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Comparative analysis of zygotic developmental genes in *Rhodnius prolixus* genome shows conserved features on the tracheal developmental pathway

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ABSTRACT

Most of the in-depth studies on insect developmental genetic have been carried out in the fruit fly *Drosophila melanogaster*, an holometabolous insect, so much more still remains to be studied in hemimetabolous insects. Having *Rhodnius prolixus* sequenced genome available, we search for orthologue genes of zygotic signaling pathways, segmentation, and tracheogenesis in the *R. prolixus* genome and in three species of *Triatoma* genus transcriptomes, concluding that there is a high level of gene conservation. We also study the function of two genes required for tracheal system development in *D. melanogaster* - *R. prolixus* orthologues: *trachealess* (*Rp-trh*) and *empty spiracles* (*Rp-ems*). From that we see that *Rp-trh* is required for early tracheal development since *Rp-trh* RNAi shows that the primary tracheal branches fail to form. On the other hand, *Rp-ems* is implied in the proper formation of the posterior tracheal branches, in a similar way to *D. melanogaster*. These results represent the initial characterization of the genes involved in the tracheal development of an hemimetabolous insect building a bridge between the current genomic era and V. Wigglesworth's classical studies on insects' respiratory system physiology.

1. Introduction

Although insects' embryology has been studied for a long time, comparative developmental genetics is a relatively new field (reviewed in Sander and Schmitt-Ott, 2004). Most of our knowledge on developmental genetics derives from studies in *Drosophila melanogaster* for which most of the genes and genetic pathways involved in embryonic development have already been described in detail. In addition, the advances in *D. melanogaster* genomics, including the sequencing of several species of the genus (Clark et al., 2007), provided a superb body of data. However, *D. melanogaster* is a derived holometabolous insect, which implies that the developmental processes might represent an evolved exception rather than the rule. In contrast, we have much less information about other insects' models. The genomes of new model insects such as *Tribolium castaneum* (Richards et al., 2008) or *Nasonia vitripennis* (Werren et al., 2010), both holometabolous, have been finished recently. There is also availability of some other genomes: *Apis mellifera* (Weinstock et al., 2006), *Bombyx mori* (The International Silkworm Genome Consortium, 2008), *Acyrtosiphum pisum* (The International Aphid Genomics Consortium, 2010) and many more will follow as the i5K project develops (http://www.arthropodgenomes.org/wiki/i5K).

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Insects represent the most biodiversed animal class in Earth despite the fact that the genetic pathways involved in developmental processes such as segmentation, morphogenesis, and organogenesis are highly conserved. Among the developmental processes, the formation of the tracheal system is of particular interest – it is an exclusive characteristic of Insecta and Myriapoda classes, although they did not evolve from a common terrestrial ancestor. The tracheas are composed of chitin, the material of the arthropod exoskeleton, forming a net of tubes that connect directly to the air through the spiracles. This system, although inefficient, has evolved to bring oxygen to the cells in an organism with an open circulatory system. Vincent Wigglesworth's seminal work in *Rhodnius prolixus* was the first to provide a detailed description of the physiology and morphology of the tracheal system of the insects (Wigglesworth, 1954). In this work, he also described the effect of hypoxia in the biological function and adult development of the tracheas (Locke, 1958; Wigglesworth, 1954). From that moment, there was a large time gap until the advent of *D. melanogaster* as model for developmental genetics, which led to the identification of the genes involved in the embryonic development of the tracheal system (reviewed in Manning and Krasnow, 1993; Metzger and Krasnow, 1999; Ghabrial et al., 2003; Samakovlis et al., 1996). The genetic blueprint that build the tracheal system during *D. melanogaster* embryogenesis also act in the larva to respond to hypoxia, in a process similar to angiogenesis in mammals (Carmeliet et al., 1998; Centanin et al., 2009; Lavista-Llanos et al., 2002; Pugh and Ratcliffe, 2003). Up today, functional studies on the tracheal system have remained restricted to *D. melanogaster*. The analysis of the genes that regulate the development and function of the tracheal system in other insects is largely over, despite the genome sequencing of some of them.

Here, we discover an extensive number of zygotic genes in the *R. prolixus* genome that are predicted to act in a variety of developmental pathways. Among these, we demonstrate that the genes involved in tracheogenesis in *D. melanogaster* are conserved in the *R. prolixus* genome and that the orthologues of *trachealess* (*Rp-trh*) and *empty spiracles* (*Rp-ems*) are functionally conserved in the development of the tracheal system. Our results bring *R. prolixus*, the classical model of tracheal system physiology, to a new level of analysis and provide the elements for future studies on the respiratory system evolution.

2. Material and methods

2.1. Insect rearing

The insects were obtained from a colony established in our laboratory maintained in a 12 h light/dark schedule at 30 °C and 70% humidity. In these conditions the embryogenesis takes 14 ± 1 days. The insects were regularly fed using an artificial feeder and porcine blood. When necessary, V larval instar were sexed before molting until adulthood and then mated.

2.2. Identification of developmental genes

We used the *R prolixus* genome (Mesquita et al., manuscript submitted) and the transcriptome of other triatomines – *Triatoma dimidiata*, *Triatoma infestans and Triatoma pallidipennis* – (Lavore, Martínez-Barnetche et al., manuscript in preparation) to perform an iterative search to identify developmental genes. We created a gene database related to developmental processes in *D. melanogaster*. The database consists of 1364 protein sequences derived from FlyBase (version 6.01) searching for *developmental genes* and manual curation. This database is available upon request. The search in the triatomine genome and transcriptomes was carried out using local BLASTX (Altschul et al., 1990) and a minimum e-value of 0.0001. BLAST results were classified into the known *D. melanogaster* developmental pathways and process classes.

2.3. Sequence analysis

Once we had identified the genes, we defined a predicted gene structure and open reading frames on the genomic sequence using the software package Lasergene (DNASTAR) and by manual curation. The sequences were aligned with orthologues from other arthropod species using ClustalW and the alignments were employed for phylogenetic analysis by Bayesian inference using the software BEAST (Drummond and Rambaut, 2007). The parameters used for the analysis were: Number of substitution types: 6(GTR), Substitution model: Blosum62, Number of generations: 200.000 and a Sample tree every ten generations. The trees were drawn using Figtree software (http://tree.bio.ed.ac.uk/software/figtree), burning the first five trees.

2.4. cDNA synthesis and PCR validation

Total RNA was isolated from *R prolixus* embryos at different developmental times using TRIZOL (Life Technology). cDNA was synthesized using MINT-2 Kit (EVROGEN) and employed to validate the expression of selected genes by PCR. A set of specific primers spanning the most conserved protein domains were designed:

Trh-Fw1:
CGACTCACTATAGGGTTCTGGAGTTAAGGAAAGAGAAATC
Trh-Rv1: CGACTCACTATAGGGCTGTGACAGTCCTAGATAGATG
Trh-Fw2: CGACTCACTATAGGGTCAACCCTGACGAAAAGAGG
Trh-Rv2: CGACTCACTATAGGGGCTGGCAGCAATCCATTATT
Trh-Fw3: CGACTCACTATAGGGTGGCTAAAATGTTGCCTTTACC
Trh-Rv3: CGACTCACTATAGGGTTGCGGCTATGTGAGAACTG
Ems-Fw1: CGACTCACTATAGGGAGGCGGTGGTTGTTGACTTA
Ems-Rv1: CGACTCACTATAGGGCGCACCGAATCCAGTAATGG

T7 promoter sequence was included in the 5′ end of the primers to further use the amplimers for *in vitro* transcription. The amplimers were cloned into the vector pGEM-T Easy (Promega) and several independent clones were sequenced to confirm identity.

2.5. Parental RNAi

dsRNA was produced by simultaneous T7 RNA polymerase transcription on PCR products containing T7 promoter sequences at both ends of either *Rp-Trh* or *Rp-ems*. Furthermore, dsRNA was quantified and injected in virgin females (2–4 μg per insect) as described in Lavore et al., 2012. The females were fed to induce oogenesis after injection. After mating, the eggs collected from individual females were dissected and the embryos were immediately fixed. A fraction of eggs was allowed to develop to account for lethality and/or cuticle preparation. Cuticles were prepared as described in Lavore et al., 2012. Images were acquired using Panasonic DM10 digital camera incorporated to a microscope. Every experiment included a negative control by the injection of dsRNA corresponding to the beta-lactamase of *E. coli* gene as described in Lavore et al. (2012). The statistical significance of the RNAI results was calculated using Student's *t*-test for three independent experiments (Sokal and Rohlf, 2012).

3. Results

3.1. Gene identification

Using a comprehensive protein database of 1364 sequences related to developmental processes in *D. melanogaster*, we identified 1203 hits that represent putative gene orthologues in the *R. prolixus* genome. In addition, we searched through the transcriptome of the following related species and got 993 hits for *T. infestans*, 950 for *T. pallidipennis* and 1005 for *T. dimidiata*. The identity was established by Bayesian inference when required and the resulting genes were classified according to the signaling pathways or developmental processes defined for *D. melanogaster* (Tables 1–3).

3.1.1. Signaling pathways

Here, we refer to gene networks that control development, morphogenesis and differentiation by regulating cell survival and death based on the genetic studies performed in *D. melanogaster*.

3.1.2. JAK/STAT pathway

Janus kinase/signal traducer and activator of transcription (JAK/STAT) signaling pathway is key in eukaryotes. It was first identified in vertebrates acting as intermediate in the cytokine response and it was later described in *D. melanogaster*. In the latter, JAK/STAT pathway has pleiotropic effects in segmentation, eye development, hematopoiesis, sex determination, planar cell polarity, and tracheal development (Baeg et al., 2005; Castelli-Gair Hombría and Brown, 2002). We have identified the molecular components of JAK/STAT pathway in the *R. prolixus* genome and in the transcriptomes of *T. infestans*, *T. pallidipennis* and *T. dimidiata*: *Hopscotch* (RPRC012987), *Signal-transducer and activator of transcription protein at 92E* (RPRC006502), *Suppressor of variegation 3–9* (RPRC015292), *Suppressor of variegation 205* (RPRC012579), *Ken and barbie* (RPRC004246), *Protein tyrosine phosphatase 61F* (RPRC007783), *Signal transducing adaptor molecule* (RPRC015396), and *Sex lethal* (*Sxl*). All genes were identified in *R. prolixus* but *Sxl*, which was only identified in the *T. infestans* transcriptome (Table 1).

3.1.3. Wnt pathway

In *D melanogaster Wnt* pathway regulates a diversity of responses during the whole life cycle: cell fate, planar polarity, proliferation and apoptosis, and tissue homeostasis. This pathway is highly conserved in vertebrates; it is also related to developmental processes as well as cell and tissue homeostasis (Du and Geller, 2010), associating this pathway to cancer (Logan and Nusse, 2004). The ligands and receptors of this pathway were identified and characterized in different insect species (Bolognesi et al., 2008; Kusserow et al., 2005; Shigenobu et al., 2010). Here, we identify most of the components of Wnt signaling pathway such as *Wingless* (RPRC005904), *Wnt oncogene analog 5* (RPRC002679), *Wnt oncogene analog 2* (RPRC011178), *Frizzled* (RPRC000842), *Frizzled 2* (RPRC004621), *Frizzled 3* (RPRC010057), *Dishevelled* (RPRC012022), *Armadillo* (RPRC003585), *Ovo/shavenbaby* (RPRC002781), *Nemo* (RPRC000184), *Notum* (RPRC003543), *Axin* (RPRC013448) and *Shaggy* (RPRC010782), and downstream target genes such as *Ultrabithorax* (RPRC000565, described in detail by Esponda-Behrens et al. in an accompanying manuscript) and *Decapentaplegic* (RPRC000401, described in detail by Pagola et al. in an accompanying manuscript). Among these genes orthologues, *Shaggy* (*Sgg*), *Ovo/shavenbaby* (*Svb*) and *Frizzled 2* (*Fz2*) were identified in the four triatomine species (Table 1). Wnt proteins are such similar members of a conserved family that they required thorough Bayesian inference to determine their identity. In *R. prolixus* we could distinguish *Wnt oncogene analog 2* (*Wnt2*), *Wnt oncogene analog 5* (*Wnt5*) and *wingless* (*wg*) (Supplementary Fig. S1).

3.1.4. Notch pathway

Notch signaling pathway is highly conserved in invertebrates and vertebrates, in which it acts during cell fate determination through embryogenesis and stem cells maintenance (Logan and Nusse, 2004). In intermediate and short germ band insects, such as *R. prolixus*, this pathway is also related to the sequential segments addition in the embryo (Stollewerk et al., 2003; Pueyo et al., 2008). We identified most of the components of the N pathway in the genome of *R. prolixus* and the transcriptome of the *Triatoma* species (Table 1): *Delta* (RPRC012997), *Serrate* (RPRC008507), *Notch* (RPRC008058), Suppressor of *hairless* (RPRC014254), *groucho* (RPRC015149), *C-terminal Binding Protein* (RPRC005545), *kuzbanian* (RPRC007479), *neuralized* (RPRC007349), *deltex* (RPRC006382), *hairy* (RPRC000496), *Strawberry Notch* (RPRC007715) and *Presenilin* (RPRC005509). *Groucho* (*gro*) and *C-terminal Binding Protein* (CtBP). *Notch* (*N*) was only identified in *R. prolixus*.

3.1.5. Hedgehog pathway

Hedghog (Hh) pathway is conserved in all bilaterians and might have evolved from choanoflagellates (Snell et al., 2006; King et al., 2008). In *D. melanogaster*, *Hh* was discovered as one of the segment polarity gene (Nüsslein-Volhard and Wieschaus, 1980; Lee et al., 1992); it also sets the anterior-posterior patterning in each segment as well as the imaginal discs and the adult morphogenesis (Robbins et al., 2012). The presence of *Hh* in Annelids suggests that segmentation and segment polarity have the same origin as in arthropods (Dray et al., 2010). We have identified most of the components of the Hh pathway in the *R. prolixus* genome and in the transcriptome of the *Triatoma* species analyzed (Table 1): *Hedgehog* (RPRC012384), *Smoothened* (RPRC004966), *Patched* (RPRC000561), *Interference hedgehog* (RPRC013243), *Fused* (RPRC003744), *Suppressor of Fused* (RPRC009912), *Costa* (RPRC004375), and *Cubitus interruptus* (RPRC007162). *Hedgehog* (*Hh*) and *Cubitus interruptus* (*Ci*) were identified in all triatomine species, while *Patched* (*ptc*) – the Hh receptor-was only identified in *R. prolixus*.

3.2. Developmental processes

Here, we describe among the developmental zygotic processes the ones that lead to segmentation and tracheogenesis. The analysis of maternal pathways is described elsewhere (Mesquita et al., manuscript submitted; Pagola et al., accompanying manuscript).

3.2.1. Segmentation

The segmentation process in insects occurs in two different modes (Krause, 1939): 1- long germ band embryogenesis, in which all the body segments are simultaneously specified throughout the egg, as in *D. melanogaster*; 2- short germ band embryogenesis, in which a few segments are specified early in development while the rest are sequentially added (Davis and Patel, 2002; Liu and Kaufman, 2005). During segmentation, a hierarchical gene network establishes the A-P axis (reviewed in Rivera-Pomar and Jackle, 1996). We have identified the majority of the segmentation genes orthologues in the *R. prolixus* genome and in the *T. infestans*, *T. pallidipennis* and *T. dimidiata* transcriptomes (Table 2): the gap genes *Kruppel* (RPRC000102; Lavore et al., 2014), *Giant* (RPRC001027, Lavore et al., 2012), *Hunchback* (RPRC000230), *knirps/knirps-related* (RPRC003216), *buttonhead* (*btd*), *empty spiracles* (RPRC014392 and RPRC010410), *knot* (RPRC004534), *Cap-n-collar* (RPRC011620), *orthodenticle* (RPRC000583) and *Tenascin major* (RPRC005422); the terminal gap genes *Huckebein* (RPRC014216) and *Tailless* (RPRC007025); the pair rule genes *Hairy* (RPRC000496), *runt* (*run*), *Odd skipped* (RPRC011812), *Odd paired* (RPRC013047) and *Sloppy paired 1* (RPRC000987); the segment polarity genes *Armadillo* (RPRC003585), *Wingless* (RPRC005904),

Table 1
Major component of signaling pathways in the *Rhodnius prolixus* genome and in the transcriptomes of *Triatoma infestans*, *Triatoma dimidiata* and *Triatoma pallidipennis*

Signaling pathways	*R. prolixus*				*T. infestans*			*T. dimidiata*			*T. pallidippenis*		
	% ID	Lenght mach	E-value	Gene ID	% ID	Lenght mach	E-value	% ID	Lenght mach	E-value	% ID	Lenght mach	E-value
Notch pathways													
Notch	66.74	1891	0	RPRC008058	–	–	–	–	–	–	–	–	–
Delta	52.12	401	4.00E-114	RPRC012997	35.33	184	2.00E-13	–	–	–	33.76	234	7.00E-20
Serrate	38.50	1117	0	RPRC008507	54.72	53	3.00E-09	–	–	–	–	–	–
Suppressor of Hairless	82.88	438	0	RPRC014254	–	–	–	79.29	140	3.00E-57	–	–	–
Groucho	88.71	363	0	RPRC015149	80.68	88	1.00E-38	28.09	178	2.00E-12	94.87	117	6.00E-65
C-terminal Binding Protein	90.88	318	3.00E-173	RPRC005545	94.38	178	1.00E-91	32.64	239	3.00E-24	82.86	175	1.00E-77
Kuzbanian	73.32	536	0	RPRC007479	67.52	157	3.00E-56	64.18	201	1.00E-73	–	–	–
Neuralized	43.25	689	2.00E-154	RPRC007349	41.05	95	2.00E-13	39.66	58	2.00E-06	–	–	–
Deltex	43.99	316	6.00E-68	RPRC006382	44.29	70	2.00E-08	–	–	–	–	–	–
Hairy	68.46	130	7.00E-41	RPRC000496	77.27	66	6.00E-18	–	–	–	39.47	114	2.00E-15
Strawberry notch	81.78	450	0	RPRC007715	64.75	139	3.00E-48	54.55	55	6.00E-11	–	–	–
Presenilin	57.80	455	7.00E-133	RPRC005509	55.56	63	5.00E-09	75.56	90	7.00E-26	–	–	–
Hh pathways													
Hedgehog	49.21	315	2.00E-73	RPRC012384	63.75	80	7.00E-23	64.56	79	7.00E-24	60.61	66	2.00E-15
Interference hedgehog	37.40	639	3.00E-91	RPRC013243	34.67	75	8.00E-08	–	–	–	–	–	–
Smoothened	52.08	626	2.00E-177	RPRC004966	–	–	–	43.40	106	4.00E-17	–	–	–
Fused	52.43	288	3.00E-93	RPRC003744	–	–	–	–	–	–	32.64	242	1.00E-30
Patched	47.44	1174	0	RPRC000561	–	–	–	–	–	–	–	–	–
Suppressor of fused	41.23	422	5.00E-85	RPRC009912	–	–	–	–	–	–	45.00	80	3.00E-11
Costa	29.50	400	5.00E-37	RPRC004375	–	–	–	41.61	149	1.00E-19	26.44	208	3.00E-07
Cubitus interruptus	62.07	174	3.00E-59	RPRC007162	48.47	196	1.00E-45	50.45	111	1.00E-27	69.39	98	4.00E-34
Wnt pathways													
Wingless	63.69	336	3.00E-119	RPRC005904	39.01	182	2.00E-30	40.43	94	1.00E-14	–	–	–
Wnt oncogene analog 5	63.25	166	6.00E-66	RPRC002679	–	–	–	–	–	–	–	–	–
Wnt oncogene analog 2	46.79	327	1.00E-75	RPRC011178	–	–	–	50.00	58	1.00E-10	–	–	–
Frizzled 2	63.17	562	0	RPRC004621	70.95	241	5.00E-88	73.68	38	9.00E-08	82.02	89	3.00E-42
Frizzled	57.85	382	6.00E-125	RPRC000842	–	–	–	–	–	–	55.42	83	2.00E-19
Frizzled 3	30.41	467	3.00E-42	RPRC010057	–	–	–	–	–	–	–	–	–
Dishevelled	69.60	250	1.00E-99	RPRC012022	–	–	–	–	–	–	–	–	–
Armadillo	86.48	725	0	RPRC003585	85.64	181	2.00E-83	–	–	–	92.11	38	2.00E-12
Ovo/shavenbaby	85.90	156	5.00E-82	RPRC002781	40.00	75	2.00E-10	35.29	85	1.00E-10	38.00	100	7.00E-14
Nemo	88.25	315	9.00E-169	RPRC000184	–	–	–	–	–	–	82.88	111	3.00E-45
Notum	58.81	318	3.00E-111	RPRC003543	–	–	–	50.44	113	2.00E-24	–	–	–
Axin	36.41	725	1.00E-95	RPRC013448	–	–	–	–	–	–	62.16	74	5.00E-22
Pangolin	75.44	114	1.00E-37	RPRC000333	–	–	–	–	–	–	34.62	104	8.00E-08
Shaggy	85.55	353	0	RPRC010782	86.40	272	1.00E-139	81.82	264	7.00E-128	86.43	339	6.00E-177
JAK/STAT pathways													
Hopscotch	46.77	263	4.00E-60	RPRC012987	–	–	–	–	–	–	–	–	–
STATprotein at 92E	37.22	771	7.00E-116	RPRC006502	49.63	270	3.00E-66	–	–	–	–	–	–
Suppressor of variegation 3–9	92.54	67	8.00E-29	RPRC015292	42.70	185	1.00E-28	92.54	67	2.00E-28	–	–	–
Suppressor of variegation 205	40.68	177	2.00E-25	RPRC012579	–	–	–	41.24	177	1.00E-23	–	–	–
Ken and barbie	34.42	276	6.00E-34	RPRC004246	–	–	–	67.11	76	1.00E-20	65.79	76	1.00E-19
Protein tyrosine phosphatase 61F	60.66	244	1.00E-84	RPRC007783	–	–	–	61.69	248	5.00E-86	55.81	86	2.00E-22
Sex lethal	–	–	–	–	32.14	140	9.00E-12	45.68	81	3.00E-16	37.30	185	3.00E-32
Signal transducing adaptor molecule	51.10	409	7.00E-105	RPRC015396	–	–	–	52.36	275	1.00E-64	49.66	149	1.00E-34

Table 2
Genes involved on the segmentation process that were identified in the *R. prolixus* genome and in the transcriptomes of *T. infestans*, *T. dimidiata* and *T. pallidipennis*

Segmentation	*R. prolixus*				*T. infestans*			*T. dimidiata*			*T. pallidippenis*		
	% ID	Lenght mach	E-value	Gene ID	% ID	Lenght mach	E-value	% ID	Lenght mach	E-value	% ID	Lenght mach	E-value
Gap genes													
Kruppel	79.03	124	2.00E-57	RPRC000102	47.93	121	3.00E-29	–	–	–	–	–	–
Knirps	85.11	94	6.00E-47	RPRC003216	–	–	–	–	–	–	43.66	71	8.00E-14
	*	*	*	no ID									
Giant	72.34	47	5.00E-13	RPRC001027	48.53	68	1.00E-10	60.98	41	3.00E-09	–	–	–
Tailless	42.55	423	1.00E-84	RPRC007025	–	–	–	–	–	–	–	–	–
Hunchback	66.25	160	5.00E-67	RPRC000230	–	–	–	33.63	113	3.00E-13	34.44	90	1.00E-12
Huckebein	69.03	113	3.00E-45	RPRC014216	–	–	–	–	–	–	–	–	–
Empty spiracles	86.54	52	6.00E-20	RPRC010410	84.09	44	5.00E-15	–	–	–	–	–	–
Knot	58.03	193	3.00E-52	RPRC004534	58.93	56	5.00E-12	–	–	–	–	–	–
Orthodenticle	84.72	72	3.00E-31	RPRC000583	–	–	–	–	–	–	–	–	–
Buttonhead	*	*	*	no ID	–	–	–	63.24	68	4.00E-20	–	–	–
Cap-n-collar	57.36	197	5.00E-53	RPRC011620	–	–	–	66.67	99	3.00E-31	43.01	186	3.00E-20
Tenascin major	69.86	2349	0	RPRC005422	66.25	160	5.00E-59	74.88	203	1.00E-90	65.60	218	2.00E-69
Pair rule genes													
Hairy	68.46	130	7.00E-41	RPRC000496	–	–	–	–	–	–	39.47	114	2.00E-15
Odd skipped	62.92	178	7.00E-63	RPRC011812	–	–	–	–	–	–	–	–	–
Odd paired	68.46	130	8.00E-45	RPRC013047	–	–	–	–	–	–	55.67	97	7.00E-26
Runt	*	*	*	RPRC000116	–	–	–	–	–	–	–	–	–
Sloppy paired 1	71.82	110	9.00E-48	RPRC000987	–	–	–	–	–	–	–	–	–
Segment polarity genes													
Armadillo	86.48	725	0	RPRC003585	85.64	181	2.00E-83	–	–	–	–	–	–
Decapentaplegic	45.73	363	3.00E-80	RPRC000401	50.97	206	2.00E-55	–	–	–	35.24	105	3.00E-11
Wingless	63.69	336	3.00E-119	RPRC005904	–	–	–	40.43	94	1.00E-14	–	–	–
Cubitus interruptus	62.07	174	3.00E-59	RPRC007162	48.47	196	1.00E-45	50.45	111	1.00E-27	69.39	98	4.00E-34
Hedgehog	49.21	315	2.00E-73	RPRC012384	63.75	80	7.00E-23	64.56	79	7.00E-24	–	–	–
Fused	52.43	288	3.00E-93	RPRC003744	–	–	–	–	–	–	32.64	242	1.00E-30
Smoothened	52.08	626	2.00E-177	RPRC004966	–	–	–	43.40	106	4.00E-17	–	–	–
Engrailed	76.79	112	7.00E-44	RPRC003110	–	–	–	–	–	–	–	–	–
Rasp	32.98	282	9.00E-42	RPRC014615	–	–	–	33.33	156	7.00E-17	–	–	–
Pangolin	75.44	114	1.00E-37	RPRC000333	–	–	–	–	–	–	34.62	104	8.00E-08
Gooseberry	84.17	120	2.00E-58	RPRC008887	–	–	–	–	–	–	–	–	–
Tout-velu	60.57	317	2.00E-102	RPRC000548	78.31	166	1.00E-64	–	–	–	37.82	238	3.00E-31
Terminal genes													
Tailless	42.55	423	1.00E-84	RPRC007025	48.28	87	8.00E-20	–	–	–	–	–	–
Huckebein	69.03	113	3.00E-45	RPRC014216	–	–	–	–	–	–	–	–	–
Torso-like	44.14	333	4.00E-81	RPRC006513	–	–	–	–	–	–	–	–	–
Pipsqueak	81.96	194	9.00E-97	RPRC012968	73.45	113	1.00E-45	–	–	–	–	–	–

(*) genes not identified by BLAST using protein databases but annotated using WGST and pending of approval and ID by Vector Base.

Cubitus interruptus (RPRC007162), *Hedgehog* (RPRC012384), *Fused* (RPRC003744), *Smoothened* (RPRC004966), *Engrailed* (RPRC003110), *Rasp* (RPRC014615), *Pangolin* (RPRC000333), *Gooseberry* (RPRC008887) and *Tout-velu* (RPRC000548). Most of these genes form part of the signaling pathways described in Sections 3.1.2–3.1.5 above. We have additionally identified genes involved in the proper formation of the most anterior and posterior structures of *D. melanogaster* embryo: *Pipsqueak* (RPRC012968) and *Torso-like* (RPRC006513). It is noteworthy that two different *ab initio* gene predictions in the *R. prolixus* genome show high score of similarity to the transcription factor *empty spiracles*: RPRC010410, which was identified in our iterative BLAST search, and RPRC008906 identified by manual curation and genome annotation of the *R. prolixus* genome. This will be discussed in detail in Section 3.4. Hox genes cluster structure and their function are described elsewhere (Esponda-Behrens et al., accompanying manuscript).

3.2.2. Tracheogenesis

Wigglesworth described in detail the physiology and morphology of *R. prolixus* tracheal system (Wigglesworth, 1954). Several decades after that, a detailed study of *D. melanogaster* anatomy was carried out and the genetics and molecular events related to tracheal morphogenesis were clarified, making this insect the only and most important model for branching morphogenesis (Metzger and Krasnow, 1999). So as to take advantage from *R. prolixus* as an amenable model for tracheal development, in which grafting and ablation experiments were described by Wigglesworth, and having now the genomic information available, we aimed at the discovery of genes involved in tracheal development. We identified in the *R. prolixus* genome and in the transcriptomes of *T. infestans*, *T. pallidipennis* and *T. dimidiata* most of the orthologue genes that were linked to the development and physiology of the tracheal system in *D. melanogaster*: *Trachealess* (RPRC008906 and RPRC000129), *Branchless* (RPRC014942), *Spalt* (RPRC000397), *Ventral veins lacking* (RPRC014454), *Thickveins* (RPRC011471), *HIF prolyl hydroxylase* (*Hph*), *Tango* (RPRC004351), *Pointed* (RPRC011695), *Stumps* (RPRC004180), *Sprouty* (RPRC000892), *Rhomboid* (RPRC008474), *Tramtrack* (RPRC013755), *Dumpy* (RPRC001058), *Multiple edematous wings* (RPRC015251), *Blistered* (RPRC001752), *Escargot* (RPRC005707), *Ribbon* (RPRC007714), *von Hippel-Lindau* (*VHL*), *similar* (*sima*, a HIF-hydroxylase), and *Grainy head* (RPRC012881). All the key regulatory genes but *breathless* were identified in *R. prolixus* genome. However, as we could identify a bona fide orthologue in the transcriptome of *T. pallidipennis*, we consider that this is a consequence of gaps in the current assembled version of the genome rather than a gene loss (Table 3).

It is remarkable that, different to the gene pathways described in the previous sections, the pathway involved in the development of the tracheal system had never been studied in any insect species other than *D. melanogaster*. Therefore, and considering that

Table 3
Genes involved during the tracheogenesis that were identified in the *R. prolixus* genome and in the transcriptomes of *T. infestans*, *T. dimidiata* and *T. pallidipennis*

Tracheas development														
	R. prolixus				T. infestans			T. dimidiata			T. pallidippenis			
	% ID	Lenght mach	E-value	Gene ID	% ID	Lenght mach	E-value	% ID	Lenght mach	E-value	% ID	Lenght mach	E-value	
Trachealess	58.97	351	1.00E-111	RPRC008906	–	–	–	–	–	–	–	–	–	
	*	*	*	RPRC000129										
Branchless	51.35	74	2.00E-16	RPRC014942	–	–	–	55.56	108	2.00E-32	–	–	–	
Breathless	–	–	–	–	–	–	–	–	–	–	33.44	308	1.00E-40	
Spalt	73.58	159	1.00E-67	RPRC000397	–	–	–	38.38	99	4.00E-13	36.89	103	1.00E-13	
Ventral veins lacking	75.46	273	2.00E-95	RPRC014454	–	–	–	39.81	103	2.00E-14	–	–	–	
Thickveins	60.32	499	6.00E-168	RPRC011471	–	–	–	–	–	–	83.43	175	1.00E-78	
HIF prolyl hydroxylase	*	*	*	no ID	68.42	114	1.00E-41	65.08	126	4.00E-45	–	–	–	
Tango	62.68	560	0	RPRC004351	–	–	–	–	–	–	–	–	–	
Pointed	64.14	198	6.00E-59	RPRC011695	–	–	–	–	–	–	66.85	181	2.00E-57	
Stumps	38.52	418	3.00E-79	RPRC004180	–	–	–	–	–	–	–	–	–	
Similar	*	*	*	no ID	–	–	–	–	–	–	–	–	–	
Von hippel lindau	*	*	*	RPRC009933	–	–	–	–	–	–	–	–	–	
Sprouty	41.80	122	2.00E-23	RPRC000892	–	–	–	53.57	56	4.00E-12	–	–	–	
Rhomboid	58.52	229	2.00E-69	RPRC008474	56.76	74	3.00E-15	56.25	144	3.00E-33	–	–	–	
Tramtrack	59.17	240	5.00E-68	RPRC013755	–	–	–	54.46	112	6.00E-36	56.14	114	2.00E-33	
Dumpy	47.79	3861	0	RPRC001058	78.43	153	2.00E-61	–	–	–	–	–	–	
Multiple edematous wings	40.37	1038	0	RPRC015251	–	–	–	–	–	–	–	–	–	
Blistered	65.22	92	5.00E-23	RPRC001752	76.92	91	5.00E-31	–	–	–	–	–	–	
Escargot	84.06	138	1.00E-66	RPRC005707	84.09	132	4.00E-62	73.91	161	1.00E-65	–	–	–	
Ribbon	47.29	203	9.00E-32	RPRC007714	–	–	–	–	–	–	–	–	–	
Grainy head	61.96	531	0	RPRC012881	–	–	–	60.69	145	9.00E-46	–	–	–	

(*)genes not identified by BLAST using protein databases but annotated using WGST and pending of approval and ID by Vector Base.

R. prolixus tracheal system is an excellent model to study the respiratory system morphology and physiology, we investigated the function of both *Rp-thl* and *Rp-ems*.

3.3. *Rp-trh is required as branching factor*

Two genes predictions with high similarity to *D. melanogaster trh* were identified in *R. prolixus* genome: RPRC008906 (Supercontig GL561638), found by BLAST search, and RPRC000129 (Supercontig GL563088), by manual curation and annotation. The alignment of the predicted coding protein sequences to other arthropod orthologues showed that RPRC000129 encodes the classical HLH and PAS domain of *Trh*, while RPRC008906 encodes a putative protein that contains only *Rp-trh* PAS domain (Fig. 1A). Phylogenetic analysis using the predicted sequences for *Rp-trh* demonstrated that they cluster with high posterior probability with the other orthologues (Fig. 1B). Paradoxically, the *ab initio* annotation of RPRC000129 predicts a non translated coding sequence. However, the scaffold GL563088, which contains RPRC000129, has a large region with low quality sequence between the contigs 17,967.42 and 17,967.44. In order to determine the gene structure, we aligned contigs 17,967.42, 17,967.44, 17,967.45 and 17,967.46 (corresponding to RPRC000129) with contig 16,516.1 (corresponding to RPRC008906). The alignment showed that contigs 17,967.42 (RPRC000129) and 16,516.1 (RPRC008906) are identical (Supplementary Fig. S2). This indicates that RPRC000129 and RPRC008906 are not duplicated genes but an assembly error in this genomic region, which corresponds to the *bona fide Rp-trh* locus. Thus, we designed specific primers to amplify the coding sequences corresponding to the HLH, PAS domain, and an additional overlapping region (Supplementary Fig S3A). The regions were successfully amplified by RT-PCR using cDNA derived from embryos, demonstrating the expression of *Rp-trh* during the embryogenesis (Supplementary Fig S3B). These amplicons were used to generate dsRNA for subsequent RNAi experiments to determine the function of *Rp-trh*.

To determine *Rp-trh* lack-of-function phenotype we injected virgin females with 3 ug of dsRNA. In three independent experiments, the injected females laid a total of 200 eggs (Table 4). The eggs were allowed to complete their development and developmental defects in the tracheal system were analyzed. 5% (n = 10/200) of the embryos did not hatch and showed an interfered phenotype, while 95% (n = 190) hatched and showed the wild type phenotype (Fig. 2A–C).

R. prolixus tracheal wild type phenotype was described by Locke (1958). It is characterized by a segmental tracheal network distributed in the first and second thoracic segments and in the abdominal segments A1 to A8.

Rp-thl knockdown embryos showed an unequivocal abnormal phenotype, in which the tracheal systems failed to develop (Fig. 2 D–F). These embryos only developed the spiracles and the spiracular branch, but they did not form primary branches (Fig. 2E). In some segments, the tracheal phenotype was more severe showing only the spiracle -represented by a superficial outline (Fig. 2 D). The observed *Thl*-RNAi phenotype was statistically significantly for three independent experiments, compared to the control ($P = 0.0194$). These results support our hypothesis that *Rp-trh* is required at early stages of tracheogenesis and the conservation of the function of the orthologue.

3.4. *Rp-ems is required for the formation of posterior spiracles*

A bioinformatic analysis lead to the identification of two genes in the *R. prolixus* genome similar to *D. melanogaster ems* predicted: RPRC014392 and RPRC010410. These transcripts contain the 5′ and 3′ regions of *Rp-ems* respectively, suggesting that a single gene is present in the genome but split into two different scaffolds: GL562267 and GL562614. The alignment of the coding sequence with other arthropod orthologues showed three conserved domains: the homeodomain (HD) and two other domains that had not been described before, named here Box A and Box B (Fig. 3A). Phylogenetic analysis using the predicted sequence of *Rp-ems* demonstrated that the sequence clusters with high posterior probability with other orthologue genes sequences (Fig. 3B).

Previous work on *D. melanogaster* showed that *ems* acts as a cephalic gap gene first in segmentation and, later on, in the tracheal system development (Dalton et al., 1989). In order to investigate the

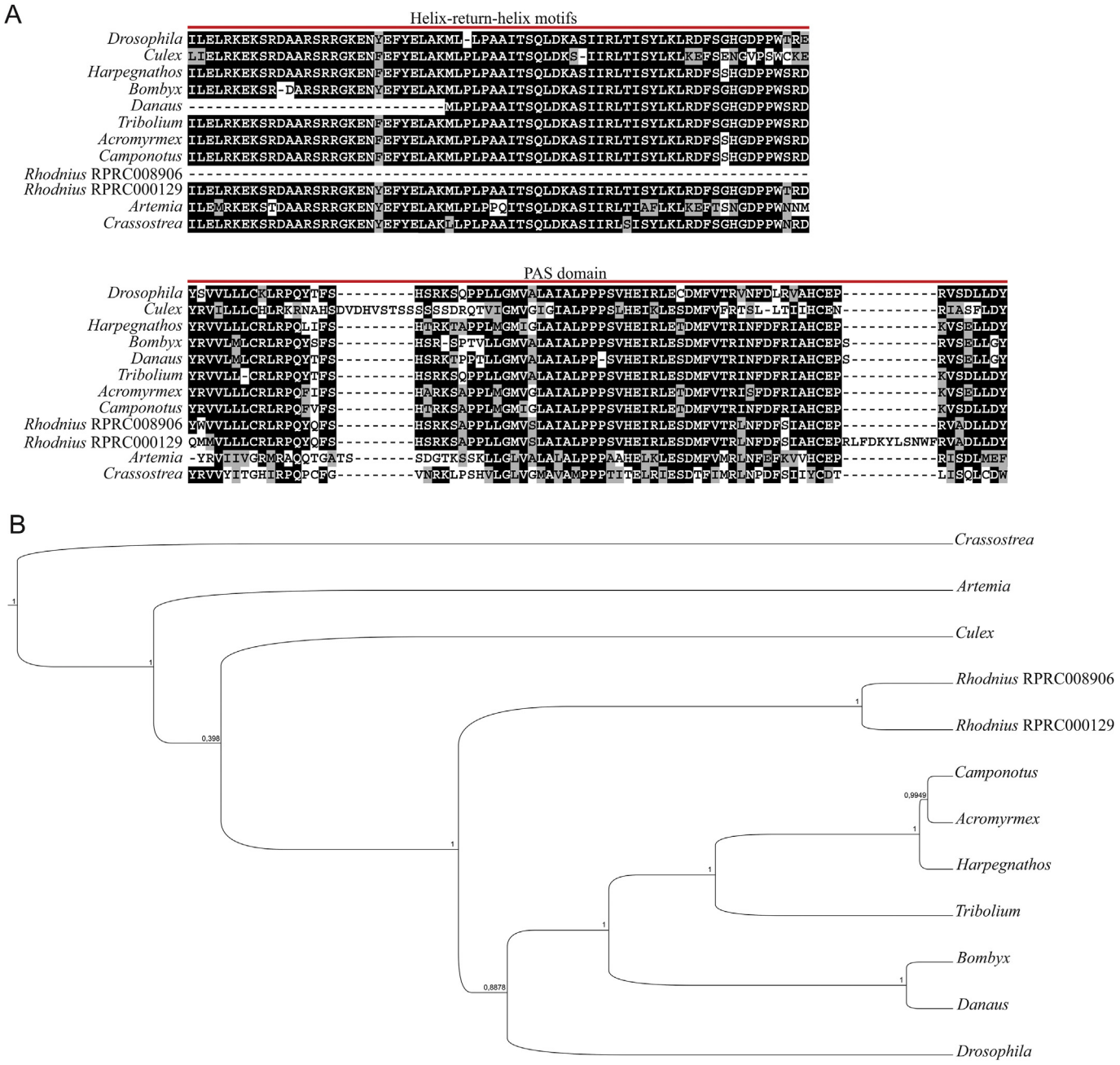

Fig. 1. *Rp-trh* gene analysis. A. Alignment of the protein sequence of *Rp-trh* with other insect orthologues. The black boxes indicate complete amino acid identity and the gray boxes similar amino acid composition. B. Phylogenetic analysis of *Rp-trh*. The tree was generated by Bayesian inference; the node values indicate the posterior probabilities.

Table 4
Parental RNAi experiments: *Rp-Thl*, *Rp-Ems* and control.

	Thl RNAi				*Ems* RNAi				B-iactamase			
	Egg analyzed	Wt phenotype	Interfered phenotype	Lethality (%)	Egg analyzed	Wt phenotype	Interfered phenotype	Lethality (%)	Egg analyzed	Wt phenotype	Interfered phenotype	Lethality (%)
Exp1	40	38	2	5	20	7	13	61.5	24	24	0	2.3
Exp2	106	101	5		30	12	18		27	26	0	
Exp3	54	51	3		15	6	9		36	35	0	
Total	200	190	10*		65	25	40*		87	85	0	

*$P \leq 0.05$.

function of *Rp-ems*, we performed parental RNAi by injecting virgin females with dsRNA corresponding to the nucleotides 11-348 of *Rp-ems* RPRC014392. The fragment used for RNAi was outside the HD to prevent off-target effects. From a total of 65 eggs 38% (n = 25/65) developed wild type hatchlings. 62% (n = 40/65) developed up to pre-larval stage, but did not hatch (Table 4). Those embryos, which

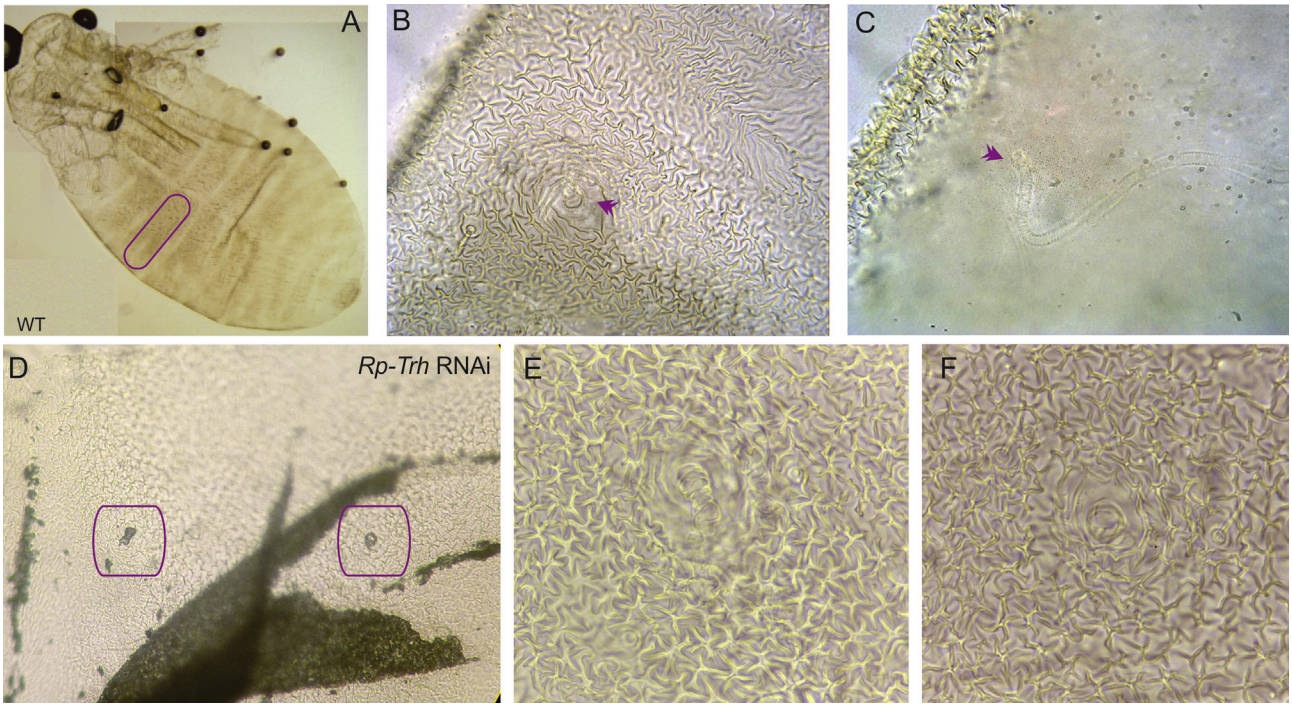

Fig. 2. *Rp-trh* RNAi phenotype. A–C. Wild type larva phenotype. A. Larva in ventral view, in which the rectangle indicates the magnification shown in B and C. B. Inset of the 4th abdominal segment, in which the spiracle and vestibule tracheal are shown. C. Here, the tracheal branch inside the segment is shown. D–F. Interfered embryo for *Rp-trh*. D. Abdominal segments with defects in the tracheal system. E. Magnification of the rectangle on the left, showing the spiracle and the rudimentary spiracular branch. F. Magnification of the rectangle on the right, showing only the spiracle shape, represented by a superficial outline.

had formed the cuticle, were dissected from the egg and analyzed for defects in the segmentation pattern and in the tracheal system. After a thorough examination of *Rp-ems*^{RNAi} embryos, we were not able to determine alterations in the cephalic segmentation pattern – where *ems* has been reported to function –, which was indistinguishable from the wild type embryos. However, we observed that *Rp-ems*^{RNAi} embryos, compared to the wild type and control ones, failed to develop a normal tracheal system in the posterior segments (Fig. 4). In *Rp-ems*^{RNAi} embryos the 7th abdominal segment (A7) developed a normal spiracle but not the trachea associated to it, while A8 segment lacks both the spiracle and the trachea (Fig. 4D–F). In a few cases ($n = 2$), the defects were also evident in A6 segment. The RNAi experiments were statistically significant as judged by t-test ($p = 0.045$). Taken together, the results support a tracheogenic function for *Rp-ems*.

4. Discussion

4.1. Conservation of zygotic developmental genes in triatomines

Comparative genomics is becoming an essential analysis to comprehend biological processes (Tatusov et al., 1997). It is also a mean to study genome evolution by extrapolating information from well studied animal models to non-conventional species (Jiang et al., 2013). Here, we identify in the *R. prolixus* genome the most significant genes that act during *D. melanogaster* development, thus to set the basis for further functional studies. Comparative analysis among different genomes of insects reveals that most of the signaling pathway components are highly conserved (Dearden et al., 2006; Harker et al., 2013; Ewen-Campen et al., 2011; Shigenobu et al., 2010; Behura et al., 2011).

A key component of Hh pathway is the Hh receptor *ptc*. A *ptc* orthologue was described in the annelid *Platynereis dumerilii* (Dray et al., 2010) and characterized both in *D. melanogaster* (Behura et al., 2011; Hoopper and Scott, 1989; Schuske et al., 1994) and *T. castaneum* (Farzana and Brown, 2008). In hemimetabolous, *ptc* was identified in the embryonic trascriptome of the hemipteran *Oncopeltus fasciatus* (Ewen-Campen et al., 2011), and here in *R. prolixus*, but it has not been identified in *A. pisum* (Shigenobu et al., 2010).

The Wnt pathway was widely characterized in insects (Bolognesi et al., 2008; Kusserow et al., 2005; Shigenobu et al., 2010). The hemipterans *A. pisum* and *O. fasciatus* (Shigenobu et al., 2010; Ewen Campen et al., 2011), as well as the species analyzed here, show conservation of the Wnt signaling pathway. However, *Wnt* family is highly conserved within quite similar members, which makes the assignment of orthology difficult and uncertain. In *R. prolixus* and *O. fasciatus Wnt2* is present, but it was not reported in *A. pisum*, which suggests that more redundant sequencing of the genome of *A. pisum* might yield a *Wnt2* orthologue.

The Notch signaling pathway is highly conserved in invertebrates and vertebrates (Logan and Nusse, 2004), with the exception of *Hairless* (*H*), which is a Notch antagonist in *D. melanogaster* (Maier et al., 2008; Pace et al., 2014). *H* was identified in the genome of *A. mellifera* (Maier et al., 2008) and in the *O. fasciatus* embryonic transcriptome (Ewen Campen et al., 2011). *H* could neither be identified in the present work nor in the *A. pisum* genome (Shigenobu et al., 2010) or in the acarus *Tetranychus urticae* (Pace et al., 2014). The lack of evidence for *H* homologues as defined by sequence similarity could be due to high sequence divergence rather than to gene loss.

JAK/STAT signal transduction pathway is highly conserved throughout evolution; moreover, functional homologue components were identified in vertebrates and invertebrates (Zeidler et al., 2000). One exception is *Unpaired* ligand (in their two paralogs, *upd-2* and *upd-3*), only present in *D. melanogaster* and considered to be a fast evolving gene (Harrison et al., 1998;

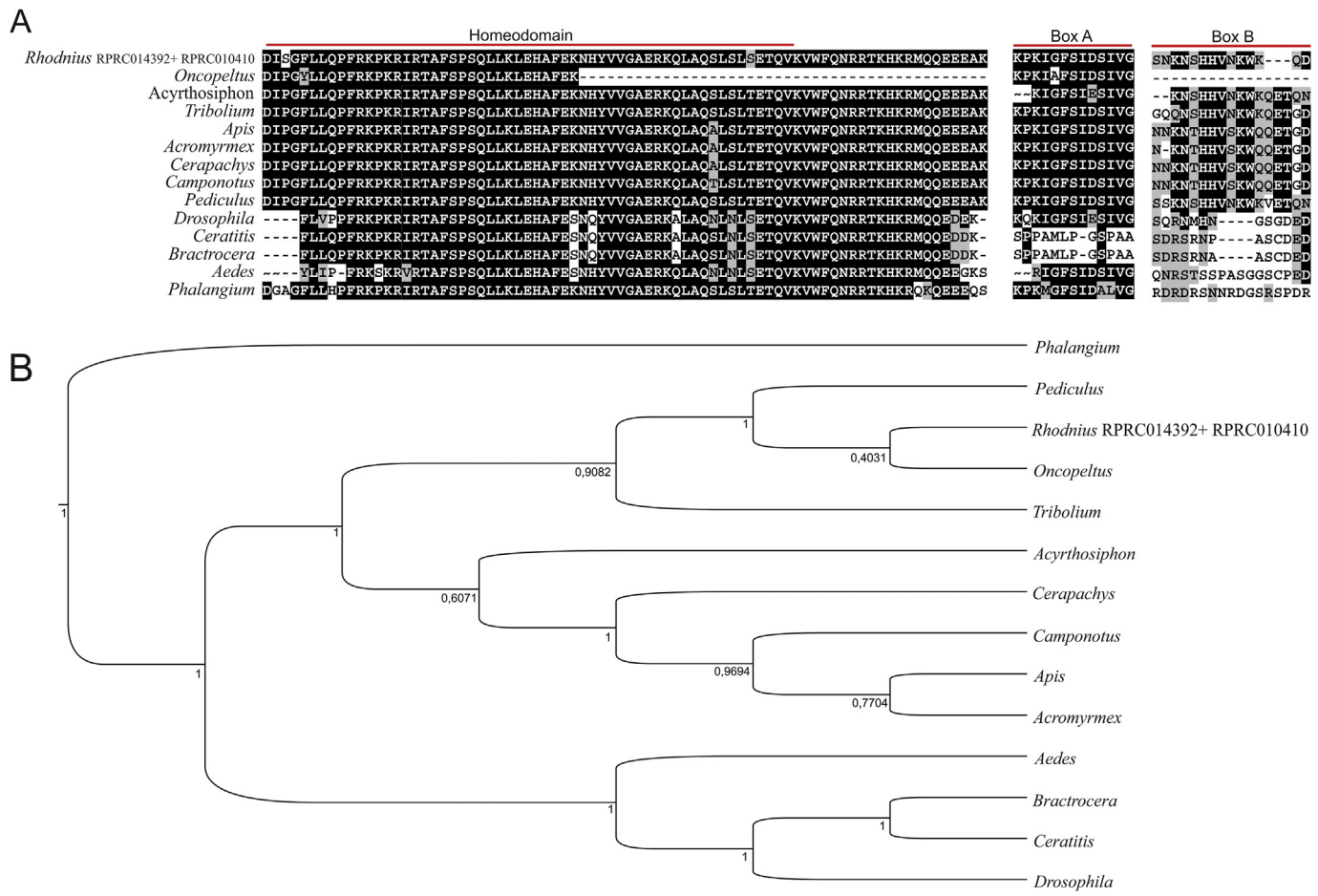

Fig. 3. *Rp-ems* gene analysis. A. Alignment of *Rp-ems* protein sequence of *Rp-ems* with other insect orthologues. The black boxes indicate complete amino acid identity and the gray boxes similar amino acid composition. B. Phylogenetic analysis. The tree was generated by Bayesian inference; the node values indicate the posterior probabilities.

Shigenobu et al., 2010), which agrees with the data presented here. *Sxl*, another target gene in this pathway, could not be identified in the current version of the *R. prolixus* genome, but in the transcriptome of *T. infestans*. This could be due to a genome gap or sequence divergence, as it was shown by phylogenetic analysis among species of the order Diptera (Traut et al., 2006).

In summary, most of the developmental genes are conserved, which is consistent with the idea that the ground condition of the basic processes is present in all organisms. However, we cannot conclusively state that the absence of any gene in our study is due to gene loss - caused by high sequence divergence in rapidly evolving genes- or to an incomplete genome coverage.

4.2. *R. prolixus: the comeback of a model for tracheal function*

The tracheal system is characteristic of myriapods and insects. Despite its importance in insect physiology and, therefore, adaptation, a comprehensive search for genes required for tracheal development and function had never been carried out in any organism but *D. melanogaster*. Functional analysis of the tracheal system is also very scarce (Beerman and Schröder, 2008; Matsunami et al., 1999; Mitcheal and Crews, 2002). *R. prolixus* is a superb model to study the tracheal system as well as the changes that occur after molting and in response to hypoxia (Locke, 1958; Wigglesworth, 1954). Therefore, the available genome and the molecular techniques we set to study developmental genes (Lavore et al., 2012, 2014 and accompanying manuscripts) have prompted us to investigate the molecular processes involved.

The work presented here is the first attempt to fill a gap in the knowledge of this basic process and provides the initial evidence of the conservation of the pathway that acts in tracheal development in hemipterans. Here, we have identified most of the genes related to tracheogenesis in *R. prolixus*. Gene orthologues such as *sima*, *fatiga* and *von hipel lindau* lacked *ab initio* protein models but we could identify the orthologues by manual curation of the genome. This implies that the genetic network underlying tracheogenesis and response to hypoxia has an almost complete conservation in triatomines.

In *D. melanogaster*, three transcription factors set the early tracheal events: *trachealess* (*trh*), *ventral veinless* (*vvl*) and *knirps/knirps-related* (*kni/knrl*) (Boube et al., 2000; Chen et al., 1998; Llimargas and Casanova, 1997). *trh* encodes a bHLH-PAS transcription factor. *Dm-trh* mutant embryos fail to form the tracheal tube precursor (Isaac and Andrew, 1996; Wilk et al., 1996). *trh* was studied in the crustacean *Artemia salina* – which lacks a tracheal system, in which it is expressed in the salt gland and thoracic epipods and functions in osmoregulation (Mitchell and Crews, 2002; Wang et al., 2012). It was also studied in the silk worm *B. mori*, in which *Bm-trh* is expressed in the silk gland and in the tracheal placodes, but no functional assays are available yet (Matsunami et al., 1999). Our results suggest that *Rp-trh* has a central role during the early trachea formation since the interfered embryos fail to develop the primary tracheas and in a more expressive phenotype, the formation of the tracheal pits also fails. We support the notion that the placodes are set in the proper position but the invagination of the trachea is aborted. Interestingly,

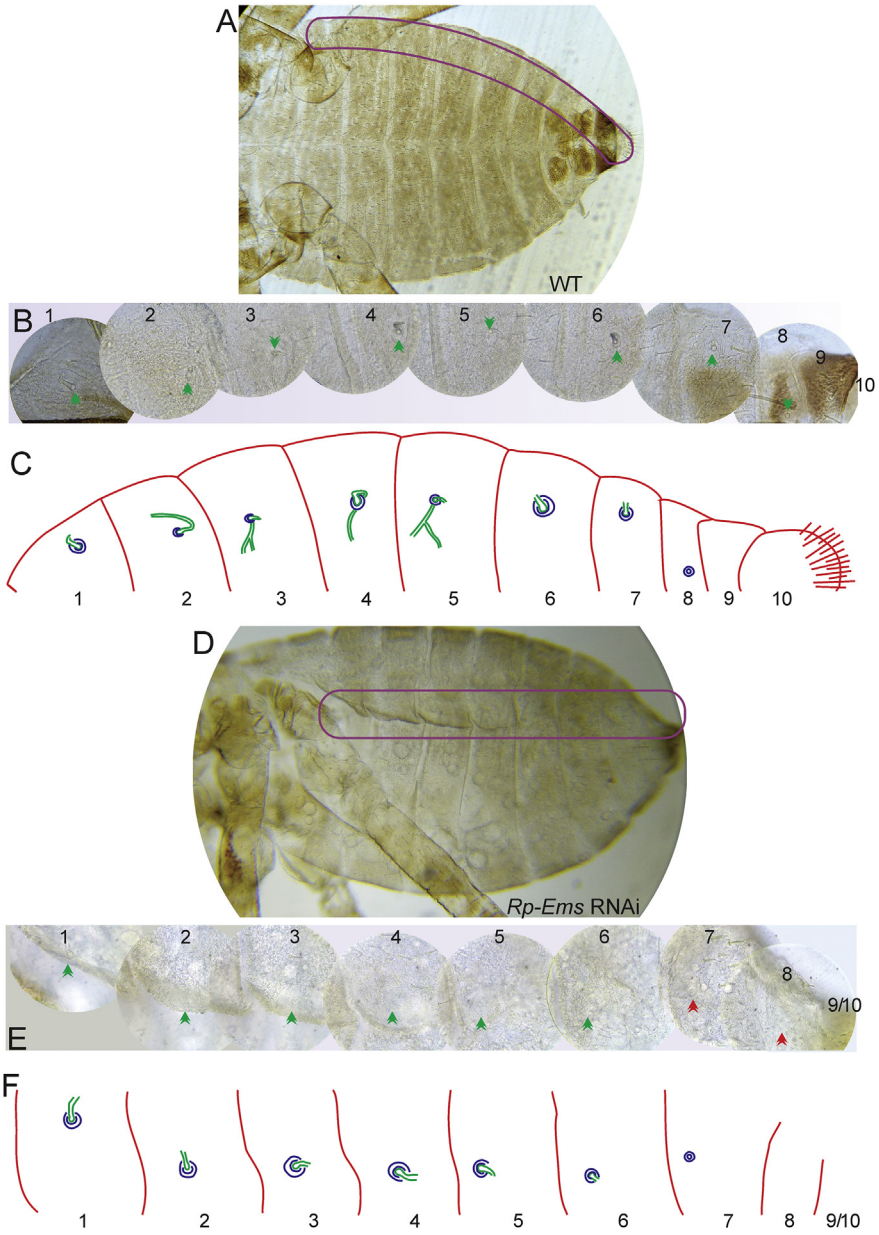

Fig. 4. *Rp-ems*^{RNAi} phenotype. A–C. Wild type larval phenotype. A. Larva in ventral view, in which the rectangle indicates the region where the spiracles are localized. B. Inset of the abdominal segments, in which the tracheas for each segment can be seen (spiracles are indicated by a green arrow). C. Scheme of the tracheas showed in B. D–F. Interfered embryo for *Rp-ems*. D. Larva in lateral view, in which the rectangle indicates the region where the spiracles are located. E. Inset of the abdominals segments, in which the green arrows show the presence of spiracle and trachea, while the red arrows show the tracheal defects. F. Scheme where the tracheas visualized in figure E are represented. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

our results agree with the phenotype observed in *trh* mutants in *D. melanogaster* and provide evidence of a conserved function.

The gap gene *Ems* encodes an HD-containing transcription factor identified in Nüsslein-Volhard and Wieschaus's genetic screenings (1980). *Dm-ems* mutants failed to form the antennal and mandibular segments and the mutant larvae lacked the trachea in the 8th abdominal segment so the connection of the dorsal trachea with the posterior spiracle was missing (Dalton et al., 1989). The analysis of *ems* orthologues in *O. fasciatus*, *T. castaneum* and *A. mellifera* also suggested an anterior role, but probably not as a gap gene. *Tc-ems* is expressed in the antennal and ocular segments and when it is missing there is a failure to establish the limit between the antennal and the ocular segments (Schinko et al., 2008). In *O. facsiatus* and *A. mellifera* only the expression pattern has been analyzed. *Of-ems* is expressed in the antennal segment and, at later stages, in lateral spots along all segments. *Am-ems* is expressed in the antennal, mandibular and maxillary segments (Dearden, 2014). RNAi experiments in *Of-ems* did not yield a phenotype or perhaps it was too subtle or with very low penetrance or expressivity (Birkan et al., 2011). Although our RNAi experiments also resulted in a low penetrance, we observed a consistent and reproducible phenotype. However, we did not observe defects in the cephalic region, as reported for *O. faciatus*. This favors the idea that *empty spiracles* function as a gap gene is a derived character of highly evolved insects such as *D. melanogaster*. Therefore we hypothesize that the requirement of *Rp-ems* for posterior trachea (A7 and A8 segments) formation, which agrees with the tracheal phenotype in *D. melanogaster*, might represent the ancient function of *ems*.

However, further functional studies in other species are required.

Our results present the initial evidence of conservation of the genes that set the tracheal system. The genes identified here will set the ground for a comprehensive and systematic analysis of the genetic network and molecular mechanisms underlying the function and evolution of insects' respiratory system, which was pioneered by the founder of insect physiology, Sir Vincent Wigglesworth.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2015.06.012.

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