Analysis of four achaete-scute homologs in Bombyx mori reveals new viewpoints of the evolution and functions of this gene family
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Abstract

Background: achaete-scute complexe (AS-C) has been widely studied at genetic, developmental and evolitional levels. Genes of this family encode proteins containing a highly conserved bHLH domain, which take part in the regulation of the development of central nervous system and peripheral nervous system. Many AS-C homologs have been isolated from various vertebrates and invertebrates. Also, AS-C genes are duplicated during the evolution of Diptera. Functions besides neural development controlling have also been found in Drosophila AS-C genes.

Results: We cloned four achaete-scute homologs (ASH) from the lepidopteran model organism Bombyx mori, including three proneural genes and one neural precursor gene. Proteins encoded by them contained the characteristic bHLH domain and the three proneural ones were also found to have the C-terminal conserved motif. These genes regulated promoter activity through the Class A E-boxes in vitro. Though both Bm-ASH and Drosophila AS-C have four members, they are not in one by one corresponding relationships. Results of RT-PCR and real-time PCR showed that Bm-ASH genes were expressed in different larval tissues, and had well-regulated expressional profiles during the development of embryo and wing/wing disc.

Conclusion: There are four achaete-scute homologs in Bombyx mori, the second insect having four AS-C genes so far, and these genes have multiple functions in silkworm life cycle. AS-C gene duplication in insects occurs after or parallel to, but not before the taxonomic order formation during evolution.

Background

Transcriptional factors of the bHLH (basic helix-loop-helix) family play important roles in the development of metazoan, taking part in the regulation of neurogenesis, myogenesis, haemopoiesis and so on [1-5]. The achaete-scute complex (AS-C), a group of four bHLH genes, has been found to be involved in the determination of Drosophila neural precursors [6-9].
AS-C proteins interact with another bHLH protein Daughterless (Da) to form a dimer, and bind Class A E-boxes CAGG/CTG [10]. AS-C/Da heterodimers’ binding sites were found in the upstream regions of hunchback (hb) [10] and phyllopod (phyl) [11]. In Drosophila, they were also found in the upstream region of ac itself, and required for auto- and cross-regulation by ac and sc [12].

The main functions of AS-C genes are regulating the formation and patterning of precursors of central nervous system (CNS) and peripheral nervous system (PNS). During neural development, AS-C genes are expressed in one or more cells within the proneural clusters, which are committed to the neural precursor fate, and the remaining cells in the cluster differentiate to be epidermal cells or are eliminated by apoptosis [8,13,14]. Tribolium castaneum ASH (Tc-ASH) and Hydra vulgaris cnidarian ASH (Cn-ASH) show similar functions during the formation of sensory organs in the transgenic Drosophila [15,16]. Also, it was found that Cn-ASH protein formed heterodimers with Drosophila Da (Dm-Da) protein in vitro, and these dimers specially bound to the consensus E-boxes [15]. Besides regulation of neurogenesis, AS-C genes have other functions. ac takes part in the development of Malpighian tubule by expressing in the tip cell and regulating the sequential fates of the organ [17]. sc regulates sex determination by its different expression dosage between females and males, but neither ac nor lsc can substitute for sc in this function [18-20]. lsc expression is necessary for muscle founder cells segregating from the somatic mesoderm. Loss of expression of lsc leads to a loss, whereas over expression causes a duplication of muscles and founder cells [21].

According to the sequence and functional analysis in different species, AS-C genes are conserved during evolution. The four genes of Drosophila AS-C are achaete (ac), scute (sc), lethal of scute (lsc) and asense (ase). The former three are defined as proneural genes and the last one as neural precursor gene, basing on their expression patterns and functions in proneural clusters. AS-C homologs have been isolated from various invertebrates and vertebrates [15,16,22-34]. They all have a conserved bHLH domain which distinguishes them from other bHLH proteins. Using the amino acid sequence of Drosophila Ac (Genbank: AAF45498) to blast the silkworm EST database (see Materials and Methods section), we obtained an EST sequence (GenBank: CK537057) encoding a conserved AS-C bHLH domain. Primers were designed based on the EST sequence, and RACE assay was carried out using the midgut total RNA of 3d 5th instar larva as the template. After sequencing and assembling, we gained a cDNA of 1,332 bp and named it Bm-ASH1 (Genbank: DQ350889). Bm-ASH1 gene contains a 582 bp ORF region (including the stop codon) and encodes a 193 aa protein.

Then we screened the Bombyx mori genome database using the 193 aa Bm-ASH1 protein sequence, and found genes with conserved AS-C bHLH region in four more contigs (Genbank: AADK01030307, AADK01036667, AADK01011379 and BAA01105243), besides the two (Genbank: AADK01005140 and BAA01089921) corresponding to Bm-ASH1. BAA01105243 is part of AADK01011379. Each of them contained a deduced ORF region, and then we cloned the ORF regions by RT-PCR methods and they were sequenced. Primers for 3'-RACE were designed basing on the sequences of the ORF regions, and 3'-RACE assay was processed using the total RNA from 1 d pupal wing as the template. The segments gained by 3'-RACE were sequenced and assembled with each corresponding ORF sequence. The final cDNA sequences were 1,449 bp, 990 bp and 1,695 bp long, and were named Bm-ASH2 (Genbank: EF620927), Bm-ASH3 (Genbank: EF620928) and Bm-ase (Genbank: EF620929).
respectively. Bm-ASH2 gene contained a 720 bp OFR region (including the stop codon) and encoded a 239 aa protein, Bm-ASH3 gene contained a 726 bp OFR region (including the stop codon) and encoded a 241 aa protein, and Bm-ase gene contained a 1,215 bp OFR region (including the stop codon) and encoded a 404 aa protein.

Alignment results with Drosophila AS-C proteins showed that all four proteins encoded by Bm-ASH genes contained the AS-C characteristic one basic and two helix domains flanking a non-conserved loop domain (Fig. 1). Bm-ASH1, Bm-ASH2 and Bm-ASH3 also had the C-terminal 16–17 aa conserved motif, but Bm-Ase did not. There was a highly conserved short motif (SPxxS, x means uncertain amino acid) around the region of PEST domain [36-38]. In the bHLH region, Bm-ASH1 shares 74.6%, Bm-ASH2 shares 72.1% and Bm-ASH3 shares 72.1% amino acid identity with Dm-L’sc, and Bm-Ase shares 74.3% with Dm-Ase (Table 1). The data above indicate that Bm-ASH1, Bm-ASH2 and Bm-ASH3 are proneural genes and Bm-ase an asense homolog.

We compared the sequences of ASH proteins from silk-worm and some other insects, and found that either at whole amino acids level or within the bHLH region, Bm-ASH1 had a higher identity with non-lepidopteran insects'...
Table 1: Homology comparison of proteins encoded by the Bombyx ASH genes with other insect ASH proteins.

| ASH Name | Percent Identity (%) |
|----------|----------------------|
| Bm-ASH1  |                      |
| Bm-ASH2  |                      |
| Bm-ASH3  |                      |
| Bm-Ase   |                      |

The number in the bracket is the identity percent between two whole protein sequences, and the number before the bracket is the identity percent within the bHLH regions.

ASH proteins than the other two Bombyx proneural proteins, Bm-ASH2 and Bm-ASH3 (Table 1). Among the silkworm ASH proteins, Bm-ASH2 and Bm-ASH3 are most identical, showing 91.3% identity within the bHLH region. It is surprising that Bm-ASH2 and butterfly Ac-ASH gene were not introns (Fig. 3). The expression of Bm-ASH2 and Bm-ASH3 was widely expressed in all the investigated samples. The expression of Bm-ASH1 and Bm-ASH2 was also detected in Bm-ASH1 and Bm-ASH2, however there was no intron in either Bm-ASH3 or Bm-Ase (Fig. 3).

**Bm-ASH genes regulating promoter activity via E-box in Bm-N cells**

AS-C genes have self-regulating functions by E-boxes in their promoter regions [12]. We cloned a 1,178 bp DNA fragment upstream of the ORF region of the Bm-ASH2 gene. At positions 188–194 bp and 791–797 bp upstream of ATG codon, there are two Class A E-boxes (CAGG/CTG). The promoter segments were cloned into pGL3-Basic vector with a luciferase reporter gene, and Bm-ASH and da genes were cloned into the modified transient expression vector pBacPAK8-ie1.hr3 [40]. Recombined plasmids were co-transfected into Bm-N cells by different assayed ways. We found that the promoter had no activity without the co-transfection of Bm-ASH or da genes' transient expression vectors (Fig. 4). The promoter had a significantly higher activity by co-transfection with Bm-ASH1 and the da genes' transient expression vectors than by other co-transfections. Besides, the promoter activity was weaker when it was only co-transfected into cells with Bm-ASH genes than when also with da (ex., promoter activity of ASH2P+ASH1 was weaker than that of ASH2P+ASH1+da, Fig. 4).

In order to study whether the bHLH transcriptional factors regulate the promoter by the E-box region, we changed one or both of the deduced E-boxes to *aAGG/CcG* by site-directed mutation (Fig. 5A). Then the mutated promoters were cloned into pGL3-Basic vector as described above, and co-transfected Bm-N cells with each Bm-ASH and da genes' transient expression vectors, respectively. The results showed that E-box mutation significantly reduced the activity of the promoter when co-transfected with silkworm proneural genes (Fig. 5B, C, D; p < 0.01). However, the activation of Bm-ase did not seem to be related with either of the two deduced E-boxes (Fig. 5E; p > 0.05). These data suggest that proteins encoded by Bm-ASH genes have similar functions to the AS-C transcriptional factors in *Drosophila*, and they synergize with Da protein.

**Expression distribution of Bombyx ASH genes in larval tissues**

To understand the potential in vivo regulations of ASH genes, we detected the expression status of Bombyx ASH genes in different tissues of the 5th instar larvae on the 3rd and the 8th days (just after spinning) (Fig. 6). The data showed that Bm-ASH1 and Bm-ASH2 were expressed in organs derived from all the three cellular derms: ectoderm, mesoderm and endoderm; whereas Bm-ASH3 and Bm-ase were only expressed in tissues derived from ectoderm and mesoderm. Bm-ASH1 was expressed in all organs except silk glands (S-G) and hemocytes (He), and at a low level in 5 d and had no expression in 8 d fat body (FB). Bm-ASH2 was widely expressed in all the investigated samples. The expression of Bm-ASH3 and Bm-ase
were more tissue specific, and the level of Bm-ase was relatively lower. Bm-ASH1 and Bm-ASH2 had high expression levels in gonads (G), while Bm-ASH2 and Bm-ASH3 had high expression levels in tracheal clusters (Tc). All of the data suggested that the four Bm-ASH genes might play various roles in the development of silkworm larva. We should note that all three proneural genes expressed highly in WD, and Bm-ase had a relatively higher expression level in 8 d WD. These implied that Bm-ASH genes might be important for the development of the wing disc.

Developmental changes of Bombyx ASH genes in the embryo and in the pupal wing

Functional genes play their roles by expression in special tissues and at special time. We detected the expression level of Bombyx ASH genes in the embryo and pupal wing in order to study the function of these genes during silkworm development. Total RNA was extracted from 1 d to 9 d embryos or from wings/wing discs (from spinning to the adult phase), and used for reverse transcription. cDNA synthesized from about 10 ng total RNA was taken as the template for each PCR reaction. We calculated the gene

![Phylogenetic status of silkworm ASH proteins in insect](image)

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Figure 2
Phylogenetic status of silkworm ASH proteins in insect. The support values are given at each node (100 replications). Branch lengths are not drawn to scale. Letters upside of the branch lines show the duplication events as clarified by Wheeler et al. [16] (see discussion for detailed description). It is obvious that all of the Aense proteins are grouped to one clade and the proneural genes are grouped to another within the insect group. Bm-ASH2 and Bm-ASH3 are grouped to a sub-clade parallel the one which Bm-ASH1 is sorted in. Ag-ASH, Anopheles gambiae Achaete-Scute homolog (Genbank: AAK97461); Am-ASH,Apis mellifera Achaete-Scute homolog (Genbank: XP_393665); B-ASH1, Butterfly (Genbank: Precis coenia) Achaete-Scute homolog 1 (Genbank: AAC24714); Bm-ASH1, Bombyx mori Achaete-Scute homolog 1 (Genbank: NP_001037416); Bm-ASH2, Bombyx mori Achaete-Scute homolog 2 (Genbank: EF620927); Bm-ASH3, Bombyx mori Achaete-Scute homolog 3 (Genbank: EF620928); Cn-ASH, Hydra vulgaris cnidarian Achaete-Scute homolog (Genbank: U36275); Cs-ASH1, Cupiennius salei Achaete-Scute homolog 1 (Genbank: CAC27516); Cs-ASH2, Cupiennius salei Achaete-Scute homolog 2 (Genbank: CAC27517); Dm-ac, Drosophila melanogaster Achaete (Genbank: AAF45498); Dm-sc, Drosophila melanogaster Scute (Genbank: AAF45499); Dm-lsc, Drosophila melanogaster Lethal of scute (Genbank: AAF45500); Pc-ASH1, Podocoryne carnea Achaete-Scute homolog 1 (Genbank: AAN5110); Tc-ASH, Tribolium castaneum Achaete-Scute homolog (Genbank: AAQ23386); Ag-ase, Anopheles gambiae Asense (Genbank: AAB01008963); Am-ase,Apis mellifera Asense (Genbank: XP_393664); Bm-ase, Bombyx mori Asense (Genbank: EF620929); Dm-ase, Drosophila melanogaster Asense (Genbank: AAF45502); Tc-ase, Tribolium castaneum Asense (Genbank: AAQ23387).
copy number corresponding to $10^3$ Bm-actin A3 copies at each developmental point in the embryo and $10^6$ in the wing/wing disc. The results showed that during the embryo development, both Bm-ASH2 and Bm-ase had two expression peaks, one being both on 3 d, and the other being on 5 d for Bm-ASH2 and on 6 d for Bm-ase (Fig. 7). However, there was only one peak for each of the other two Bm-ASH genes, Bm-ASH1 on 5 d and Bm-ASH3 on 4 d. Although the expression levels of Bm-ASH3 showed a peak from 3 d to 5 d, its expression level was always lower than the other three genes.

During the spinning (wandering) and pupal ages, the silk-worm wing disc/wing form changes obviously by extension, turning and scale foundation. The results of quantitative RT-PCR showed that all four genes had relatively high expression levels from 0 d to 3 d pupae (Fig. 8). The result of Bm-ASH1 was similar to our previous results [41]. Bm-ASH1, Bm-ASH2 and Bm-ASH3 all reached their

Figure 3
Genomic structure of Bm-ASH genes. The red region stands for the ORF, and the yellow region stands for non-coding regions; gDNA stands for genomic DNA; (+) and (-) show the published genomic sequences [39] being forward or reverse compared with the cDNAs, "?" shows the region unsequenced. The 6 bp in Bm-ASH1 indicates that there is a 6 bp uncoding region in the first exon of the gene just following the coding region, so is the 4 bp in Bm-ASH2. Genomic sequences are all from SilkDB [39]. The name of the genomic DNA fragment in Bm-ASH1 is Scaffold002070, in Bm-ASH2 is Scaffold007910, in Bm-ASH3 is Scaffold013050, and in Bm-ase is Scaffold000880, respectively.
peak values on P-1 d, whereas Bm-ase peaked one day later. Flanking these days, expression levels of the genes were significantly lower. During the spinning period, expressions of all four genes were at low levels, with Bm-ase being undetectable. It was notable that the expression levels of the three proneural genes decreased from S-0d to S-3d. Bm-ASH3 and Bm-ase were not express anymore after P-3d pupa, while both Bm-ASH1 and Bm-ASH2 had a low but noticeable peak in 5 d pupa.

Discussion
Our results showed that there were four AS-C homologs in Bombyx mori: Bm-ASH1, Bm-ASH2, Bm-ASH3 and Bm-ase. The first three were proneural genes, and the last one was a neural precursor gene. The proneural genes enhanced the activity of the Bm-ASH2 promoter by binding its E-boxes in Bm-N cells, which was one of the AS-C homolog characteristics (Figs. 4 &5). The four genes had various expression profiles in silkworm larval tissues (Fig. 6), and further studies showed that they have important roles during the development of the embryo and the wing (Fig. 7 &8).

Figure 4
Activity analysis of ASH2P promoter by co-transfected with Bm-ASH genes. In the figure, ASH2P stands for the recombinanted plasmid pGL3-Basic-ASH2P; ASH1, ASH2, ASH3, ase and da respectively stands for the modified transient expression vector pBacPAK8-ie1-hr3 containing Bm-ASH1, Bm-ASH2, Bm-ASH3, Bm-ase and Dm-da (daughterless) ORFs as transcriptional factor genes. At least three independent repeats were carried out for each treatment.

AS-C genes duplication in insects occurs after or parallel but not before the taxonomic order formation during evolution
Homologs of AS-C genes exist in various animals from low-grade coelenterate to high-grade mammals, including human being [30]. It has been proposed that AS-C gene has several independent duplication events in Arthropoda, resulting in the plasticity of the gene number. In this model, Cn-ASH, the AS-C homolog in Hydra is supposed as the ancestral one. Parallel but independent duplication events occurred in insects and chelicerates (Fig. 2,a &2b). Within the Diptera two more duplication events happened during evolution. So the present most derived dipteran, Drosophila melanogaster, has the four AS-C genes achaete, scute, lethal of scute and asense [16,30]; while lower derived dipteran, such mosquito, has only two [31].

We found that there were four AS-C homologs in the silkworm, Bombyx mori, a model lepidopteran. Also, Bombyx is the only insect besides Drosophila found with four AS-C genes so far. Among the three proneural AS-C genes in Drosophila, l'sc is the most original one, which is more...
identical to the proneural genes in other insects than ac and sc (Table 2). Like l’sc, Bm-ASH1 is more identical to non-lepidopteran insect proneural genes than other silk-worm achaete-scute homologs (Table 2). What’s more, l’sc and Bm-ASH1, and other sole proneural genes from other insects were sorted into one clade in the phylogenetic tree (Fig. 2). The data imply that Bm-ASH1 is the most original one in Bombyx. But homogeneous and phylogenetic analysis could not match each Bombyx proneural gene to those of Drosophila one by one, and phylogenetic result sorted ac/sc and Bm-ASH2/Bm-ASH3 into two independent branches. So we suggest that the generations of ac/sc from l’sc and Bm-ASH2/Bm-ASH3 from Bm-ASH1 are two parallel and independent events, that is, further duplication of proneural genes occurs after or parallel to but not before the split of Diptera and Lepidoptera during evolution.

The medfly Ceratitis capitata, a member of family Tephritidae, has three AS-C homolog genes Cc-sc, Cc-l’sc and Cc-ase. The bHLH domains of proteins coded by Ceratitis capitata achaete-scute homologs are highly conserved and display 95%, 91.5% and 90% identity with the Drosophila counterparts, respectively [28]. Only one AS-C homolog, B-ASH1, has been isolated from the Nymphalidae insect P. coenia [26]. B-ASH1 protein has a surprising identity with Bm-ASH2, 100% within the bHLH domains and
90.3% within the whole protein sequences. It is even higher than that between Bm-ASH2 and Bm-ASH3, 91.3% within the bHLH domains and 49.8% within the whole protein sequences (Table 1). Moths first appeared on earth between 100 and 190 million years ago, and butterflies appeared about 40 million years ago, based on fossilized evidence. All above suggest that there are at least three AS-C genes in *P. coenia*, corresponding with Bm-

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**Figure 6**

**Tissue expression distribution of Bm-ASH genes in silkworm larvae.** RT-PCR templates including: ectoderm tissues H (head), WD (wing disc), S-G (silk gland), MT (Malpighian tubule), TC (trachea cluster), BW (body wall); mesoderm tissues He (hemocyte), FB (fat body), G (gonad), T (testis), O (ovary); and endoderm tissue MG (midgut). V3d stands for 3-day-old larvae of the 5th instar, and S stands for 8-day-old larvae of the 5th instar (begin spinning). Amplification cycles were 30 for Bm-ASH1, Bm-ASH2, Bm-ASH3 and Bm-ase, and 25 for the internal control gene Bm-actin A3.

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**Figure 7**

**Developmental changes of Bm-ASH genes in silkworm embryo.** The numerical value are copy number of ASH cDNA per 10^3 Bm-actin A3 cDNA copies. At least three independent repeats were carried out for each developmental stage.

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**Figure 8**

**Developmental changes of Bm-ASH genes in silkworm wing disc/wing.** The numerical value are copy number of ASH cDNA per 10^6 Bm-actin A3 cDNA copies. S0-S3 represent days after the beginning of spinning, and P0-P7 represent days after pupation. At least three independent repeats were carried out for each developmental stage.
Table 2: Homology comparison of proteins encoded by the Drosophila AS-C genes with some of the other insect ASH proteins.

| ASH name | Percent Identity (%) |
|----------|----------------------|
|          | Dm-L’sc | Dm-Sc | Dm-Ac |
| Dm-L’sc | /       | /     | /     |
| Dm-Sc   | 73.5 (34.6) | 66.7 (33.8) | /     |
| Dm-Ac   | 69.1 (35.8) | 66.7 (33.8) | /     |
| Ag-ASH  | 79.4 (43.2) | 73.5 (29.0) | 75.0 (37.3) |
| Am-ASH  | 72.1 (26.6) | 69.6 (28.9) | 66.7 (33.3) |
| B-ASH1  | 73.5 (31.9) | 62.3 (25.2) | 66.7 (29.9) |
| Bm-ASH1 | 74.6 (40.9) | 68.7 (42.0) | 68.7 (31.6) |
| Bm-ASH2 | 72.1 (31.0) | 62.3 (27.2) | 65.7 (28.9) |
| Bm-ASH3 | 72.1 (34.9) | 58.0 (27.4) | 65.2 (30.3) |
| Tc-ASH  | 70.6 (34.2) | 65.2 (33.2) | 63.9 (33.8) |

The number in the bracket is the identity percent between two whole protein sequences, and the number before the bracket is the identity percent within the bHLH regions.

ASH1, Bm-ASH2 and Bm-ase, respectively, and Bm-ASH3 might be the most recent one among the silkworm proneural genes.

**AS-C genes have a broad expression distribution in insect tissues**

AS-C genes are important for the development of the nervous system, and have key roles in regulating the formation and patterning of neural precursors. They are specially expressed in most of the proneural clusters during the development of either the central (CNS) or peripheral nervous system (PNS) in arthropod, such as flies, butterflies, beetles, bees, spiders, chilopods, etc. Other functions of these genes have also been found in *Drosophila*. l’sc participates in the specification of muscle progenitors [21], sc functions in sex determination [18] and ac regulates the development of Malphigian tubules [17].

In the present paper, we studied the expression of *Bm-ASH* genes in various silkworm larval tissues using RT-PCR methods. These genes are expressed in most organs derived from all three derms, showing they might have multiple functions during silkworm development. They have higher expression levels in the head, wing disc, tracheal cluster, body wall or gonad, and all of them are expressed in the organs though lower at some developmental points. This indicates that the genes might have overlapping functions, just as in *Drosophila* [42]. Expression profiles during the development of the embryo and the pupal wing also suggest co-operation characters of Bm-ASH genes. During silkworm embryo development, neurogenesis takes place around 3 d-old, and trachea, bristle and appendage occur on 5 d and 6 d. The correspondence between the two stages and those when the two expression peaks of Bm-ASH genes occur in the embryo (Fig. 7) suggests that these genes regulate the development of PNS and CNS. Expressions in wings of all the four genes are significantly higher on 1 d or 2 d after pupation than during other stages. Wing scale precursor cells form around the 2 d-old pupal stage [41], and our further studies on the scaleless wings mutant silkworm strongly proved the key role of *Bm-ASH2* in the formation of wing scales (Zhou et al., unpublished).

Although we have known many important functions of AS-C, the analysis of the structures and evolution of these genes may suggest some of their unknown functions. We compared the protein sequences of 38 AS-C homologs from vertebrate and invertebrate. There are conserved domains besides the bHLH and C-terminal motifs, especially within vertebrates. This conservation even exists between vertebrate and invertebrate organisms. A ~20 amino acid conserved domain (corresponding to PEM-RCKRRINFAQLGYNLPQ of Asp-ASH) was found in ASH genes from vertebrates and myriapod animals *Lithobius forficatus* (Lf-ASH, Genbank: AAT99570) and *Archispirostreptus* (Asp-ASH, Genbank: AJ536345). Together with the broad expression of Bm-ASH genes in tissues outside of the nervous system, they show that this gene family has other important functions waiting for exploration.

**Conclusion**

In this work, we isolate and identify four *achaete-scute* homologs from *Bombyx mori*. So far, *Bombyx mori* is the second insect which has been found to have four AS-C genes. During organism evolution, genes are duplicated with conserved domains to gain more special functions. Results of phylogenetic and gene expression analysis show that during evolution, AS-C genes duplication in insects occurs after or parallel to but not before the taxonomic order formation and functions of these genes are broad during insect development.

**Methods**

**Insects and cell culture**

The silkworm stock Jingsong × Haoyue was maintained in our laboratory. The insects were reared on an artificial diet at 25°C with 70%–80% relative humidity. Bm-N cells derived from silkworm were cultured at 27°C in TC-100 insect medium containing 10% heat-inactivated (56°C, 30 min) fetal bovine serum (Invitrogen). Cell culture details were the same as Summers and Smith [43].

**RNA isolation and RT-PCR**

Silkworm tissues were dissected out at different stages and the total RNA was extracted with TRIZOL Reagent (Invitrogen) according to the standard protocol, and whole embryo total RNA was extracted using the acid-guanidine method [44]. One microgramme of total RNA from each sample was used to synthesize first-strand cDNA using M-
MLV Reverse Transcriptase (Promega) as the protocol described. PCR with proper program was performed using the reverse transcription product as template. Sequences of all primers used in this paper are available upon request.

**Rapid amplification of cDNA ends (RACE)**

One microgramme of total RNA was used for RACE cDNA synthesis (BD SMART™ RACE cDNA Amplification Kit, Clontech), according to the user’s manual. PCR was performed with primer1 and Universal Primer A Mix (UPM, Clontech), then a nest PCR was processed with primer2 and NUP using the suitable diluted former PCR product as the template. Each PCR reaction was carried out under the following conditions: one cycle of pre-denaturing for 5 min at 95°C; and 30 cycles of 94°C for 40 s, 60°C for 40 s, 72°C for 3 min, then followed by 10 min incubation at 72°C.

**Database blast, protein sequences alignment and phylogenetic analysis**

We used the amino acid sequence coded by *Drosophila achaete* (Genbank: AAF45498) to blast the silkworm EST database with the blastx program on the NCBI web site [45], by limiting the organism with "*Bombyx mori*". As formerly described [46], insect ASH protein sequences were aligned with CLUSTALX [47] and revised manually with Gendoc software. Then a neighbor-joining (NJ) tree based on amino acid sequences was constructed using the PHYLIP software package (100 bootstrap replicates) [48].

**Dual-Luciferase Reporter Assay**

Promoter segments were cloned into Luciferase Reporter Vector pGL3-Basic separately, and the ORF region of silkworm ASH and *Drosophila da* (GenBank: Y00222) genes were cloned into the modified pBacPAK8 vector with an IE1 promoter and a hr3 enhancer [40]. Then the plasmids were transfected into Bm-N cells as described formerly [49]. 0.1 µg of pRL-CMV Vector was co-transfected as an internal control reporter for each transfection. After incubating for 48 hours, the cells were collected by centrifugation at 10,000 rpm for 1 min at 4°C. Then the cell lysates were prepared using the passive lysis buffer, and 10 µg of each lysate was used for the dual-luciferase reporter assay according to the protocol (Dual-Luciferase® Reporter [DLR™] Assay System, Promega). Firefly luciferase activity and Renilla luciferase activity were determined with 20/20® Luminometer (Turner BioSystems, Inc., USA). Each treatment was repeated at least three times.
Introduction of point mutation

Primers P-F and P-R were designed at the terminals of the target segment sequence. Reversed primers m-R and m-F with the mutated bases were designed around the site where the point mutation would be introduced. PCRs were carried out with P-F pairing m-R or m-F paring P-R. After purification, the two PCR products were mixed, and denatured, renatured and extended for three cycles without any primers. Three cycles after running, P-F and P-R were added and the PCR was continued for 30 cycles of amplification. All of the PCRs were processed with pfu DNA polymerase.

Quantitative real-time PCR

Q-PCR was used to determine the changes of silkworm ASH genes expression during embryo development and wing development. Primers were designed based on the cDNA sequence and a segment around 200 bp would be specially amplified for each gene. The housekeeping gene Bm-actin A3 was used as the internal control. A 20 µl volume containing cDNA produced from about 10 ng of total RNA, 5 pmol of each primer, and 10 µl of SYBR Green Realtime PCR Master Mix (Toyobo Co., Ltd., Japan) was used for each PCR reaction. Then the PCR was processed on a Chromo4 Four-Color Real-Time System (Bio-Rad [formerly MJ Research]) under the following program: one cycle of 95°C for 3 min; then 40 cycles of 95°C for 15 sec, 60°C for 15 sec and 72°C for 30 sec. The melting curve was established from 60°C to 95°C. Three independent repeats were carried out for each reaction. Threshold cycle values were used for the further analysis.

Standard curves were constructed using serial dilutions of the ORF segments of the genes recombined into pMD18-T vectors (Takara, Japan). The log ranges of the standard curves were from 106 to 102 copies. The data curve and the T vectors (Takara, Japan). The log ranges of the standard curve was established from 60°C to 95°C. Three independent repeats were carried out for each reaction. Threshold cycle values were used for the further analysis.

Theoretic copy of each sample was calculated with the linear equation for each gene, and trendlines for Bombyx ASH genes during the development course of silkworm embryo and pupal wing were constructed.

Authors’ contributions

ZQX carried out most of the work and wrote the paper. ZIY isolated Bm-ASH1 and helped write the paper. YLL took part in RT-PCR and helped analyzing the data. YYZ cultured the cells. XWH and ZZL conceived the project and draft the manuscript. All authors read and approved the final manuscript.

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