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Microstructure of the biological attachment devices in the ladybug *Harmonia axyridis* (Coleoptera: Coccinellidae)

Myung-Jin Moon*, Hyo-Jeong Kim, Hoon Kim and Jong-Gu Park

Department of Biological Sciences, Dankook University, 29 Anseo-dong, Cheonan 330-714, Korea

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Biological attachment device is optimized in insect legs for attachment onto the variety of natural substrate. We have studied the microstructural characteristics of the tarsal appendages in the ladybug *Harmonia axyridis* using scanning electron microscopy to reveal the attachment system of their legs. The attachment devices are composed of claws and adhesive pads. The claws are connected with pretarsal segment, and their apical diverged hooks are developed to hold rough substrates. In contrast, the adhesive pads have an adhesive function onto smooth surface. The pads are interspersed at the ventral part of each tarsomere, and are composed of two kinds of hairy setae. The discoid tip seta (DtS) has a spoon-shaped endplate usually with a rounded concave structure, whereas the pointed tip seta (PtS) has a pointed tip, usually with a hooked endplate. While the PtS is broadly localized concentrically on the marginal area of both the proximal and distal pads, the DtS can be seen at the central areas of each adhesive pad except for the hind legs. Our findings demonstrate the presence of the direction-dependence pattern of the fibrillar system as well as a functional modification of the tenent setae to achieve proper contact with almost any kind of substrates.

Keywords: attachment device; *Harmonia axyridis*; fine structure; ladybug; seta

Introduction

Animals such as insects, spiders, and geckos have adopted biological attachment systems on their feet to move on smooth surface and even ceilings (Arzt et al. 2003; Gorb 2008; Moon and Park 2009). The attachment devices in arthropods can be basically divided into two principal groups, smooth or hairy (fibrillar) types (Huber et al. 2005; Autumn et al. 2006; Kim and Bhushan 2007) according to their independent evolutionary background (Irschick et al. 1996; Beutel and Gorb 2001). The smooth and hairy systems which generate strong friction and adhesion (Irschick et al. 1996; Autumn and Peattie 2002; Gao and Yao 2004; Huber et al. 2005; Kim and Bhushan 2007; Gorb 2008) have been described in different taxa (Beutel and Gorb 2001; Gorb 2008).

It has been known that some of these attachment pads are supplemented with various kinds of fluids (wet adhesion; Federle et al. 2002; Vötsch et al. 2002), but some are not (dry adhesion; Autumn and Peattie 2002; Kesel et al. 2003; Huber et al. 2005). Among these attachment systems, the wet adhesion system using adhesive fluid is seen in animals such as flies and beetles (Gorb 1998; Niederegger et al. 2002; Gorb and Gorb 2004), whereas gecko lizard (*Gecko gecko*) and spiders use dry adhesion system by the van der Waals forces and electrostatic interactions between the cuticular hairy elements and the substrate (Autumn and Peattie 2002; Kesel et al. 2003; Gao and Yao 2004).

The hairy adhesive system is thought to have a proper contact with almost any kind of natural substrates (Irschick et al. 1996; Autumn and Peattie 2002; Gao and Yao 2004; Huber et al. 2005; Kim and Bhushan 2007; Gorb 2008) with a number of specific advantages including energy-efficient detachment (Autumn et al. 2006; Federle 2006; Bullock and Federle 2009) and specialized self-cleaning (Hansen and Autumn 2005; Gorb 2008). Previous works have shown that this hairy adhesive device is optimized in insect legs, and this system also requires the use of various kinds of fluids (Gorb 1998; Federle et al. 2002; Vötsch et al. 2002; Betz 2003; Langer et al. 2004) unlike the dry adhesives of spiders (Kesel et al. 2003) and geckos (Autumn and Peattie 2002; Huber et al. 2005).

Although significant progress has been made to show efficiency of the hairy attachment systems by several researchers (Gorb 2008), however, the detailed mechanisms are still not sufficiently understood in either smooth or fibrillar systems. In addition, both the morphological and fine structural properties of these attachment systems have been studied only in representative species of selected taxa among the vast field of arthropod systematics. Thus, we have investigated the attachment pads on the tarsal appendages of the ladybug *Harmonia axyridis* using field emission scanning electron microscopy (FESEM) to reveal the fine structural characteristics of its hairy attachment system.

Materials and methods

Ladybugs or ladybird beetles are actually not bugs (Hemiptera) but beetles (Coleoptera). They are insects

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*Corresponding author. Email: moonmj@dankook.ac.kr

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of the beetle family Coccinellidae that have a bright colored body with small black spots on their elytra. Nearly all ladybugs, both larvae and adults, are predators on destructive, plant-eating insects such as aphids, mites, and scale insects (Frank and Mizell 2009). *H. axyridis* is a large coccinellid beetle, nearly hemispherical in shape with very short legs. As these beetles have adopted fibrillar pads on their feet to achieve the extraordinary adhesion for vertical walls and ceilings, they can form groups that tend to stay in upper corners of windows (Koch 2003).

Adult *H. axyridis* were collected from sites near the Cheonan campus of Dankook University and brought back to the laboratory where they were used for the experiment. The beetles were anesthetized with CO_2 and dissected under a dissecting light microscope in a drop of insect Ringer's solution consisting of 128 mM NaCl, 18 mM $CaCl_2$, 1.3 mM KCl, and 2.3 mM $NaHCO_3$, pH 7.4.

For light microscopic observation, their six appendages were gently removed and fixed in a Bouin's fixative solution consisting of picric acid, glacial acetic acid, and formaldehyde in an aqueous solution. The specimens were then dehydrated in ascending concentrations of ethanol and embedded in Poly/Bed 812-Araldite mixture (Polysciences Inc., Warrington, PA, USA) via propylene oxide. Semithin sections, 0.5–1.0 μm thick, were obtained using an LKB Ultratome V (LKB, Stockholm, Sweden). These plastic sections were stained with 1% toluidine blue (dissolved in 1% borax), and were photographed using Zeiss Axiophot microscope (Carl Zeiss, Jena, Germany) coupled with Motic digital imaging system (Motic Instruments Inc., Richmond, Canada).

For scanning electron microscopic examination, whole body of the ladybug beetle was fixed in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde buffered with 0.1 M phosphate buffer at pH 7.4. Postfixation was performed with 1% osmium tetroxide in the same buffer and washed several times in 0.1 M phosphate buffer following fixation. The resulting specimens were dehydrated in ascending concentrations of ethanol 30–100% (30 minutes at each concentration, with one repeat at 100% ethanol). These samples were then either critical point-dried or transferred to hexamethyldisilazane and allowed to air dry. All samples were coated to a thickness of approximately 20 nm with gold–palladium alloy using a sputter coater, and they were examined on a Hitachi S-4300 (Hitachi Co., Tokyo, Japan) FESEM operated with accelerating voltages of 5–15 kV.

Results

The tarsal formation in *H. axyridis* is cryptotetramerous and is equipped with a pair of terminal claws (Sasaji 1971). The claws are quite sharp, elongated, and curved ventrally, and the hook structure is adapted to move on course or rough surfaces (Figure 1A,B). Two claws on each leg are symmetrical in size and shape, and flexibly articulated with the terminal part of the fourth tarsal segment. The fourth tarsal segment is long and cylindrical, and many trichoid sensilla are scattered on the surface of the sclerotized structure of this segment, whereas both the first and second tarsal segments were dilated and relatively broadened compared to that of the terminal segment with a dense brush of hairs (Figure 1C,D).

In *H. axyridis*, all feet were equipped with tibial spur structures. The distal part of tibia is enlarged and bears numerous spurs on the lower surface (Figure 1E). Each of the tibial spur is quite a sharp spine with a smooth surface, and arranges compactly along the marginal area of the distal edge of tibia (Figure 1F). The average size of the spur is 40 μm, and the average diameter of the base is 10 μm. The total number of the spurs found in each tibia is approximately 50 (Figure 1G). A series of longitudinal ridges were observed on the surface of the spurs (Figure 1H).

Legs of the ladybug have been equipped with a characteristic locomotory adhesion device to move on both smooth and rough surfaces. They attach to rough substrates using their pretarsal claws; however, attachment on smooth surfaces is achieved by means of a tuft-like tarsal attachment pad. Although the two adhesive pads were morphologically distinct in shape and dimension, no significant differences were observed among the three pairs of feet. No additional apparatus or other types of adhesion devices were observed for walking on a smooth surface such as arolium or pulvilli (Figure 2A–C).

A pair of special heavy spines was found at the apical region in the ventral part of tibia where the first tarsomere and serrate tibial spurs are attached. In particular, these heavy spines were greatly expanded on the hind leg; however, no heavy spines were observed on the foreleg. The heavy spines are long and strong cuticular protrusions with smooth surface acuminating toward the tip. The average length is approximately 100 μm, in contrast to the length of the tibial spurs, which is 50 μm (Figure 2D–F).

On the basis of its morphological and fine structural characteristics, it has finally been revealed that the attachment pads in *H. axyridis* belong to the fibrillar type among the diverse adhesion systems of the insect foot. Each leg has two groups of attachment pads which are covered by numerous tiny tenent setae at the ventral surfaces of the first and second tarsomeres. The first tarsal segment is relatively longer and slender than second segment, and the first (proximal) and second (distal) tarsal adhesive pads are separated by

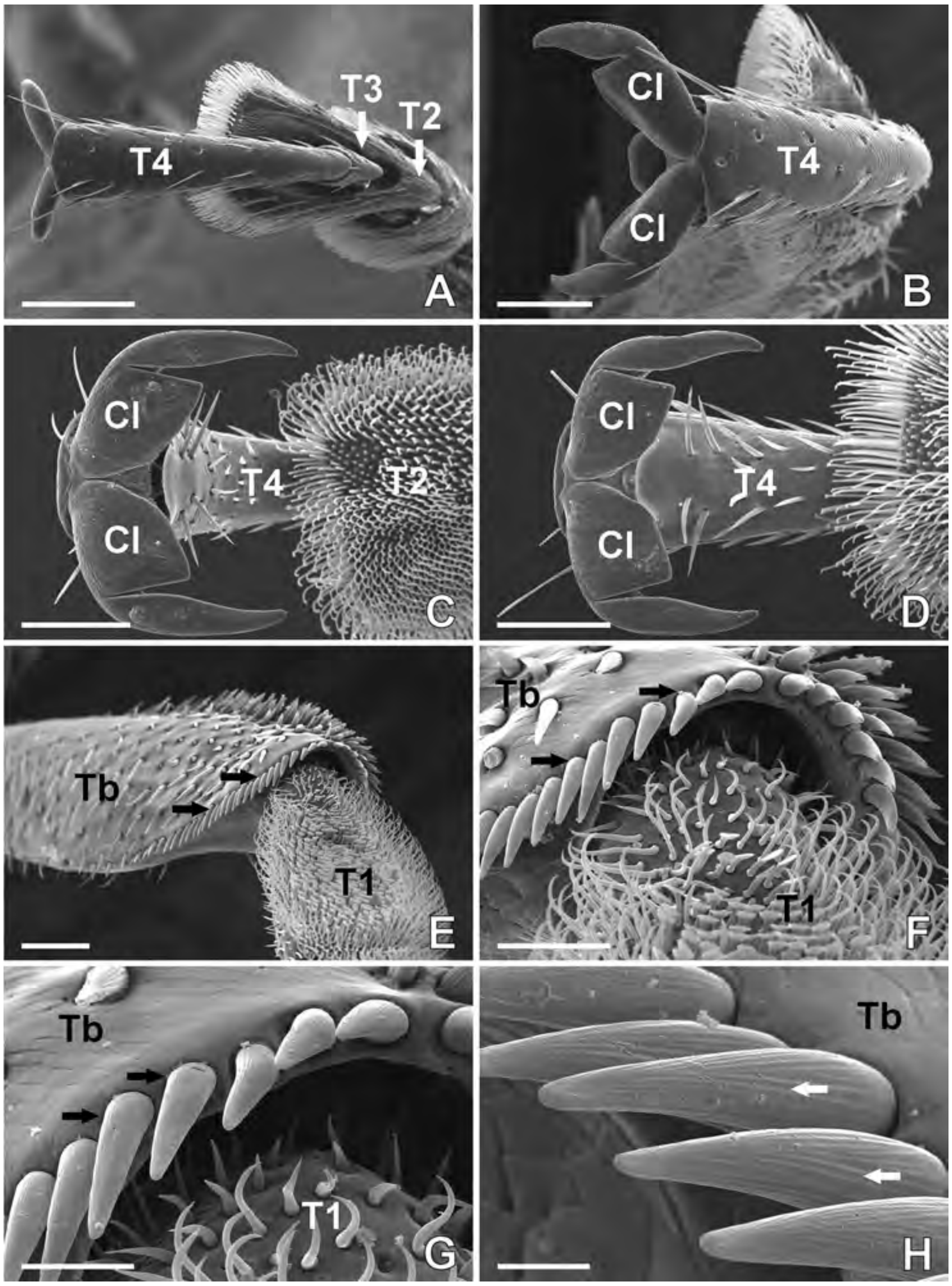

Figure 1. Scanning electron micrographs of the pretarsal claws and tibial spurs in the ladybug *Harmonia axyridis.* (A,B) The tarsus is composed of four tarsal segments and is equipped with a pair of terminal claws (Cl). (B) The claws on the end of the last tarsal segment (T4) are quite sharp and adapted to walk on coarse or rough surfaces. (C,D) The second tarsal segments (T2) are dilated and relatively broadened compared to the terminal segment with dense brush hairs. (E) The distal part of tibia (Tb) bears tibial spurs (black arrows) on the lower surface. (F) Each of the spur is quite a sharp spine with a smooth surface, and arranges compactly along the marginal area of the distal tibia. (G) The average length and base diameter of the spur is 40 and 10 μm. (H) A series of longitudinal ridges (white arrows) are observed on the surface of the spurs. T1, First tarsomere; T3, third tarsomere. Scale bar indicates 200 μm (A), 100 μm (B–E), 50 μm (F), 25 μm (G), and 10 μm (H), respectively.

membranous sclerites and are being articulated with one another by means of hinge joints (Figure 3A).

As the pad itself appeared to be a simple hairy structure under light microscopy, the FESEM examination showed dynamic microstructures of the fine fibrillar tenent setae. The biological attachment devices in *H. axyridis* consist of fine hairy setae with various contact sizes depending on their cuticular substructure. Basically, two different types of tenent setae were distinguished as follows: seta with a pointed tip, usually

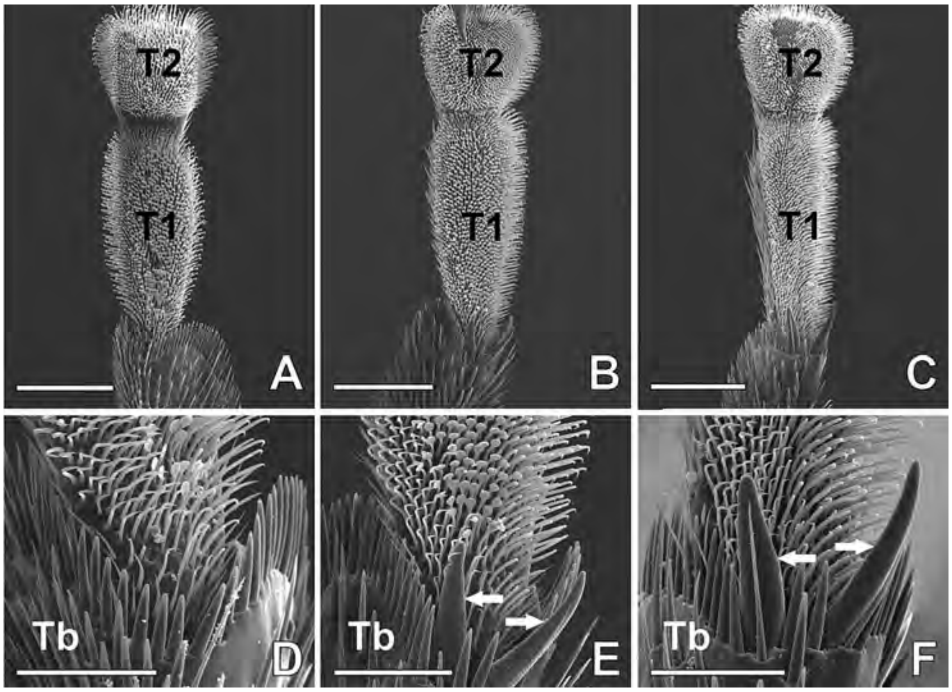

Figure 2. Scanning electron micrographs of the adhesive pads at the first (T1) and second tarsomeres (T2) and heavy spines (arrows) at the apical tibia (Tb). (A–C) Surface structure of each pad had some local variations in shape and dimensions resulting from the difference in the contact position of the feet with the substrates. (D–F) The heavy spines were greatly expanded on the hind leg; however, no heavy spines were observed on the foreleg. (A,D) Hairy adhesive pads at proximal and distal tarsomeres of the foreleg. (B,E) Pads of the mid-leg. (C,F) Hind leg pads. Scale bar indicates 250 μm (A–C) and 100 μm (D–F).

with a hooked endplate (pointed tip seta, PtS), and seta with a flat discoid tip, usually with a spoon-shaped endplate (discoid tip seta, DtS; Figure 3B).

Although two types of setae can be seen both proximal and distal pads in *H. axyridis*, the distribution pattern of each seta type is somewhat different. The PtS is a dominant type of seta distributed at the marginal regions of all tarsal pads. These setae are long and strong bristles acuminating toward the tip. Tips on the ventral side of the setae are bent toward their distal end, forming hooked endplates (Figure 4A,B). The PtS is broadly localized concentrically on the peripheral area of both the proximal and distal pads of all six feet, whereas these hairs are only observed at the restricted central area of the proximal pad of the hind leg (Table 1). The length of the PtS is mostly 30–40 μm (Figure 4C,D).

The DtS has the most distinct and conspicuous cuticular substructure, which represents the direct point of contact with the substrate. These setae are distributed at the central areas of the proximal pads of both the fore- and mid-legs. In addition, they are also found at the restricted area on the central region of the distal pad of the mid-leg; however, no DtS was found either proximal or distal pads of the hind leg (Table 1). As orientation of the DtS shows the direction-dependence pattern, most setae stand closer within horizontal rows but further apart in the perpendicular direction (Figure 5A,B). Average size of the seta stalk was mostly identical to that of PtS, and the average diameter of the discoid endplates was approximately 10 μm (Figure 5C,D).

The direct point of contact with the substrate was seen on the apex of this seta. There were some variations among setae according to their location on the pad. The ventral surface of the DtS endplate which distributed along the longitudinal axis of each tarsomere was perfectly discoid and either flat or concave (Figure 5E). There was some transition of the DtS which located peripheral region of the axis. The ventral surface was slightly expanded, and had fine grooves ran parallel to the longitudinal axis of the hair shaft (Figure 5F,G). The distal part of the seta shaft located just under the plate is thickened, but any specialized microstructures are not observed at their tips (Figure 5H).

Discussion

As feet are one of the primary contact points between an animal and its environment, they bear elaborate locomotory attachment devices, which have featured morphological adaptations required for survival of an

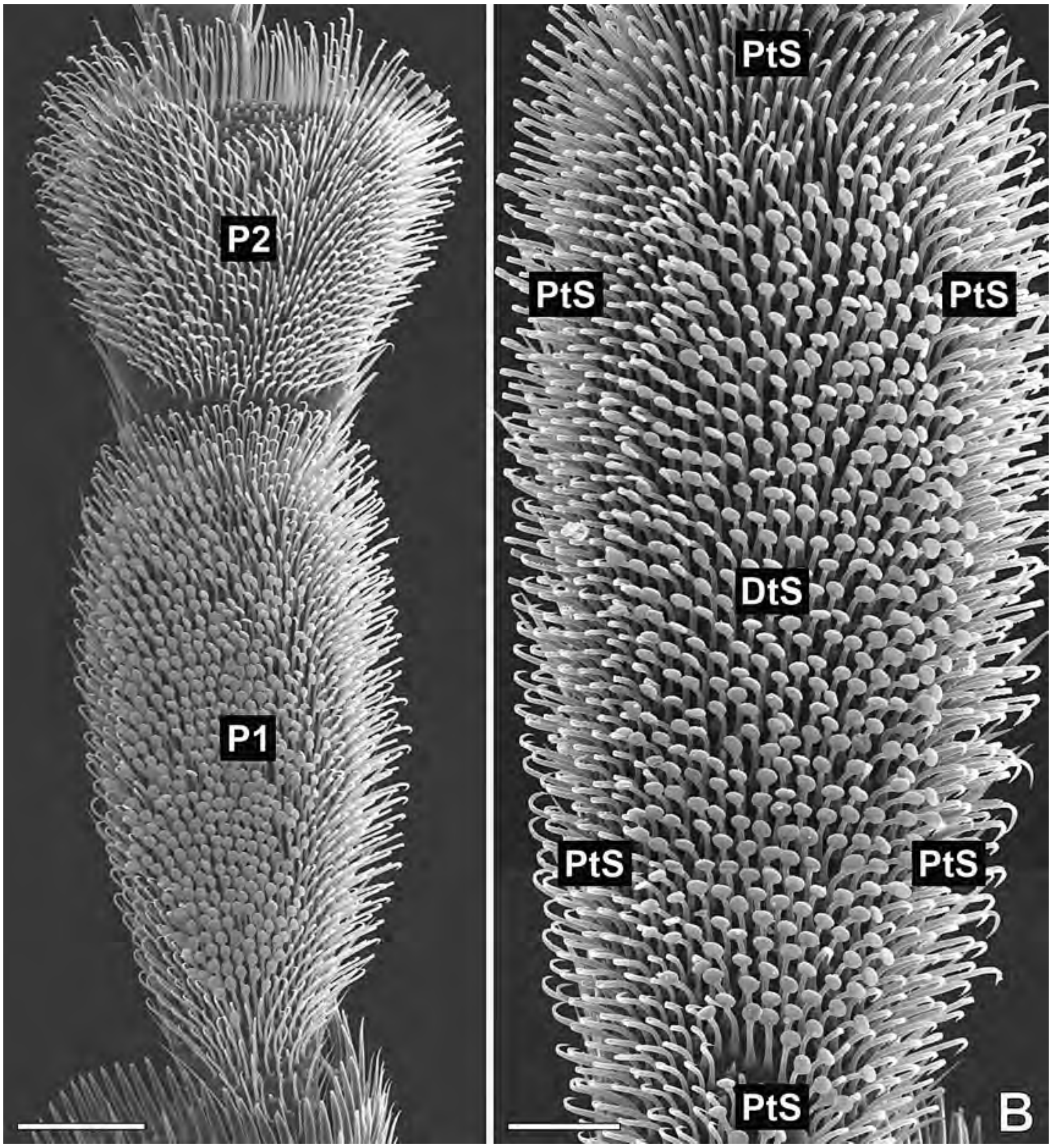

Figure 3. Scanning electron micrographs of the fine fibrillar tenent setae at the adhesive pads in *Harmonia axyridis*. (A) Each leg has two groups of attachment pads which are covered by numerous tiny tenent setae. The proximal pad (P_1) is relatively long and slender than distal pad (P_2), and they are separated by membranous sclerites. (B) All six feet were equipped with the hairy-type locomotory devices, and two different types of tenent setae were distinguished. The setae with pointed tips are distributed at peripheral region of pad, whereas the setae with flat discoid tips appeared at the central region. All scale bars indicate 50 μm.

animal in its natural habitat (Kesel et al. 2003; Autumn et al. 2006; Federle 2006). Since many functional solutions have evolved independently in different lineages (Irschick et al. 1996; Beutel and Gorb 2001), the evolutionary background influences the specific composition of attachment systems in each particular organism. Therefore, larger animals often minimize friction between the feet and substrate during locomotion, but smaller animals maximize friction for locomotion and attachment to smooth surfaces, and a variety of attachment devices have evolved accordingly (Kesel et al. 2003).

It has been known that a broad diversity of coupling and clamp like structures that ensure attachment to rough surfaces can be found (Stork 1983; Gorb 2008), and most insect locomotory systems bear pretarsal organs that provide mechanical interlocking on substrates (Dai et al. 2002). In this study, the locomotory attachment device of *H. axyridis* to move on rough surface was found on the end of the last tarsal

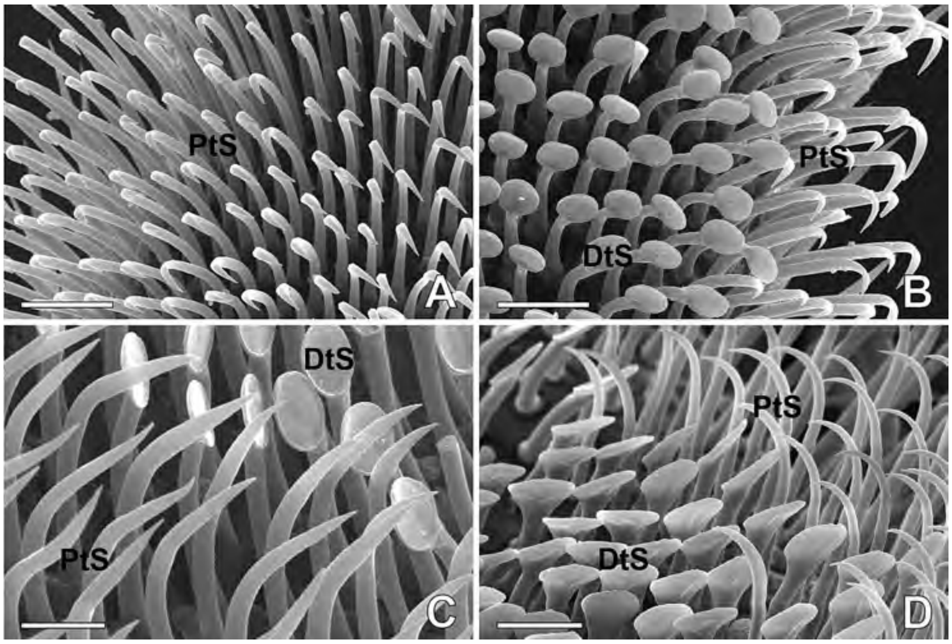

Figure 4. Scanning electron micrographs of the pointed tip setae (PtS) in *Harmonia axyridis*. (A) The PtS is the most dominant type of seta distributed at the marginal regions of all tarsal pads. (B) Tips on the ventral side of the setae are bent toward their distal end, forming hooked endplates. (C) This setae are long and strong bristles acuminating toward the tip with the length of 30–40 μm. (D) The PtS is broadly localized concentrically on the peripheral area of the adhesive pads of all six feet. DtS, discoid tip seta. Scale bar indicates 20 μm (A,B) and 10 μm (C,D).

segment of each leg. The pretarsal claws were quite sharp and flexibly articulated to walk on coarse or rough surfaces. Different hook-like attachment systems can be subdivided into two main functional groups depending on the time scale they are operating (Gorb 2008). The first type of these systems is mainly reported in parasitic animals, adapted for long-term attachment to surfaces of the host body (Gorb 2008). The tarsal claw is the most common example of the hook-like interlocking device used for short-term attachment during locomotion (Gorb 2008).

Although a pair of heavy tibial spines and serrate tibial spurs were found at the legs of this ladybug additionally, they are not homologous to claws, but serve a similar function. Following measurements of array stiffness and performance on rough surfaces of individual adhesive pads in dock beetle *Gastrophysa viridula*, Bullock and Federle (2009) showed that other parts of the tarsus and pretarsus may be used during free walking on a rough surface. Previous study has also shown that the pretarsal claws in all insects contribute to pulling (Dai et al. 2002), and distally oriented, stiff spines on the tibia, which regularly contact the ground, are probably used for pushing (Burrows 2006).

Other devices enable attachment to comparably smooth surfaces and usually involve microstructural modifications. They can be categorized into wet adhesion and dry adhesion systems. The former is present in many insects and in grasshoppers (Slifer 1950), flies (Walker et al. 1985), cockroaches (Frazier et al. 1999), and ants (Federle et al. 2002). On the other hand, dry adhesion is found in geckoes (Stork 1983; Autumn et al. 2006) and salticid spiders (Kesel et al. 2003).

The ultrastructure of the adhesive device has been investigated using SEM in several insect groups, such as Coleoptera (Betz 2003), Hymenoptera (Federle et al.

Table 1. Microstructural properties of the tarsal adhesive pads in *Harmonia axyridis.*

Leg	Pad	Region	Seta
Fore (1st)	Proximal	Central	DtS
		Peripheral	PtS
	Distal	Central	PtS
		Peripheral	PtS
Mid (2nd)	Proximal	Central	DtS
		Peripheral	PtS
	Distal	Central	DtS+PtS
		Peripheral	PtS
Hind (3rd)	Proximal	Central	PtS
		Peripheral	PtS
	Distal	Central	PtS
		Peripheral	PtS

DtS, discoid tip seta; PtS, pointed tip seta.

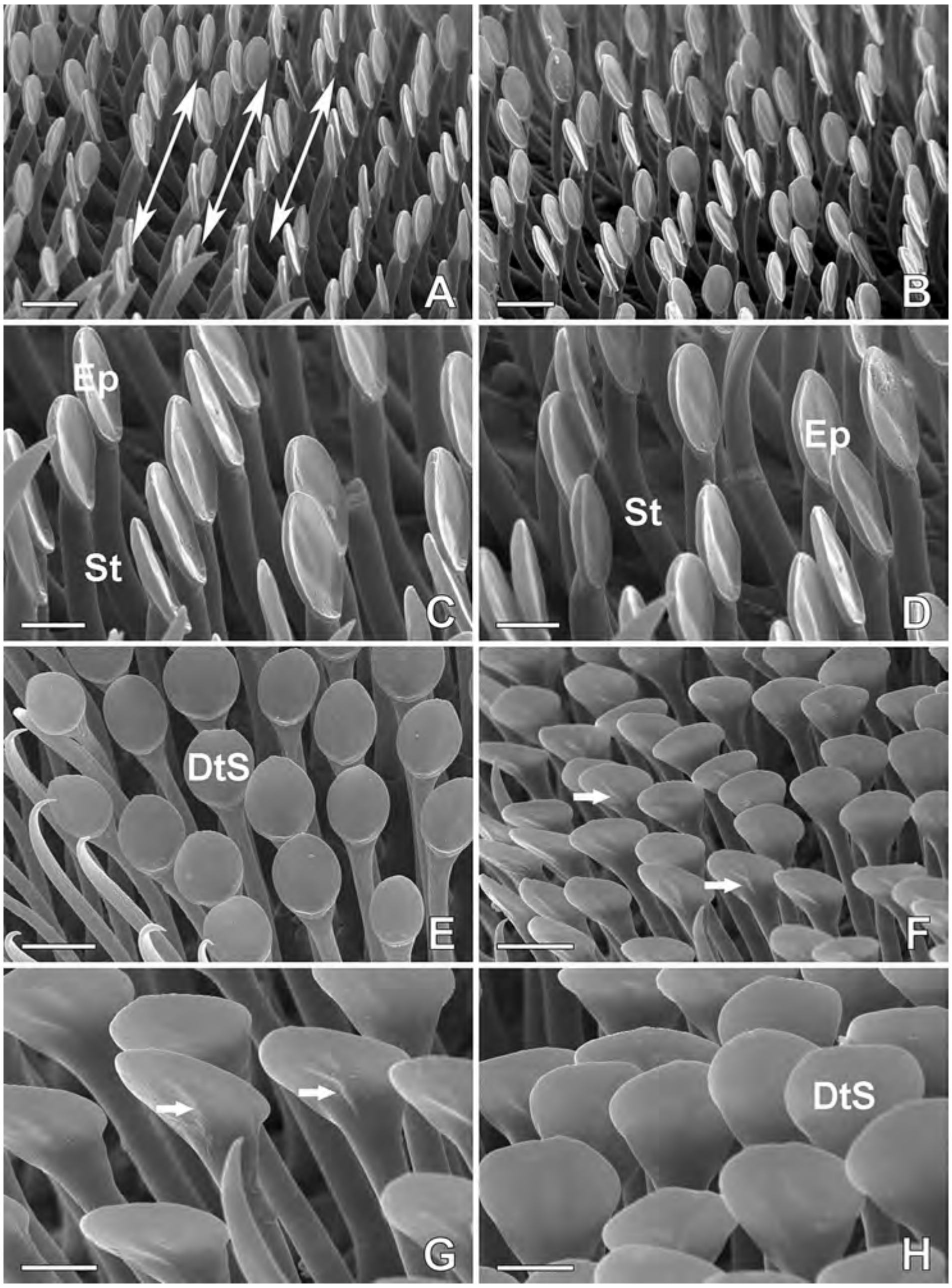

Figure 5. Scanning electron micrographs of the discoid tip setae in *Harmonia axyridis*. (A,B) As orientation of the discoid tip seta (DtS) shows the direction-dependence pattern (double arrows), setae stand closer within the horizontal rows but further apart in the direction perpendicular to it. (C,D) This type of seta is the most dominant type of setae and was composed of an elongated stalk (St) and a flat and discoid spoon-shaped endplate (Ep). (E) The surface of the DtS endplate was perfectly discoid, either flat or concave. (F,G) The direct point of contact with the substrate was seen on the apex of this seta. The ventral surface was slightly expanded, and had fine grooves (arrows) ran parallel to the longitudinal axis of the hair shaft. (H) The distal part of the seta shaft located just under the plate was thickened in some transitional DtS. Scale bar indicates 10 μm (A,B,E,F) and 5 μm (C,D,G,H).

2002), Diptera (McAlpine 1991; Gorb 1998; Niederegger et al. 2002), and Heteroptera (Gorb and Gorb 2004). These researches have demonstrated that insects have developed two distinct mechanisms to attach themselves to a variety of smooth substrates using either smooth or hairy pads (Beutel and Gorb 2001). Our FESEM investigation showed smooth surface attachment is achieved by means of a hairy device at the ventral surface of the first and second tarsal segments, and each individual pad was covered by numerous fine cuticular processes referred to as tenent setae. On the basis of a precise examination of its fine structural characteristics, this tarsal pad belongs to the hairy type of locomotory devices in insect feet.

Previous research has shown that the tarsus of *G. viridula* consists of five segments with pretarsal claws, and the proximal three tarsomeres bear the adhesive pads. The three adhesive pads of the same tarsus were not only composed of morphologically different types of setae, but also differed in their effective elastic modulus and adhesive performance (Bullock and Federle 2009). However, the tarsus of the ladybugs consisted of three tarsomeres and only the proximal two (first and second) tarsomeres bear ventral side adhesive pads. Nevertheless, the basic design of the tarsal adhesive pad tenent setae is surprisingly similar, because most setae commonly have tube-like elongated stalks and cuticular grooves at the ventral surface of their endplates regardless of their type except for some local variations in size and shape.

In the present study, a fine structural examination showed two remarkable features of the tenent setae: PtS and DtS. The DtS represent the prevalent design in ladybugs and exhibit similar properties to those of other ladybird beetle *Coccinella septempunctata* (Gorb et al. 2010) and *G. viridula* (Bullock and Federle 2009). This type of tenent setae was also described previously among flies with smooth adhesive pads, including the syrphid fly *Episyrphus balteatus* (Gorb 1998) and the blowfly *Calliphora vicina* (Niederegger et al. 2002); however, their fine structural characteristics were not exactly coincident with the ladybug hairy adhesive pads. The diameter of ladybug DtS was five times larger than those measured *E. balteatus*, and there was no openings under the endplate where secretions were released (Gorb 1998). It has been reported that the density of surface hairs increased with the body weight of the animal, and size variations of fibrillar structures on animal feet were ranging from a few hundred nanometers to a few micrometers, depending on the species (Gao and Yao 2004). Therefore, beetles had the largest size of tenent seta, and large-sized geckos had the highest density among all animal species that have been studied (Gorb 2008).

In addition, video recordings of walking beetles climbing upward and downward confirmed that proximal and distal pads are used differently and selectively during locomotion. Feet below the center of gravity primarily used their proximal pads, whereas tarsi placed above the body center of gravity mainly made contact using the distal pads. This suggests that the proximal pads were used when legs have to push laterally or away from the body, whereas distal pads were mainly used for pulling and adhesion (Bullock and Federle 2009). In *H. axyridis*, the DtS was distributed at the central areas of the proximal pads of both the fore- and mid-legs; however, no DtS were found either proximal or distal pads of the hind leg. In addition, orientation of the DtS showed the direction-dependence pattern, setae stood closer within horizontal rows but further apart in the direction perpendicular to it. Therefore, our findings also indicate that the fibrillar adhesive pads on the tarsal segments of *H. axyridis* have developed a direction-dependence of shear forces similar to that recently reported for the smooth attachment pads of dock beetles (Bullock and Federle 2009) as well as stick insects (Clemente et al. 2010).

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