our view, an improbable explanation of the observed pattern.

Our observations invite comparison with the process of segment generation in *Drosophila*. There, a pattern of double segment periodicity is first generated and then subdivided to yield the final single segment repeat. However, the generation of the "pair-rule" pattern in *Drosophila* shows few if any similarities with the early stages of segmentation in *Strigamia. Drosophila* subdivides the entire body axis into unique domains by activating "gap genes" under the influence of maternal gradients and then uses the complex promoters of the pair-rule genes to compute a repetitive pattern of gene activity from this underlying aperiodic pattern. The generation of this pattern is almost static with respect to the forming cells of the blastoderm. In *Strigamia*, the initial patterns of *odr1* expression are not static with respect to the underlying cells. We suggest that the patterns of *odr1* gene expression are oscillations of cell state, coordinated as waves that move across the population of cells in the blastodisc, sharpening to encompass fewer cells and stabilizing to double segment periodicity. Thus, despite the fact that *odd-skipped* is one of the genes expressed in a pair-rule pattern during *Drosophila* segmentation, we think it likely that the processes that generate this pair-rule pattern are different in the two species.

Interestingly, *odd-skipped* family members are downstream targets of the Notch signaling pathway during *Drosophila* limb segmentation [10, 12]. Recently, it has been shown that the *Notch* ligand *Delta* and its target *hairy* are expressed in a striped pattern during early development and segmentation in the embryo of the spider *Cupiennius salei* [13, 14]. Stollewerk et al. [13] suggest that Notch signaling in the spider is generating a reiterated pattern through a mechanism analogous to that shown for vertebrate segmentation [15]. These two observations, taken together, suggest the possibility that the *odd-skipped* family in *Strigamia*, and possibly in other arthropods, is modulated through a Notch-Delta-mediated oscillator to generate the first serially repeated pattern that begins the segmentation process.

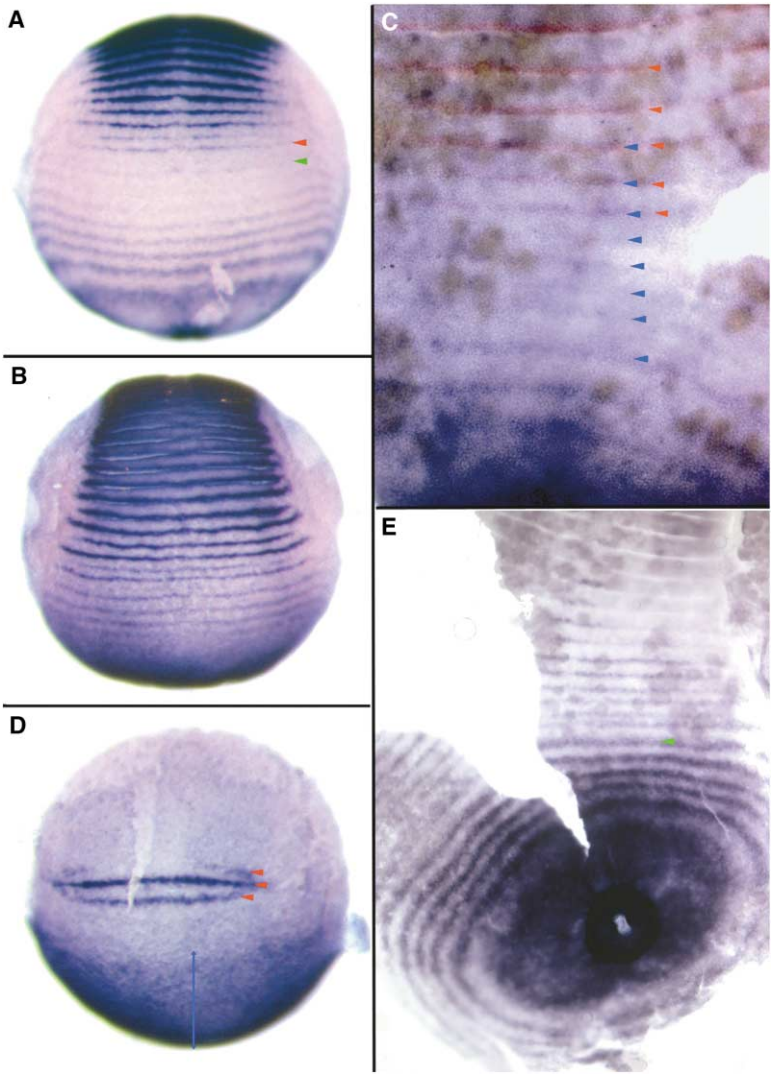

Figure 3. Double Stained Embryos of *Strigamia maritima*

(A) Single color double in situ hybridization for *Stmodr1* and *Stmen* in an embryo with ~20 segments, viewed from above, anterior to the top. *Stmodr1* rings can be seen in the posterior of the embryo and *Stmen* stripes in the anterior, continuing beyond the visible area. The most anterior *Stmodr1* ring is marked with a green arrowhead and the posterior *Stmen* stripe is marked with a red arrowhead.

(B) Single color double in situ hybridization for *Stmcad* and *Stmen* in an embryo with ~30 segments, viewed from above. Anterior to the top. The stripe pattern of the two genes overlaps in the area where new segments are being formed. In this preparation it is not possible to state unambiguously which of the stripes represent the overlap between the two genes, but see next panel.

(C) Flat-mounted embryo with ~25 segments, stained for *Stmcad* (blue) and *Stmen* (red). Blue and red arrowheads indicate *Stmcad* stripes and *Stmen* stripes, respectively. Analysis of comparable embryos stained for each of the genes separately suggests that the overlap between the two genes is more extensive (up to 10 segments); however, with our double staining protocol, the two genes can only be seen to overlap in 3–4 segments.

(D) A young embryo with no morphologically distinct segments, stained for *Stmcad* and *Stmen*. Three *engrailed* stripes can be seen in the anterior (marked with red arrowheads), but there are no *caudal* stripes anterior to the continuous expression domain (marked with a double-headed blue arrow), suggesting that *caudal* expression is not modulated during the formation of the first few segments.

(E) Flat-mounted embryo with ~20 segments, stained for *Stmcad* and *Stmodr1*. The *Stmcad* stripes and *Stmodr1* rings cannot be distinguished, indicating that they overlap in the domain where they are co-expressed. The most anterior *Stmodr1* ring is marked with a green arrowhead.

A separate and unresolved issue is whether there are similarities between the process that resolves the pair-rule repeat of *Drosophila* into a single segment pattern and the process whereby secondary *caudal* stripes intercalate between primary stripes to generate the single segment repeat in *Strigamia*. The possibility that such "frequency doubling" processes may be widespread among the arthropods is supported by the observation of analogous phenomena in chelicerates and short germ insects: in the mite *Tetranychus urticae*, expression of the *paired* gene in the prosoma is initially at double segment intervals, with secondary stripes intercalating between them to generate the single segment repeat [16]. In the growing abdomen of the grasshopper *Schistocerca americana*, *paired* gene expression also shows a transition from double to single segment periodicity, though in this case the process is one of stripe splitting rather than intercalation [17]. However, in other cases, either no such pattern has been described or the periodicity of gene expression is not yet clear. Of particular relevance in this context is a recent study of segmentation gene expression in the lithobiomorph centipede, *Lithobius atkinsoni* (only distantly related to *Strigamia*) [3]. The expression of *even-skipped* in the posterior of the *Lithobius* germ band shows broad rings around the proctodeum that could reflect dynamic expression, resolving into stripes. However, there is as yet no evidence of subsequent frequency doubling.

Conclusions

Our results provide a possible explanation for the observation that, in nature, centipede segment number varies in two-segment increments. We propose that variation in segment number among centipedes is caused by variation in the number of cycles of a primary segmentation oscillator, each cycle of which generates two segments. The anteroposterior range of this process may well extend beyond the trunk to include the poison claw and parts of the head and genital regions. Therefore, the occurrence of odd rather than even numbers of leg-bearing segments is not incompatible with our explanation.

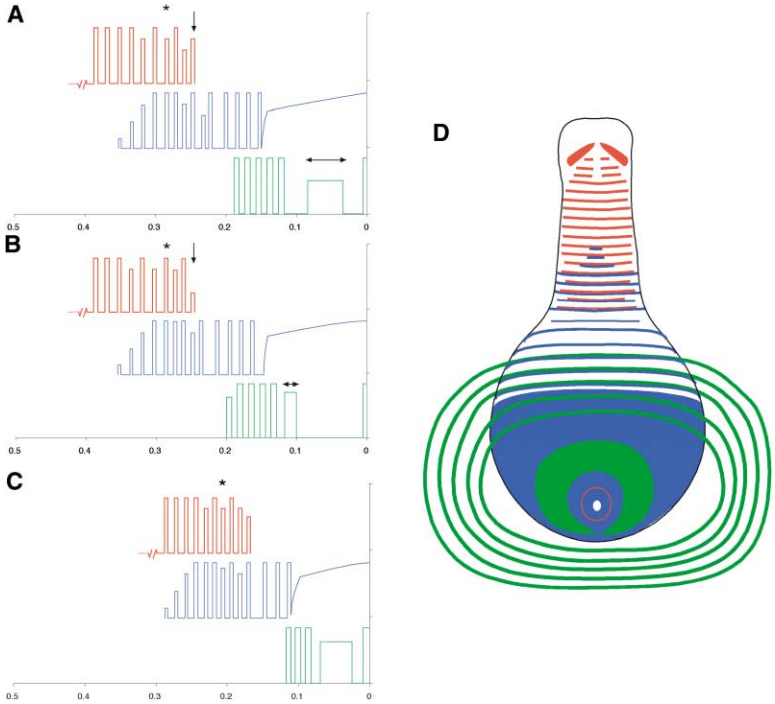

Figure 4. A Diagrammatic Representation of the Spatial and Temporal Relationships of *odr1, caudal*, and *engrailed*

The diagram is based on observations of a sample of ~50 single and double stained embryos, on 12 of which the expression profiles were measured on photographs of whole or flat-mounted embryos.

(A–C) Expression profile of the three genes along the embryonic midline. The x axis represents distance from the center of the proctodeum, given as a proportion of the circumference of the egg. The y axis is a subjective estimate of the intensity and breadth of the expression domain. *odr1* is in green, *caudal* in blue, and *engrailed* in red. Only the 10 youngest (i.e., most posterior) *engrailed* stripes are shown. The posterior-most morphologically distinct segment is marked with an asterisk.

(A and B) Two phases of expression seen in embryos with a similar number of segments (19–20 *engrailed* stripes). In (A), the most recently formed *engrailed* stripe corresponds to a primary *caudal* stripe (marked by an arrow). There is a diffuse but broad *odr1* expression domain within the terminal disc (marked by a double headed arrow). In (B) the most recently formed narrow *engrailed* stripe (marked by an arrow) corresponds to a secondary *caudal* stripe that is already evident in (A). The broad *odr1* domain is narrower, sharper, and farther from the proctodeum. We have drawn the diagram assuming that (B) is half an *odr1* cycle later than (A). The stripes of *caudal* and *engrailed* expression are shown as having moved anteriorly by a segment's width, reflecting the inferred forward extension of the germ band.

(C) Expression profiles in an embryo with ~30 segments. The terminal disc has contracted and all expression domains have shifted posteriorly. The spacing between consecutive *engrailed* stripes (and the corresponding *caudal* stripes) is approximately the same as in younger embryos. There are fewer *odr1* rings.

(D) Schematic flattened projection of the expression patterns of the three genes in an embryo of the same stage as that represented in (A). *odr1* rings extend onto the reverse hemisphere of the egg. For clarity, overlapping stripes have been portrayed as adjacent.

Experimental Procedures

Embryo Collection

Embryos were collected in the wild and fixed as described previously [5].

Cloning of *Strigamia maritima odd-skipped related-1*

A 230 bp fragment (183 bp without primers) of *odd-skipped* was recovered fortuitously by RT-PCR using specific primers designed to clone out the *S. maritima Krüppel* gene (unpublished data). The primers used were TTGCAGAATCACGAGCGAACGCAC (forward primer) and TTCTCGTCCTTCGGGCACTTGTGA (reverse primer), which correspond (partially) to the conserved zinc finger motifs in the *odd-skipped* gene family. An RNA probe was made using this sequence and proved sufficient for in situ hybridization experiments. Later, this gene sequence was extended using 3′ RACE. A probe generated using the 442 bp RACE fragment gave the same expression pattern as that given by the shorter fragment.

Cloning of *Strigamia maritima caudal*

A 248 bp fragment of *caudal* was recovered by RT-PCR using the degenerate primers CGNTCCCCNTAYGARTGGATGA (forward primer) and TTCTTGTTCTGCTTNCKYTCYT (reverse primer). This fragment was extended using 3′ RACE to 342 bases of coding sequence. Specific primers were used to clone out a 339 bp cDNA sequence, which was used to make a labeled RNA probe for in situ hybridization. The specific primer sequences were AAGCCATCT TACCAAACACAGTGC (forward primer) and GTCCAAACTAATCAT CTTATTGCC (reverse primer). Sequences for both genes can be found in GenBank Accession #AY562125 and AY562126.

In Situ Hybridization

Staining was carried out according to published protocols [5]. Single color double staining was done by simultaneous hybridization and detection of two probes labeled with Digoxygenin. Two-color staining was done by simultaneous hybridization of two probes, one stained with Digoxygenin and one with fluorescein. Antibody binding was done sequentially, with a 0.1 M glycine (pH 2.2) wash between the two antibodies, using the alkaline phosphatase substrates BM-purple (Roche) and Fast-Red (Roche).

Supplemental Data

Two supplemental figures are available online at http://www.current-biology.com/cgi/content/full/14/14/1250/DC1.

Acknowledgments

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