The achaete—scute complex contains a single gene that controls bristle
development in the semi-aquatic bugs

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The semi-aquatic bugs (Heteroptera, Gerromorpha) conquered water surfaces worldwide and diversified to occupy puddles, ponds, streams, lakes, mangroves and even oceans. Critical to this lifestyle is the evolution of sets of hairs that allow these insects to maintain their body weight on the water surface and protect the animals against wetting and drowning. In addition, the legs of these insects are equipped with various grooming combs that are important for cleaning and tidying the hair layers for optimal functional efficiency. Here we show that the hairs covering the legs of water striders represent innervated bristles. Genomic and transcriptomic analyses revealed that in water striders the achaete—scute complex, known to control bristle development in flies, contains only the achaete—scute homologue (ASH) gene owing to the loss of the gene asense. Using RNA interference, we show that ASH plays a pivotal role in the development of both bristles and grooming combs in water striders. Our data suggest that the ASH locus may have contributed to the adaptation to water surface lifestyle through shaping the hydrophobic bristles that prevent water striders from wetting and allow them to exploit water surface tension.

1. Introduction

The semi-aquatic bugs (Hemiptera: Gerromorpha) are freshwater or marine insects that occupy various water surface niches worldwide [1–4]. These insects are thought to have derived from a terrestrial ancestor that evolved the ability to stand and generate efficient movement on the water [5]. Early-diverging lineages occupy transitional zones and walk both on land and water, whereas derived lineages evolved rowing as a novel mode of locomotion on the open-water surface [1,4]. Water surface invasion is directly associated with the ability of these insects to support their body weight on the water–air interface by exploiting surface tension [5–7]. An important adaptive morphological trait that has been critical to this transition is the evolution of hydrophobic leg hairs, whose density, morphology and orientation contribute to water repellency [6,8,9]. In addition, the complex body hair layers confer hydrophobicity to the semi-aquatic bugs and protect the animal from wetting and drowning. The body of the semi-aquatic bugs is generally covered by two hair layers [10]: a dense layer of microtrichiae (short hairs) close to the body surface is thought to play the role of waterproofing in case of submergence, whereas stiff macrotrichiae (long hairs) are thought to play the role of rainproofing [11]. The semi-aquatic bugs maintain the anti-wetting properties of the hair architecture through meticulous care using grooming combs consisting of rows of stiff hairs that are present on the distal tibiae [12]. In spite of their ecological importance, the nature of the hairs covering the leg and those forming the grooming combs remains elusive. These hairs might alternatively be
innervated mechanosensory structures (bristles) or non-innervated cuticular projections (trichomes) [13].

In the fly Drosophila, the four genes of the achaete–scute family encode basic helix–loop–helix factors that are master regulators of bristle development [14,15]. Achaete (ac), scute (sc) and lethal of scute (lsc) have redundant pronuclear functions and promote the formation of neural precursors, whereas ase (ase) is expressed in neuronal precursors. Loss-of-function mutant flies for ac and sc display a notum devoid of bristles [16]. Similarly, loss of lsc leads to the loss of body bristles whereas ectopic expression of lsc in the fly notum produces additional bristles [17]. All four genes are intronless, oriented in the same orientation and clustered in a 100 kb region containing numerous shared and interspersed cis-regulatory elements [18,19]. Genetic re-arrangements within the complex lead to mutant phenotypes owing to disruption of the cis-regulatory organization [20].

The ancestral achaete–scute complex (AS-C) in insects contained two genes: an ase gene and an achaete–scute homologue (ASH) gene which has undergone independent duplications in the Diptera [21]. In the flour beetle Tribolium castaneum, Tc-ASH is required to promote neural precursor formation and could play the role of multiple duplicated proneural ac–sc genes present in species such as Drosophila [22]. Moreover, the expression pattern of Tc-ase is highly conserved between Drosophila and Tribolium suggesting a conserved ancestral function for ase genes [22]. In addition to the conservation of their function, ac–sc genes show a conserved genomic structural organization between Diptera and Coleoptera [21]. It is widely thought that the organization of the complex and the presence of shared cis-regulatory regions prevent separation of the genes.

Given the key role that hydrophobic bristles play in water strider biology, we hypothesized that changes to the ac–sc gene family could have occurred during the evolution of the Gerromorpha lineage. To address this question, we investigated the genomic organization of ac-sc genes in 14 species of the Gerromorpha in comparison with other insect lineages. We generated and compiled a unique dataset of ACSC-proteins across insects, including available transcriptomes and genomes of early-diverging lineages. Furthermore using RNA interference (RNAi), we examined the role of this complex and the presence of shared cis-regulatory elements [18,19]. Genetic re-arrangements within the 100 kb region containing numerous shared and interspersed cis-regulatory elements [18,19]. Genetic re-arrangements within the complex lead to mutant phenotypes owing to disruption of the cis-regulatory organization [20].

2. Material and methods

(a) Data collection

Phylogenetic markers were identified in available genomes or transcriptomes by tBLASTn using a set of selected Drosophila melanogaster genes as a probe. Species names are indicated by the following prefixes Aae: Aeaces agypti, Aca: Aphisissi californica, Aga: Anophelesgambiae, Ame: Apis mellifera, Apa: Aquariris paludum, Bge: Blattella germanica, Bmo: Bombyx mori, Bta: Bemisia tabaci, Caq: Catanajus aquilonaris, Cca: Ceratitis capitata, Cle: Cinex lectularius, Cpa: Cyrtodanus palmaris, Csa: Cupeniussalei, Dci: Diaphorosa citri, Dma: Daphnia magna, Dme: Drosophila melanogaster, Dpo: Dendroctonus ponderosae, Dpu: Daphnia pulex, Eda: Ephemerapanaica, Eaf: Eurytemoraaffinis, Foc: Frankliniella occidentalis, Gbu: Gerris bueno, Hha: Halysponenta halys, Hst: Hydrobiapaus, Htu: Husseymellanus, Hvi: Homalodisca vitripennis, Hvu: Hydra vulgaris, Lde: Leptinotarsa decemlineata, Ldi: Linneagum ponscianus, Lfu: Lulena fulva, Lmu: Limnephilus lunatus, Lni: Locusta migratoria, Mam: Microvelia americana, Mdo: Muscondomestica, Mes: Medauradeaextradentata, Mbl: Mesoxella furcata, Mbe: Metortes hesperius, Mio: Microvelia longipes, Mmu: Mesoxella mulsanti, Mmu: Musnasculus, Nlu: Nilaparauutegens, Nvi: Nasonia vitripennis, Oci: Orchesella cincta, Ocu: Oixeumcucumcumumana, Ofa: Oncopeltus fasciatus, Pac: Paracera conata, Pap: Pseudorhoporis apetris, Pba: Pogonomynchus barbatius, Prb: Platypedia brachialis, Pbu: Paranevia bullialata, Pdu: Platynereis dumerilii, Phe: Peliculus humanus, Pve: Pachypulla venusta, Ran: Rhagovelia antileana, Rob: Rhagoveolia obesa, Rpr: Rhodiusprolixus, Rze: Rhagoletiszerphyrila, Sma: Stigmamaria maritima, Smi: Stegoptyus mimosarum, Ste: Sturidulevialigristiga, Ste: Sturidulevialigristiga, Tca: Triboliumcastaneum, Tlo: Triopslongicaudatus, Tla: Xenopus laevis, Zne: Zooteornopsis nevadensis. The novel sequences generated for this analysis have been deposited in the EMBL database with specific accession numbers (electronic supplementary material, table S1).

(b) Phylogenetic analysis

Nucleotide sequences were aligned with MUSCLE [23], manually adjusted and selected blocks were used for phylogenetic reconstruction. Maximum-likelihood (ML) searches were performed using RAxML v.8 [24] under the site-homogeneous LG+Gamma model. One hundred bootstrap replicates were conducted for support estimation. Bayesian phylogenetic analyses were performed using MrBayes 3.2 [25] under the GTR+Gamma model. We ran two chains for at least 1,000,000 generations and removed the first 250,000 generations as burn-in. The different nucleotide sequence alignments and tree files are available from the Dryad Digital Repository at: http://dx.doi.org/10.5061/dryad.rc454pc [26].

(c) Ancestral state reconstruction

Ancestral reconstruction of the number of grooming combs on the left legs was performed using R software. ML methods were adapted to discrete characters (ace, package ape [27]) and the package phytools [28] was used for generating graphical representations. The simplest model ‘ER’, with equal transition rates across all categories, was the best both with Akaike information criterion and likelihood comparisons.

(d) Microscopy

For transmission electron microscopy (TEM), adult tarsi were transversally cut into several pieces and fixed in 2% glutaraldehyde in 75 mM sodium cacodylate buffer (pH 7.3) overnight at 4°C. Samples were washed in cacodylate buffer and post-fixed in 1% osmium tetroxide for 1 h. Samples were then dehydrated in ethanol and embedded in epoxy resin. Samples were sectioned on a Leica UC7 ultramicrotome, stained with uranyl acetate and lead citrate and imaged with a Philips CM120 TEM at 80 kV. For scanning electron microscopy (SEM), adult bugs were fixed in 4% paraformaldehyde:heptane (ratio 1:3) for 20 min at room temperature. Samples were examined by using a Hitachi S800 SEM.

(e) In situ hybridization

Dissected embryos were fixed in 4% paraformaldehyde:heptane (ratio 1:3) for 20 min at room temperature, washed several times in cold methanol and then rehydrated through a methanol series to phosphate buffered saline-Tween 20 0.05%. Embryos were prehybridized for an hour at 60°C in hybridization buffer (for composition, see [29]) prior to addition of digoxigenin.
(DIG)-labelled RNA probe overnight at 60 °C. Blocking step was performed in 1% bovine serum albumin prior to incubation with anti-DIG antibody coupled with alkaline phosphatase for 2 h at room temperature. Embryos were washed several times before revelation with nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate in alkaline phosphatase buffer. Embryos were mounted on slides in Hoyer’s medium and photographed on a Zeiss Axi observer microscope. The polymerase chain reaction primers we used in this study are listed in the electronic supplementary material, table S2.

(f) Parental RNAi
Gene knockdown of ASH using parental RNAi was conducted following the protocol described in [29]. To obtain stronger phenotypes, Mesovelia mulsanti embryos were placed at 30–31°C which allowed them to develop faster. Technically, this method shortens the delay between the injection of the double-stranded RNA and the late expression of ASH transcripts.

3. Results and discussion

(a) Nature and structure of leg hairs
The legs of water striders are covered by layers of hairs of different sizes, which form a cushion between the leg and the water surface thus preventing the leg from breaking surface tension [6,9,10,30]. Although some authors informally refer to these structures as hairs/setae [5,6,8,9], bristle-like setae [31] or bristles [32], their cellular origin is unknown. In the water strider Gerris buenoi (Gerridae), leg tarsi are covered with at least two types of ‘hairs’ based on their size and that are regularly arranged along the leg (figure 1a). This feature is shared by the microveliid Microvelia americana (figure 1b) but not the mesoveliid Me. mulsanti where only one ‘hair’ type can be detected (figure 1c). Differences in the density of hairs are also observed across species (figure 1a–c). SEM images show a cuticle protuberance at the base of each hair (figure 1f), reminiscent of the socket cell of innervated bristles in insects [33] (figure 1g). TEM imaging on leg hairs revealed the presence of an outer dendritic segment underneath every single hair (figure 1d,e,g) and electronic supplementary material, S1. We therefore conclude that the numerous structures found on Gerromorpha legs are innervated bristles.

(b) Diversification of grooming combs in Gerromorpha
Grooming, or active cleaning of body parts with specialized grooming structures, is a critical activity in the life of insects [34]. Grooming allows care of the body surface through the removal of contaminants [35,36], parasitoids [37] and pathogens [38]. Grooming also facilitates the distribution of hydrocarbons and antiseptic secretions on the body surface [39,40], as well as displacement behaviour in stressful conditions [41]. In Gerromorpha, grooming combs are of critical importance as they are used to keep the hair layers tidily arranged to prevent the leg from breaking water surface tension and the bug from drowning [5,12] (electronic supplementary material, videos S1–S3).

We found tibial grooming combs to be present in all semi-aquatic bugs we have investigated in this study. However, the number of grooming combs varies between legs in a given individual, as well as across species (figure 2). First instar nymphs have two grooming combs on the foreleg and one grooming comb in midleg tibiae in all Gerromorpha, except in Hydrometra stagnorum which has one grooming comb in the foreleg and none in the midleg. The reconstruction of the plesiomorphic state predicts that the common ancestor of Gerromorpha had two grooming combs in the forelegs and a single one in the midlegs. This result suggests that the loss of one of the two grooming combs of the forelegs and that of the midlegs occurred in the lineage leading to Hy. stagnorum (figure 2). This species is known to preferentially live on solid substrates [4], which might explain the loss of grooming combs.

The presence/absence pattern of the hindleg grooming comb is more labile across the species we investigated. The
eight Gerromorpha species that lack grooming combs on the hindleg are not clustered in the phylogenetic tree, which argues for independent events during the course of evolution. However, statistical tests did not support either of the two possible hypotheses over the other: the ancestor had a hindleg grooming comb versus the ancestor lacks a hindleg grooming comb. In the event of hindleg grooming comb present in the ancestor, at least three independent losses would have occurred during the diversification of Gerromorpha (figure 2). Conversely, if the ancestor was missing hindleg combs, at least three independent gains took place in semi-aquatic bugs. According to published work in true bugs (Hemiptera: Heteroptera), the presence of tibial grooming combs on all legs is regarded as the most plesiomorphic state [42]. Moreover, the loss of grooming combs on the different legs appears to be a very common trend in terrestrial bugs [42]. It is therefore tempting to envision that the last common ancestor of extant Gerromorpha had a hindleg comb that has been independently lost in several lineages.

(c) Conservation of achaete-scute complex genomic architecture

The structures covering the legs of the Gerromorpha being innervated bristles, it is reasonable to consider bristle specification genes as candidates to explain the specific characteristics of these leg bristles. The analysis of the AS-C gene complex is essential to understanding the contribution that proneural genes have made to the evolution of epidermal structures in insects. Whereas AS-C genes have been extensively investigated in flies, little is known about the molecular evolution of these genes across insects. To evaluate how conserved the extended genomic locus is in other insect genomes, we conducted both content and synteny analyses of this locus in two hemipterans (G. buenoi and Homalodisca vitripennis), one thysanopteran genome (Frankliniella occidentalis) and one blattodean genome (Blattella germanica). Strikingly, we failed to detect a sense in the genome of G. buenoi, Ho. vitripennis and F. occidentalis species. To confirm the absence of a sense in these genomes, we performed synteny analysis, and we took advantage of the fact that the extended genomic locus of the whole AS-C complex exhibited a set of conserved features across the Holometabola (figure 3). In the fly D. melanogaster, the four AS-C genes are flanked by the genes yellow (y) and Cytochrome P450–4g1 (Cyp4g1) and are present in the same position in many other insects [21] (figure 3). First, the two genes y and Cyp4g1 that constitute the boundary markers in the Holometabola have not been identified in the vicinity of the AS-C in the three non-holometabolan genomes we investigated (figure 3). Conversely, we identified several other markers, especially major facilitator superfamily genes, whose homologues are present within the AS-C locus in most insect species (figure 3). Second, we showed that genes which flank the position of the missing a sense gene (ASH in the 5’ region; major facilitator superfamily genes in the 3’ region) are conserved across insects. This result suggests that a sense was lost in the lineage leading to the Hemiptera (G. buenoi, Ho. vitripennis) and the Thysanoptera (F. occidentalis), and this loss is not associated with a reshuffling of the surrounding genomic locus.
To further confirm this lineage-specific loss of *ase*, we searched for putative orthologues of *ase* in a broader sampling among insects. Using publicly available insect transcriptomes and in-house generated transcriptomes of 14 species of the Gerromorpha, we assembled sequence data of 77 AS-C genes and performed ML and Bayesian phylogenetic reconstruction. This sequence-based phylogenetic reconstruction identified both orthologues of *ASH* and *ase* genes in most insect lineages (figure 4, supplemental material, figure S3). Scale bar indicates a number of changes per site.

**Figure 3.** Syntenic organization of the *achaete–scute* locus in representative insects. The *D. melanogaster* ac–sc locus, which is used as a reference, is arbitrarily limited to 12 genes with specific colours. Single-copy genes depicted with colour combinations in other species mean that duplication(s) of this gene occurred in the lineage leading to the Diptera. Yellow bar: gene duplications specific to *D. melanogaster*; red star: loss of *ase*.

**Figure 4.** Phylogram of the 77 taxon analyses obtained through maximum-likelihood and Bayesian reconstructions were conducted using LG + Γ and GTR + Γ models, respectively. (a) Full tree depicting all insect taxa used in this analysis. (b) Zoom on Gerromorpha clade. Support values are shown in the electronic supplementary material, figure S3. Scale bar indicates a number of changes per site.
electronic supplementary material, table S3). Furthermore, both ASH and aSense clades include sequences from early-diverging lineages of insects and hexapods but exclude non-hexapod sequences. Non-hexapod genes grouping closely to both ASH and aSense clades are probable pro-orthologues of the ASH and aSense duplicated genes.

We conclude with confidence that the last common ancestor of the extant insects possessed at least two AS-C genes, corresponding to the precursors of the ASH and aSense clades. This transcriptome analysis, together with the characterization of the AS-C genomic locus, suggests that the aSense gene has been lost in the lineage leading to both Hemiptera (true bugs) and Thysanoptera (thrips) (Condylognatha). Both of these groups have sucking mouthparts and their monophyletic relationship was recently confirmed [45]. This finding represents, to our knowledge, the first reported case of aSense gene loss in insects.

(e) Expression of achaete-scute homologue in developing Gerromorpha embryos

To determine the role of the unique AS-C gene during development in the Gerromorpha, we investigated the expression of ASH by in situ hybridization in G. buenoi, M. mulsanti and M. americana. At mid-embryogenesis, Mmu-ASH is expressed in ectodermal cell clusters throughout the central nervous system (CNS), especially in the head and abdominal segments (figure 5a,b). These cell clusters are likely to correspond to the presumptive neural precursors, suggesting that Mmu-ASH acts as a proneural gene in M. mulsanti. Because we also detected CNS expression for Mam-ASH and Gbu-ASH (data not shown), it is reasonable to conclude that ASH has a proneural function in all Gerromorpha. These expression domains mirror those of ac-sc genes known in Coleoptera [22], Diptera [46,47] and Lepidoptera [48].

We also detected ASH expression in transverse stripes in all legs in post-katatrepsis embryos. In G. buenoi, the stripes are first sharp and narrow (figure 5e) before the domain of expression expands throughout leg segments (figure 5f). The expression of Gbu-ASH eventually becomes restricted to numerous dots in the legs (figure 5g,h), each dot likely to prefigure the position of a future bristle. This expression pattern recapitulates only the early, but not the late, expression of ac and sc found in D. melanogaster legs [49]. In D. melanogaster, the predominant expression from 3 to 5 h after puparium formation is in discontinuous transverse stripes that encircle the tarsal segments. At approximately 5 h ac and sc expression in longitudinal stripes begins to appear in conjunction with the transverse stripes, and by 6 h the longitudinal stripes in each segment replace the transverse stripes [49]. We have not detected any expression in longitudinal stripes either in G. buenoi, M. mulsanti or M. americana.
In late embryos, the expression of ASH is restricted to discrete regions such as the distal tip of the tarsus (Mam-ASH, figure 5d) that prefigures the location of the future claw.

(f) Achaete-scute homologue gene regulates neural precursors in the Gerromorpha

We used RNAi to deplete ASH function in the embryos. In *M. mulsanti*, we observed altered bristle development in 82% of ASH RNAi-treated embryos (n = 32 out of 39), ranging from a weak to a dramatic reduction in the number of bristles in the thorax and the abdomen (figure 6a–d), as well as in the legs (data not shown) in late embryos. The variability we observe in the knockdown phenotype is a consequence of the RNAi technique as reported in previous studies in the Gerromorpha [29,50,51]. In *Mi. americana*, only mild alterations of bristle development have been obtained (40% of embryos affected, n = 12 out of 30), showing missing bristles in the first two thoracic segments (figure 6e,f). In *G. buenoi*, we observed an altered phenotype in 97% of ASH RNAi-treated embryos (n = 208 out of 214), consisting of reduced bristle number on the whole body (figure 6g–j). ASH depletion, even subtle, consistently affected abdominal bristles (figure 6g,h), thoracic and head bristles secondarily and leg bristles (figure 6i,j). The variability in RNAi leg phenotype also ranges from mild to strong (electronic supplementary material, figure S4). The bristles that are missing or altered are not consistently the same ones between RNAi-treated embryos. However, all bristles can be affected independently of their location. It is reasonable to hypothesize that ASH controls the specification of all leg bristles and that the variability we see results from the partial efficiency of knockdown methods to silence the ASH gene.

Another important role of ASH, as revealed by RNAi, is the control of grooming comb development in all legs.
Whereas wild-type G. buenoi embryos have two grooming combs on the forelegs, and one comb on mid and hindlegs (figure 2), ASH RNAi-treated embryos are devoid of grooming combs altogether (figure 6) and electronic supplementary material, figure S4). Previous studies have shown that the knockdown of the gene Ultrabithorax (Ubx) does not affect grooming combs on any of the legs in the Gerridae [50,52]. However, Ubx RNAi leads to the development of an ectopic hindleg comb in Microvelia and Mesovelia both of which lack hindleg combs otherwise [50]. This suggests that the absence of hindleg grooming combs in some Gerromorpha is owing to ASH repression by Ubx. Further experiments would be required to test this possible interaction between Ubx and ASH genes.

ASH RNAi-treated embryos also lack claws (figure 6)). This phenotype is observed for the first time in the context of an altered ac–sc complex. In Drosophila, mutation of one or several genes of the ac–sc complex does not affect the tarsal claw. However, the proneural gene amos, whose post-embryonic expression prefigures the anlage of the innervated tarsal claw, is thought to be involved in the formation of sensory organ precursors in the tarsal claw [53]. Previous studies have shown that amos prevents bristle formation through the repression of scute function in Drosophila [54]. As a future direction, it might be interesting to test whether the genes ASH and amos act in the same developmental network during tarsal claw development in Gerromorpha.

4. Conclusion

The evolution of a higher density of hydrophobic leg hairs accompanied the invasion of water surface by semi-aquatic bugs. Variation in the density of bristles and the number of grooming combs found among the Gerromorpha might have evolved as an adaptation to the diversity of habitats these bugs live in [4]. We have shown these leg hairs to be bristles representing innervated structures that are known to act as mechanoreceptors. The massive number of leg bristles is associated with the semi-aquatic bugs’ lifestyle that involves detection of prey trapped on the water surface, as well as detection of predators. We therefore hypothesize that the increase in leg bristle density during the course of evolution could have played a dual role: exploiting water surface tension for locomotion and developing a higher sensitivity to detect various activities through vibrations of the water–air interface including prey and predator attacks [55]. Finally, we uncovered the pivotal role of the gene ASH in bristle and grooming comb specification in Gerromorpha. We identified unexpected changes at the genomic ASH locus. Our results suggest that changes in the regulation of the gene ASH could have been associated with the adaptation of semi-aquatic bugs to the water surface and their subsequent diversification.

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