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**The T-box genes *H15* and *optomotor-blind* in the spiders
Cupiennius salei, *Tegenaria atrica* and *Achaearanea tepidariorum*
and the dorso-ventral axis of arthropod appendages**

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Abstract

Dorsoventral axis formation in the legs of the fly *Drosophila melanogaster* requires the T-box genes *optomotor-blind* (*omb*) and *H15*. Evolutionary conservation of the patterning functions of these genes is unclear, because data on *H15* expression in the spider *Cupiennius salei* did not support a general role of *H15* in ventral fate specification. However, *H15* has a paralogous gene, *midline* (*mid*) in *Drosophila* and *H15* duplicates are also present in *Cupiennius* and the millipede *Glomeris marginata*. *H15* therefore seems to have been subject to gene duplication opening the possibility that the previous account on *Cupiennius* has overlooked one or several paralogs. We have studied *omb* and *H15* related genes in two additional spider species, *Tegenaria atrica* and *Achearanea tepidariorum* and show that in both species one of the *H15* genes belongs to a third group of spider *H15* genes that has an expression pattern very similar to the *H15* pattern in *Drosophila*. The expression patterns of all *omb* related genes are also very similar to the *omb* expression pattern in *Drosophila*. These data suggest that the dorsoventral patterning functions of *omb* and *H15* are conserved in the arthropods and that the previous conclusions were based on an incomplete data set in *Cupiennius*. Our results emphasize the importance of a broad taxon sampling in comparative studies.

Introduction

Arthropod appendages develop along three different axes. The molecular mechanisms of appendage axis formation have been studied best in the fly *Drosophila melanogaster*. The anterior-posterior axis builds on the mechanisms that also establish the anterior-posterior polarity of the body segments (Cohen 1990). The second axis, the proximal-distal axis, is governed by a hierarchic gene cascade comprising three principal levels (e.g. Lecuit and Cohen 1997; Rauskolb and Irvine 1999; Rauskolb 2001): first, the morphogens Wingless (Wg) and Decapentaplegic (Dpp) erect a system of protein concentration gradients. These gradients are read out by the next level, the leg gap genes, that include genes like *dachshund* and *Distal-less*. The ensuing subdivision into leg segments (podomeres) is the responsibility of the lower level of the cascade that includes mostly members of the Notch signaling pathway or its target genes.

The developmental mechanism governing the formation of the third appendage axis, the dorsal-ventral axis, is poorly known even in *Drosophila*. The two morphogens Dpp and Wg that organize the proximal-distal leg axis also organize the formation of the dorsal-ventral leg axis. The *dpp* gene has been shown to activate the T-box gene *optomotor-blind* (*omb*) along the dorsal side and misexpression of *omb* has been shown to lead to dorsalizing effects (Brook and Cohen 1996; Maves and Schubiger 1998). The *wg* gene activates another T-box gene, *H15*, in a large ventral domain (Wilder and Perrimon 1995; Brook and Cohen 1996; Abu-Shaar and Mann, 1998). This ventral expression domain suggests a ventralizing role of *H15*, but experimental evidence for this is still lacking. Apart from these two T-box containing

factors no other genes have been identified yet that are involved in dorsal-ventral leg axis formation in *Drosophila*.

The mechanisms of anterior-posterior and proximal-distal axis specification have been studied in other arthropods as well and seem to be conserved to a certain extent among arthropods (e.g. Abzhanov and Kaufman 2000; Prpic et al. 2003). The patterning of the dorso-ventral leg axis, however, is not well studied in other arthropods. As a first step towards a better understanding of dorsal-ventral patterning in arthropod appendages, we have previously studied the expression of *omb* and *H15* related genes in the American wandering spider *Cupiennius salei* and the pill millipede *Glomeris marginata* (Prpic et al. 2003; Prpic et al. 2005). In both species, the *omb* gene showed a conserved expression pattern along the dorsal side of all appendages, compatible with a role in dorsal specification. With regard to the *H15* genes, we recovered two different genes in each species. Also in *Drosophila* two *H15*-type genes are present, *H15* and *midline (mid)*, but they have identical expression patterns in the legs (Prpic et al. 2003) and probably identical functions in development (e.g. Buescher et al. 2004; Reim et al. 2005; Qian et al. 2005; Miskolczi-McCallum et al. 2005; Buescher et al. 2006). Confusingly, however, the gene pairs in *Cupiennius* and *Glomeris* could neither be directly homologized with the *Drosophila* *H15*-related genes in a phylogenetic analysis, nor did they show expression patterns readily comparable with the *Drosophila* genes or with each other. The expression of the *H15*-related genes in *Cupiennius* was not compatible with a conserved function in ventral fate determination under the control of Wg signaling.

Here we present a study of *omb* and *H15* type genes in three spider species representing three different superfamilies of entelegyne spiders (see Fig. 1D): *Cupiennius salei* (Fig. 1A), *Tegenaria atrica* (Fig. 1B) and *Achaearanea*

tepidariorum (Fig. 1C). The most important finding is the discovery of a third *H15*-type gene in *Tegenaria* and its ortholog in *Achaeearanea* that show an expression pattern very similar to the *Drosophila H15*-type genes. Our new data show that our previous conclusions with respect to the *H15*-type genes were based on an incomplete data set. All available data now suggest that the dorsalizing and ventralizing roles of *omb* and *H15*, respectively, are conserved in the arthropods. Further studies are now needed in order to confirm the dorsalizing and ventralizing functions of these genes, to establish their regulation by upstream factors (e.g. *dpp*, *wg*), and to identify further genes involved in dorsal-ventral axis formation in the arthropods.

Materials and Methods

Embryo fixation and staging

Embryos of *Cupiennius* were obtained and treated as described (Damen et al. 1998; Damen and Tautz 1998). Female and male *Tegenaria* were collected at several sites in Cologne (Germany). Embryos of *Tegenaria* were obtained and treated as has been described for *Cupiennius* embryos. Embryos of *Achaeearanea* were obtained from a laboratory culture in Cologne and fixed as described by Akiyama-Oda and Oda (2003). Spider embryos develop first by germ band extension, but then undergo a process called inversion, in which the germ band splits ventrally and which precedes dorsal closure (see Prpic and Damen (2005a) for details). Embryos of all three spider species were staged roughly into stages before inversion (germ band elongation

stages) and stages during inversion. For further details about spider embryology see Foelix (1996).

Cloning of T-box genes and sequence analysis

RNA from each species was isolated from a mixture of embryos of different developmental stages using TRIZOL (Invitrogen). Poly-A RNA (PolyATtract mRNA isolation system III, Promega) was used to produce cDNA (SuperscriptIII first strand synthesis system for RT-PCR, Invitrogen). Two independent duplicates were performed for each gene cloning assay. The primers used to obtain fragments of *omb* and *H15/mid* type genes have been published previously (Prpic et al., 2003; Prpic et al. 2005). In addition, we used the following primers: *Cs-omb-2* was recovered with the primers CAN AAY GAR ATG ATH GTN CA (forward primer) and AA NGG RTT RTA YTT DAT YTT (reverse primer) in the initial PCR and the primers CAN AAY GAR ATG ATH GTN CA (forward primer) and TC RTT YTG RTA NGC NGT NAC (reverse primer) in the nested PCR. The sequences of *At-H15-1*, *At-H15-2* and *At-omb* were available from GenBank. We used gene specific primers to amplify fragments of these genes by PCR (*At-H15-1*: nt 15-610; *At-H15-2*: nt 30-619; *At-omb*: 1071-1513). Sequences were determined from both strands on an ABI-3100 automated sequencer (Applied Biosystems), using Big Dye dye-terminators (Perkin Elmer). The sequences are available from GenBank with the following accession numbers: *Cs-omb*: AJ518937; *Cs-omb-2*: AM774407; *Cs-H15-1*: AJ518938; *Cs-H15-2*: AJ518939; *At-H15-1*: AB177874; *At-H15-2*: AB177875; *At-omb*: AB177876; *Ta-H15-1*: AM404110; *Ta-H15-2*: AM404111; *Ta-H15-3*: AM404111; *Ta-omb-1*:

AM404113; *Ta-omb-2*: AM404114. Sequence analysis was performed as described previously (Prpic et al. 2005).

In situ hybridization and preparation

Whole-mount in situ hybridization was essentially performed as described for *Drosophila* (Tautz and Pfeifle 1989) with modifications for spider embryos (Damen and Tautz, 1998). We used DIG-labeled RNA probes. Embryos were analysed as whole mounts in PBST under a Leica dissection microscope equipped with an Axiocam (Zeiss). Appendages were dissected with fine tungsten needles and photographed under a Zeiss Axioplan compound stereo microscope. Brightness, contrast and color values were corrected in all images using the image processing software Adobe Photoshop (Version 7.0 for Apple Macintosh).

Results

T-box genes from the spider species *Cupiennius salei*, *Tegenaria atrica*, and *Achaeearanea tepidariorum*

From the spider *Tegenaria* we have isolated in a PCR screen two different gene fragments with high similarity to *omb* from *Drosophila*, and three different gene fragments with high similarity to *H15* and *mid* from *Drosophila*. New PCR screening in the spider *Cupiennius* resulted in the identification of a second *omb*-type gene

fragment, in addition to the previously reported *omb*-type T-box sequence and the two *H15*-related T-box sequences in this spider species (Prpic et al. 2003). In addition the sequence of one *omb*-type gene and two *H15*-type genes of the common house spider *Achaearanea* are available in GenBank (accession numbers: see methods).

In order to establish the orthology of the spider *omb* and *H15*-type genes we performed a phylogenetic analysis using all T-box genes present in the *Drosophila* genome and all *omb* and *H15*-types genes identified in the three spider species, supplemented with sequences from the pill millipede *Glomeris marginata* (Prpic et al. 2005). The phylogenetic tree (Fig. 2) shows that all *omb* related sequences form a cluster of closely related sequences. The *omb* gene of *Drosophila* forms the sister group of all other *omb* genes. It is not possible to designate one of the two *omb*-like genes from either *Cupiennius* or *Tegenaria* as being more closely related to *Drosophila omb* and we designate them therefore simply as *Ta-omb-1* and *Ta-omb-2* (in the case of *Tegenaria*) and as *Cs-omb* (this name has been given previously; Prpic et al. 2003) and *Cs-omb-2* (in the case of *Cupiennius*).

The phylogenetic analysis also shows that all *H15*-like sequences form a well-supported cluster of closely related sequences, but also shows that they cluster together according to arthropod class (chelicerate/myriapod/insect) (Fig. 2). Furthermore, the phylogenetic analysis reveals that, within the spiders, the *H15* sequences form three groups, one group consisting of *At-H15-1*, *Cs-H15-1* and *Ta-H15-1* (group-1 *H15* genes), a second group consisting of *Cs-H15-2* and *Ta-H15-2* (group-2 *H15* genes), and a third group consisting of *At-H15-2* and *Ta-H15-3* (group-3 *H15* genes). These three groups are also supported by the expression pattern analysis (see below).

Please note that the *Achaearanea* genes have been named previously (Akiyama-Oda and Oda 2004); both sequence and expression analysis suggest that *At-H15-2* despite its name is a group-3 *H15* gene. Nevertheless we decided not to rename the gene as this could lead to new confusion, because it would suggest that three *H15-type* genes have already been isolated in this species.

Embryonic expression of *omb* homologs

We next examined the expression of the *omb*-related genes in all three spider species. First we describe the general expression pattern of the genes; the expression in the prosomal appendages is reported in the following section. The body of spiders is divided into two tagmata, the anterior prosoma and the posterior opisthosoma. The prosoma bears six pairs of appendages (from anterior to posterior): the chelicerae (venom fangs), the pedipalps (mainly sensory appendages) and four pairs of walking legs. The opisthosoma bears the breathing organs (book lungs and tubular tracheae) and the spinnerets.

The expression of *Cs-omb* has been described before (Prpic et al. 2003). Here we focus on the newly isolated *Cs-omb-2* gene. The expression of *Cs-omb-2* is virtually identical to the expression of *Cs-omb*. Like *Cs-omb*, *Cs-omb-2* is expressed in the brain centers of the lateral eyes (Fig. 3A) and in a segmental pattern in the opisthosoma (Fig. 3B). When at later stages the four opisthosomal limb buds develop, *Cs-omb-2* is expressed in the dorsal portion of all of them. However, *Cs-omb-2* is also

expressed in more posterior segments that do not develop appendages or appendage-derived organs (Fig. 3B).

Like *Cupiennius*, *Tegenaria* also has two different *omb*-related genes. Both genes are expressed in the head lobes in a large region that also encompasses the primordia of the lateral eyes and corresponding brain regions (Fig. 3E,G). The expression of *Ta-omb-2* in this area is significantly weaker than the expression of *Ta-omb-1*. *Ta-omb-1* is also expressed in the labrum (not shown). Both genes are expressed in the opisthosoma in virtually identical patterns. Expression is detected in the primordia of the book lungs, tubular tracheae and spinnerets, but only in their dorsal part (Fig. 3F,H). At younger stages there is also very weak expression in opisthosomal segment 6, at a position obviously serially homologous to the expression in the breathing organs and spinnerets. However, this segment does not develop appendages or appendage derived organs.

At-omb, the only *omb*-type gene known from *Achaearanea*, is expressed similar to the *omb*-related genes from the other two spider species (see also Akiyama-Oda and Oda 2006). However, the expression in the optic lobes is very weak and restricted to a thin stripe in early stages (Fig. 3C,D). Like in the other two spider species, *At-omb* is expressed in a segmental pattern in the opisthosoma (Fig. 3C).

Expression of *omb* homologs during leg development

In the chelicerae of *Cupiennius* both *omb*-related genes are expressed along the dorsal side in early stages (Fig. 4A,G). Later, *Cs-omb* fades in the chelicerae (Fig. 4B), but expression of *Cs-omb-2* remains strong (Fig. 4H). In both *Achaearanea* (Fig. 4M,N)

and *Tegenaria* (Fig. 4S,T; Y,Z) all *omb*-related genes are expressed along the dorsal side of the chelicerae at all examined stages of development.

The pedipalps and walking legs of all three species also show expression of all *omb*-related genes along the dorsal side (Fig. 4C-F; I-L; O-R; U-X; a-d). However, some genes show additional expression features: *Cs-omb* and *Ta-omb-1* are expressed in the mesoderm in a repeated fashion at later stages (Fig. 4D, F; V, X). In addition, the dorsal expression of *Cs-omb* is weaker in late stages in both the pedipalps and the walking legs (Fig. 4D,F). In earlier stages, *Cs-omb* (Fig. 4C,E), *At-omb* (Fig. 4O,Q) and *Ta-omb-1* (Fig. 4U,W) display a discontinuous dorsal expression. Expression at the proximal and distal ends of the appendages is strong, but these expression domains are connected by weaker expression that in some cases appears to form a distal-to-proximal gradient.

Embryonic expression of *H15* homologs

The general expression of both *H15*-related genes from *Cupiennius* has been reported before (Prpic et al. 2003).

Apart from expression in the appendages that is described in the next section, both *H15*-related genes from *Achaearanea* are expressed in spots in the central nervous system (CNS) (Fig. 5A,C) and in the developing dorsal vessel (heart) of the embryos. However, the expression of *At-H15-2* in the heart is visibly weaker than the expression of *At-H15-1* (Fig. 5A,B,D).

All three *H15*-related genes of *Tegenaria* have similar but clearly different patterns of expression. *Ta-H15-1* is expressed in the labrum and the developing heart

(Fig. 5E). The gene is also expressed in the CNS: there is a small group of cells in the cheliceral neuromere (not shown) and similar spot-like expression domains are seen in all remaining prosomal neuromeres (Fig. 5F). Expression in the opisthosoma includes the primordia of the spinnerets and a segmental row of spots in the opisthosomal neuroectoderm extending into the posterior end of the germband (not shown). *Ta-H15-2* is also expressed in the labrum (not shown) and the developing heart (Fig. 5G). Also the expression in the prosomal neuroectoderm is similar to *Ta-H15-1* (Fig. 5H), but we did not detect expression of *Ta-H15-2* in the cheliceral neuromere. In the opisthosoma there is expression in the spinnerets, but expression in the opisthosomal neuroectoderm was not detected (not shown). In contrast to the other two *H15*-related genes, *Ta-H15-3* is not expressed in the labrum or the developing heart (Fig. 5I). It is expressed in the neuroectoderm of the prosoma including the cheliceral neuromere (Fig. 5J and data not shown), but in a pattern different from the point-like expression of the other two genes. The expression domain is larger and is more posterior in each hemisegmental neuromere. In the opisthosoma there is a faint segmental expression in the neuroectoderm, but this is so weak that it is hardly above the level of artificial background staining of our in situ hybridization technique; the only stronger opisthosomal expression of *Ta-H15-3* is in the primordia of the breathing organs (not shown).

Expression of *H15* homologs during leg development

As previously reported (Prpic et al. 2003), the two *H15*-related genes in *Cupiennius* are expressed in a restricted area along the proximal ventral side of the

appendages. The expression domain of *Cs-H15-1* (Fig. 6A-F) is larger and it is expressed earlier in development than *Cs-H15-2* (Fig. 6G-I). *Cs-H15-1* also has additional expression domains in the proximal dorsal area and in the ventral tip of pedipalp (Fig. 6D) and leg (Fig. 6F).

At-H15-1 has an expression pattern similar to *Cs-H15-1*. Ventral expression is restricted to the proximal portions (Fig. 6J-O) and there is an additional spot of expression in the ventral distal tip of pedipalp and leg (Fig. 6L,N). In the legs at late stages there is a repeated pattern of expression dots (Fig. 6O). By contrast, *At-H15-2* is not expressed in the ventral proximal area, but is expressed along the entire distal ventral side (Fig. 6P-U). This is similar to *H15* and *mid* in *Drosophila* and fundamentally different from both *Cupiennius* genes. It is interesting to note, that the ventral patterns of *At-H15-1* and *At-H15-2* in pedipalps and legs are complementing each other and together they extend along the entire ventral side of the appendages.

The expression of *Ta-H15-1* (Fig. 6V-Z, a) is similar to *Cs-H15-1* and *At-H15-1*. Expression is restricted to the proximal ventral side and, in the legs, there are additional expression domains proximally on the dorsal side and in the ventral tip (Fig. 6Z,a). The expression of *Ta-H15-2* (Fig. 6b-g) is similar to *Cs-H15-2*. It is expressed more weakly and more restricted than *Ta-H15-1* and lacks the additional expression domains. In the chelicerae we could not detect expression of *Ta-H15-2* (Fig. 6b,c). *Ta-H15-3* (Fig. 6h-m), finally, is expressed similar to *At-H15-2* and to *H15* and *mid* in *Drosophila*. *Ta-H15-3* is expressed in the ventral ectoderm along the entire ventral side. However, in the pedipalps and legs proximal expression is weaker than in medial and distal areas (Fig. 6j,k,l,m). In addition, there is a relatively strong expression within the appendages, especially at earlier stages (Fig. 6h, j, l).

Discussion

***H15*-related genes in three spider species from different superfamilies.**

We have isolated *H15*-type genes from three spider species that belong to three different superfamilies, the *Araneoidea* (*Achaearanea tepidariorum*), the *Amaurobioidea* (*Tegenaria atrica*) and the *Lycosoidea* (*Cupiennius salei*) (see Fig. 1 for an overview). The differences in paralog number (two each in *Achaearanea* and *Cupiennius*, and three in *Tegenaria*) might be explained simply by the evolutionary distance between the species. However, the phylogenetic analysis (Fig. 2) groups together those spider *H15*-related genes that also show similar expression patterns. Together these data strongly suggest that in the spiders there are three groups of paralogous genes that were already present in their last common ancestor (Fig. 7): Group-1 *H15* genes (*At-H15-1*, *Ta-H15-1*, and *Cs-H15-1*); group-2 *H15* genes (*Ta-H15-2* and *Cs-H15-2*) and group-3 *H15* genes (*At-H15-2* and *Ta-H15-3*). In group 2 and group 3 the respective genes from *Achaearanea* and *Cupiennius* seem to be missing (as indicated by question marks in Fig. 7). Although we cannot exclude that the group-3 *H15* gene in *Cupiennius* and the group-2 *H15* gene in *Achaearanea* have been lost secondarily, a more likely explanation for these "missing genes" is that these genes are present in the genomes, but have not yet been isolated in the PCR screens. Thus, we assume that a set of three *H15*-related genes is present in all three spider species (Fig. 7).

***omb* and *H15* in dorsoventral appendage patterning**

The data we present in this report suggests that the role of *omb* and *H15* as instructors of dorsal and ventral fate in the appendages is conserved in all arthropods. Our new data on *omb* and *H15* genes in the spiders *Achaearanea* and *Tegenaria* shows that the two T-box genes *omb* and *H15* are acting in a dorsal and ventral domain, respectively, in the legs of all arthropod groups studied so far (Figure 7).

In *Drosophila* *omb* is activated by *decapentaplegic* (*dpp*), which is expressed in a dorsal sector, and the *H15* gene is activated by *wingless* (*wg*), which is expressed in a ventral sector (e.g. Brook and Cohen 1996). In other arthropod species investigated so far *dpp* is not expressed along the dorsal side of the appendages like it is in *Drosophila*. Rather it is restricted to the distal tip early and forms rings later on (Jockusch et al. 2000; Niwa et al. 2000; Prpic et al. 2003; Prpic 2004). Thus, it is difficult to imagine how Dpp could activate *omb* expression along the dorsal side of the legs in these species. It remains unclear, therefore, whether *omb* is activated directly by Dpp in these species. However, because Dpp protein is a morphogen, the Dpp protein spreading from the tip might be sufficient to trigger dorsal *omb* expression (see also Prpic et al. 2003). Alternatively, *omb* is activated via an as yet unknown mechanism. It is important to note that in more basal insects like the beetle *Tribolium castaneum* (Sanchez-Salazar et al. 1996) and the milkweed bug *Oncopeltus fasciatus* (Angelini and Kaufman 2005a) *dpp* is expressed similar to the millipede *Glomeris* and the spiders *Cupiennius* and *Achaearanea* (Prpic et al. 2003; Akiyama-Oda and Oda 2003; no *dpp* expression data is available yet for *Tegenaria*). The expression domain of *dpp* along the entire dorsal side is thus not characteristic for insects and could be an adaptation to leg development from imaginal discs in flies. Subsequently, this dorsal *dpp* expression could have been used for activating *omb* in

the dorsal domain in flies, replacing a more ancestral system for dorsal *omb* activation.

In contrast to *dpp* expression, the expression of *wg* on the ventral side is highly conserved among all arthropods including the expression in the imaginal discs of *Drosophila*. The expression of *wg* in the legs of *Tegenaria* has not yet been studied, but if we assume it to be expressed like *wg* in the two other spider species, *Cupiennius* and *Achaearanea*, (Prpic et al 2003, N. M. F. unpublished data) then it is interesting to note that the ectodermal expression of the group 3 *H15* genes (i.e. *Ta-H15-3* and *At-H15-2*) is almost identical to the *wg* expression in the spider legs. The expression patterns thus are compatible with a role of *H15* in ventral fate determination in all arthropods and with *H15* being a regulatory target of *wg* in leg development. This is an important finding since the role of *wg* in arthropod leg development has been questioned: recent work in *Oncopeltus* could not establish a role for Wg signaling in leg development (Angelini and Kaufman 2005a). These authors propose that the involvement of Wg signaling in leg development evolved very late in arthropod evolution, probably in the lineage leading to the higher dipterans (Angelini and Kaufman 2005b). The extraordinary conservation of *wg* expression in the variety of arthropods studied so far seems to contradict this notion, as does the ventral expression of *Notum* (a regulator of Wg signaling) in *Cupiennius* (Prpic and Damen 2005b). The *H15* data shown here demonstrate that the expression of a regulatory target of Wg signaling is conserved in arthropods as distantly related as spiders and flies. This supports the notion that involvement of Wg signaling including the target gene *H15* is an ancestral feature of arthropod appendage development.

Evolution of the *H15*-related genes: how many gene duplications?

In the phylogenetic analysis the *H15* genes cluster together according to arthropod class. This indicates that the *H15* genes in each class are more closely related among themselves than to any of the *H15* genes in a different arthropod class. This suggests independent duplication events in each class. In this scenario (Fig. 7, green squares) the ancestral state is a single *H15* like gene that gave rise to the multiple *H15* genes in the fly, the millipede, and the spiders by independent duplication events. In this case an additional duplication of one of the genes must have taken place in the chelicerate lineage to arrive at the complement of three genes in spiders.

On the other hand, this scenario invokes a high number of independent gene duplication events and, therefore, is not very parsimonious. An alternative interpretation is that the sequence analysis is influenced by concerted evolution. Concerted evolution can be the result of gene conversion and has been documented for genes or portions of genes that share a high sequence similarity and that are located close to one another in the genome (e.g. Peel et al. 2006). Thus, although our phylogenetic analysis suggests independent duplications of *H15* in all arthropod classes (with high reliability values at all three edges), the analysis might be suffering from artefacts caused by concerted evolution. In the latter scenario, also a lower number of gene duplication events has to be postulated (Fig. 7, blue squares): the ancestral state for arthropods then would be two *H15*-like genes and an additional duplication event has taken place in the lineage leading to the spider species. Further work is needed to investigate this in more detail.

Conclusions

Although comparative analyses of gene expression patterns can give important clues as to the evolutionary conservation or change of gene regulation, our data indicate that the presence of multiple paralogous genes can be a potential source of misinterpretation. Our previous analysis of T-box genes in *Cupiennius* demonstrated that *omb* was expressed along the dorsal side of the appendages, compatible with a role in dorsal fate determination like in *Drosophila*. However, the two *H15*-related genes recovered from *Cupiennius* are only expressed in a very small portion of the ventral side and thus only in a fraction of *wg* expressing cells. These data argued against a general role in ventral fate determination as well as against a general activation of *H15* by Wg signaling. The discovery of a third *H15*-type gene in two other spider species, *Tegenaria* and *Achaearanea*, suggests that this conclusion presumably was premature. This third *H15*-type gene is expressed along most of the ventral side of all appendages in these two spider species much like the *Drosophila* genes and thus is fully compatible with a role in ventral fate determination downstream of Wg-signaling. It also suggests that our previous account on the *H15*-type genes in *Cupiennius* was incomplete and that the group-3 *H15* gene has not yet been isolated from *Cupiennius*. Taken together all data now suggest that the role of *omb* and *H15* in dorsal-ventral axis formation in the legs is conserved. In addition, the possible link between Wg-signaling and *H15*-type genes is at odds with the recent notion that Wg-signaling has no role in appendage development in arthropods other than *Drosophila*. More work is needed to clarify the role of this signaling pathway in the processes of dorso-ventral and proximal-distal leg axis formation in the arthropods. However, all present data firmly establish the link between *omb* expression and the dorsal surface of the appendages. Thus, *omb* expression might be a

useful marker for identifying the dorsal side in highly specialized appendages (e.g. the mandible in insects or the gnathochilarium in millipedes), where it has been difficult in the past to unambiguously identify homologies with other, more normal appendages.

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Legends to the figures

Fig. 1. Spider species used in this study. A, *Cupiennius salei*, the Central-American wandering spider, belongs to the family *Ctenidae* (Superfamily *Lycosoidea*). B, *Tegenaria atrica*, a member of the *Agelenidae* (Superfamily *Amaurobioidea*). C, *Achaeearanea tepidariorum* belongs to the *Theridiidae* (Superfamily *Araneoidea*). D, Phylogeny of the higher entelegyne spiders (simplified after Coddington and Levi 1991) illustrating the interrelationships of the spider species in this study.

Superfamilies containing a species used in this study are in bold type. The asterisk indicates that the monophyly of the *Amaurobioidea* is not firmly established. Images taken by and courtesy of Arne Nolte (A), Nikola-Michael Prpic (B), Matthias Pechmann (C). Scale bar in A, B, C is 10 mm.

Fig. 2. Phylogenetic analysis of arthropod T-box gene fragments. Shown is the unrooted Puzzle tree computed from 1000 intermediate trees produced with the Quartet Puzzling method (Strimmer and von Haeseler 1996). The numbers at the tree edges are the reliability values. For reasons of clarity the branch with the *omb* genes has been magnified (see inset). Dm, *Drosophila melanogaster*; At, *Achaeearanea tepidariorum*; Cs, *Cupiennius salei*; Ta, *Tegenaria atrica*; Gm, *Glomeris marginata*.

Fig. 3. Embryonic expression of *omb*-type T-box genes. A,B, expression of *Cs-omb*-2. A, frontal view showing expression in the optic lobes (arrows). B, view of the opisthosoma. C,D, expression of *At-omb*. C, lateral view, D ventral view; arrows

denotes expression in the head lobes. E,F, expression of *Ta-omb-1*. E, frontal view showing expression in the optic lobes (arrows). F, view of the opisthosoma. G,H, expression of *Ta-omb-2*. G, frontal view showing expression in the optic lobes (arrows). H, view of the opisthosoma. Note that the irregular black dots in E-H are unspecific precipitation of NBT/BCIP caused by the in situ procedure in *Tegenaria*. All panels: anterior to the left. A, B, and H are mid inversion stages. C, E, F, and G are early inversion stages. D is a late inversion stage. Abbreviations: ch, chelicera; pp, pedipalp; 11-14, walking legs 1 to 4; o1-o6, opisthosomal segment 2 to 6.

Fig. 4. Expression of *omb*-type genes in the developing appendages. Genes are arranged in rows and appendage types are arranged in columns and these are given to the right and on the top, respectively. Species are arranged in rows and the species names are given to the left. For each appendage type, the first column shows young appendages at early stages of germ band elongation and the second column shows old appendages at late stages of inversion. The arrowhead in some panels denotes discontinuous expression of *omb* homologs along the dorsal side. "m" denotes mesodermal expression in some appendage types. For details see text.

Fig. 5. Embryonic expression of *H15*-type T-box genes. All embryos are late inversion stage, except for A, C, D and G, which are mid inversion stage. A,B, expression of *At-H15-1*, lateral view, arrow denotes expression in the heart. C,D, expression of *At-H15-2*. A, ventral view of the prosoma. D, lateral view, arrow denotes expression in the heart. E,F, expression of *Ta-H15-1*. E, dorsal view, arrows point to expression in the heart. F, ventral view, arrows denote expression in the neuroectoderm. G,H, expression of *Ta-H15-2*. G, ventral view of the opisthosoma,

arrow denotes expression in the heart. H, ventral view, arrowheads denote expression in the neuroectoderm. Note that the third right walking leg has been removed. I, J, expression of *Ta-H15-3*. I, dorsal view. J, ventral view, arrows denote expression in the neuroectoderm. All panels: anterior to the left. Abbreviations: see Fig. 2.

Fig. 6. Expression of *H15*-type genes in the developing appendages. Genes are arranged in rows and appendage types are arranged in columns and these are given to the right and on the top, respectively. Species are arranged in rows and the species names are given to the left. For each appendage type, the first column shows young appendages at early stages of germ band elongation and the second column shows old appendages at late stages of inversion. The arrow and/or asterisk in panels D,F,L,N,Z, and a denote smaller expression domains in addition to the main ventral expression. The arrow in G denotes very weak ventral expression in the chelicera. For details see text.

Fig. 7. Alternative duplication histories of *H15*-type T-box genes in the Arthropoda. The green boxes 1-4 denote the four independent duplication events for *H15* that have to be postulated if the ancestral state for the arthropods is a single *H15*-type gene (scenario 1; this is the hypothesis compatible with our phylogenetic analysis of the gene sequences (see Fig.1)). (Note that duplication event 4 affected only one of the two *H15* genes produced in event 3). If the sequence analysis, however, is subject to artefacts due to gene conversion, then the ancestral state in the arthropods could be already two *H15*-type genes (scenario 2; light blue box at the bottom of the tree denotes duplication of *H15* before the split of extant arthropod lineages). A second duplication then occurred in the spider lineage before the split between the three

superfamilies of *Amaurobioidea*, *Araneoidea* and *Lycosoidea*. The top of the diagram depicts the simplified expression patterns of the genes in the different species. The images are based on Abu-Shaar and Mann (1998) for *H15* and *omb* from *Drosophila*; Prpic et al. (2003) for *mid* in *Drosophila*; and Prpic et al. (2005) for *H15* and *omb* genes from *Glomeris*. The question marks in the *Cupiennius* and *Achaearanea* drawings indicate that these genes have not yet been isolated from these species, but are being postulated to exist, because of the corresponding data from *Tegenaria*. See text for further discussion. Note that the arrangement of the drawings is not intended to indicate orthology of genes, except for the *H15* genes in the spiders that have been arranged in the three groups discussed in the text. The orthology of the spider *omb* genes is presently unclear; there might be a second *omb* gene in *Achaearanea* that is more similar to *Ta-omb-1* and *Cs-omb*.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7