The Evolution of Tarsal Adhesive Microstructures in Stick and Leaf Insects (Phasmatodea)

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Insects have developed specialized structures on their feet for adhering to surfaces, with stick and leaf insects or Phasmatodea exhibiting an unexpectedly high diversity of these structures. In Phasmatodea, attachment on different substrates is achieved by two types of pads on the legs: the euplantulae on the tarsomeres and the arolium on the pretarsus. The euplantulae are adhesive structures capable of adaptability to the substrate profile and generation of the required attachment strength. The diversity of euplantular microstructures of 56 species that represent all major lineages recognized within Phasmatodea and the whole biogeographical distribution of the group are examined using scanning electron microscopy (SEM). Nine different types of attachment structures can be distinguished whereby one, the nubby type, can be further divided into three different distinct types based on the specific ratio of each conical outgrowth. We mapped the morphological data from the SEM onto a phylogenetic tree we reconstructed based on molecular data. Previously, the evolution of different adhesive microstructures (AMs) on these pads has been suggested to reflect phylogenetic groups. However, different types of AMs are found within monophyletic groups, and our ancestral character state reconstruction suggests smooth euplantulae in the ground pattern of Euphasmatodea and multiple independent origins of other forms. The type of AM appears to be strongly associated with ecomorphs, e.g., smooth euplantular surfaces are more frequently found in tree-dwellers than in ground-dwellers, whilst the attachment pads of ground-dwelling species primarily bear conical cuticular outgrowths (nubby euplantulae).

Keywords: euplantulae, tarsal attachment, adhesive microstructures, phylogeny, cuticle, functional morphology

INTRODUCTION

The mesodiverse Phasmatodea are widespread, inhabiting nearly all temperate and tropical ecosystems worldwide (Günther, 1953; Bedford, 1978; Brock et al., 2017), but have only limited dispersal capabilities (Bradler et al., 2015). These exclusively phytophagous insects are well-camouflaged in their preferred habitats due to their masquerade imitating leaves or twigs (Bradler, 2009). While undergoing a fast radiation since the Cretaceous (Bradler et al., 2015) the
slowly migrating phasmds adapted to very local environmental settings. In a wide distributional range various ecological adaptations occurred independently in different lineages, causing different species inhabiting similar environments to display similar adaptations (e.g., Buckley et al., 2009; Dennis et al., 2015).

Since the wide distribution and occupancy of similar ecological niches has led to many convergences in Phasmatodea, many distinct morphological adaptations, such as specialized egg deposition modes, are more likely to form ecomorphological groups within this lineage than to reflect phylogenetic relationships (Buckley et al., 2009; Bradler et al., 2015; Goldberg et al., 2015). For instance, the exclusively ground dwelling tree lobsters (e.g., Droycoccelus, Eurycantha, Canachus) are shown to be polyphyletic, but adapted to specific environmental conditions, leading to many parallel evolutions. These formerly taxonomically associated species exhibit a very similar habitus, which is robust, dorsoventrally depressed and includes sturdy, armed legs. They dwell on the ground and all bury their eggs therein, usually with a secondary ovipositor (Buckley et al., 2009). Such a distinct ecological specification is attended by dissimilar substrata, species dwell on in their preferred habitats, and most probably includes adaptations of the attachment devices of these insects (Gottardo et al., 2015).

In order to adhere securely in the inhabited environment, phasmds possess specialized structures on their tarsi (Figure 1). Especially species living in the canopy depend on secure attachment to avoid potential damage from dropping to the ground (Schmitt et al., 2018). In addition to the pretarsal claws two main types of attachment pads are used in phasmds. The pretarsus is equipped with a pan-shaped toe pad (arolium) in every species. This particular attachment pad is suggested to create adhesion, if the tarsus is pulled from the surface (Labonte and Federle, 2013). On the proximal tarsomeres a small number of heel pads (euplantulae) are situated. Most species possess one euplantula on each of the four proximal tarsomeres enabling adjustment to the surface profile. In some species, a fifth euplantula is found on the distal tarsomere as well (Vallotto et al., 2016). In contrast to the arolium, the euplantulae generate large friction coefficients, when they are pressed onto the substrate due to shear forces, but create negligible adhesion (Busshardt et al., 2011, 2012; Labonte and Federle, 2013; Labonte et al., 2014).

Attachment in insects is achieved in general by adaption to the surface profile in order to maximize the actual contact area. This is accomplished either by flexible setose structures, or by soft cuticle layers (Gorb, 2001, 2005; Bennemann et al., 2014). Both principles appear in different groups of insects (Beutel and Gorb, 2001; Grohmann et al., 2015). Hairy systems, consisting of deformable adhesive setae, are common in spiders (Gorb et al., 2006), beetles (Stork, 1980; Gorb and Gorb, 2002; Bullock and Federle, 2011), earwigs (Haas and Gorb, 2004), and flies (Bauchhenss, 1979; Gorb, 1998; Niederegger et al., 2002; Friedemann et al., 2014). The other system consists of cuticular pads, which bear no prominent ornamentation, such as setae, but might bear different adhesive microstructures (Beutel and Gorb, 2008). This type is common in Phasmatodea (Büsch et al., 2017) and also found in Orthoptera (Gorb et al., 2000; Perez Goodwyn et al., 2006), Hymenoptera (Federle et al., 2001, 2002; Fransevich and Gorb, 2004), and Blattodea (Clemente and Federle, 2008).

For hexapods in general, the structural diversity of the attachment devices has been demonstrated to reflect phylogenetic relationships and, for the Phasmatodea and their controversially discussed sister groups, potential evolutionary scenarios were postulated based on these traits (Beutel and Gorb, 2001, 2006, 2008). The presence of smooth arolium and euplantulae, without macroscopic adhesive structures, has been discussed with respect to a phylogenetic placement of Phasmatodea as sister group to Mantophasmatodea, emphasizing the morphological similarity of adhesive systems in both groups (Beutel and Gorb, 2008). Nevertheless, recent morphological and molecular approaches support the sister group relation of Embioptera and Phasmatodea (Ishiwata et al., 2011; Friedemann et al., 2012; Letsch et al., 2012; Letsch and Simon, 2013; Misof et al., 2014). Although the attachment pads of stick insects appear to be smooth on a macroscopical level, the basal splitting of Timema and Euphasmatodea (the remaining Phasmatodea excluding Timema), is supported by the ultrastructure of the arolium, which is covered with pointed acanthae in Timema and entirely smooth in the latter. This sister group relationship of Timema and Euphasmatodea is considered well supported (e.g., Bradler, 2009). Beutel and Gorb (2008) distinguished the pointed acanthae on the euplantulae of Timema and the nubby adhesive microstructures (AM) of the euphasmatodeans Aretaon asperrimus and Neohirasea maerens. The nubs found in these two taxa were referred to as low aspect-ratio acanthae, but subsequent discussion regarding their structure used various different terms, including microtrichia (Gottardo and Heller, 2012) and bumps (Zill et al., 2014) without considering the cellular origin of these structures. Similar AM are found on the euplantulae of stick insects in a considerable diversity, as revealed in various taxonomic descriptions, biomechanic studies, and phylogenetic approaches. Five types of AM have been found so far: in addition to the pointed acanthae in Timema, nubs with two different aspect ratios are found in taxa from different lineages of euphasmatodeans. Carausius morosus possesses comparatively long nubs (Busshardt et al., 2012), while other stick insects from different lineages bear shorter nubs (Beutel and Gorb, 2008; Gottardo and Heller, 2012; Büsch et al., 2017).

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and Gorb, 2017). Some taxa possess entirely smooth euplantulae without any micromorphological ornamentation (Busshardt et al., 2012; Gottardo and Vallotto, 2014; Gottardo et al., 2015; Vallotto et al., 2016; Büscher and Gorb, 2017), and Dallaiaphasma eximius revealed a hexagonal or plateau pattern (Gottardo, 2011). Although the structural diversity of phasmatodean AMs is known (Büsch et al., 2018) and the phylogenetic clustering of similar AMs is supposed to partly reflect the evolution of Phasmatodea (Gottardo et al., 2015; Büscher and Gorb, 2017), there is no recent study approaching the phylogenetic relationships of the different AMs. We therefore examine the diversity of euplantular AMs within Phasmatodea in correlation with the phylogeny of the species examined.

MATERIALS AND METHODS

Morphology

The euplantulae of 56 adult female specimens were examined using SEM. These species represent the major lineages currently recognized in Phasmatodea (see Supplementary Data 1: Supplementary Table S1 for detailed information). Living specimens were bred in captivity until they reached adulthood. The insects were then anesthetized with CO₂ and then decapitated. The tarsi were cut off and fixated in a 2.5% solution of glutaraldehyde in PBS buffer and stored on ice on a shaker for 24 h. In the case, when no living specimens were available, dried specimens from collections were used. In order to restore the original condition of attachment pads, the cuticle has been softened. Therefore, the tarsi were removed and stored in a relaxing chamber for 24 h. Afterwards the tarsi were put into a 10% solution of lactic acid for 24–48 h until the attachment pads were restored into an adequate condition and then fixed in a 2.5% solution of glutaraldehyde in PBS buffer (Gladun and Gumovsky, 2006). Then specimens were stored in 70% ethanol, dehydrated in an ascending alcohol series and critical-point dried. Dried samples were mounted on aluminum stubs and sputter-coated with a 15 nm thick layer of gold-palladium. Samples were observed in the scanning electron microscope (SEM) Hitachi S4800 (Hitachi High-Technologies Corp., Tokyo, Japan) at 7 kV of acceleration voltage.

Phylogenetic Analysis

DNA has been extracted and sequenced for 14 stick insect specimens. We combined these data with previously sequenced taxa (Buckley et al., 2009; Goldberg et al., 2015). Sampled taxa were chosen with a focus on the different ecomorphs, covering the entire geographic distribution of Phasmatodea and all extant stick insect lineages. We amplified regions of the mitochondrial cytochrome c-oxidase subunit I (COI) and II (COII) genes, and the nuclear histone subunit 3 (H3) and ribosomal large subunit RNA gene (28S) using methods described previously (Buckley et al., 2009). DNA sequences were edited in Geneious R10 (Kearse et al., 2012). Alignments were made using Muscle (Edgar, 2004) as implemented in Geneious R10. The alignments were partitioned into four sets of characters; mitochondrial 1st and 2nd codon positions, mitochondrial 3rd codon positions, H3 gene, and 28S gene. Use of a partitioned model allows us to account for the typically different substitution patterns between different genes and codon positions, especially the increased rate at the third codon positions. Model selection using the AIC in JModelTest v.2.1.3 (Darriba et al., 2012) was then performed independently on each partition. Phylogenetic reconstruction was then performed using MrBayes v3.2.6 (Ronquist et al., 2012). For prior distributions we used a flat Dirichlet distribution (1,1,1,1) on “ratepr,” informative Dirichlet distribution (1,2,1,1,2,1) on “revmatpr,” beta distribution (1,1) on “tratiopri,” exponential distribution (1) on “shapepr,” and an unconstrained exponential distribution (1) on “brlens.” State frequencies were fixed at empirical values. We ran five independent analyses of 10 million generations, four chains, a sample frequency of 1000, and an MCMC temperature of 0.2. Output files were inspected in Tracer v1.6.0 (http://tree.bio.ed.ac.uk/software/tracer/) to ensure all effective sample sizes were >200 and MCMC chains were mixing appropriately. We also reconstructed phylogenetic relationships using maximum likelihood as implemented in Garli (Zwickl, 2006). We used the same partitioned substitution model as in the Bayesian analysis. The data were bootstrapped with 100 replicates and 1 search replicate per bootstrap. Ancestral character states were reconstructed using the Mkvc model (Lewis, 2001) as implemented in Mesquite v3.4 (Maddison and Maddison, 2018). Ancestral states were estimated on the topology and branch lengths from the Bayesian analysis. The alignment used for the phylogenetic reconstruction is provided as a Nexus input file as Supplementary Data Sheet 1.

RESULTS

Morphology

The tarsus of stick insects generally consists of five tarsomeres (Figure 1). The pretarsus is equipped with two claws and an arolium. Tarsomeres 1–4 bear distal euplantulae in all species studied. In some species, an accessory euplantula is found on the fifth tarsomere. The whole tarsal morphology reveals no intraspecific difference, neither between different specimens, nor in the micromorphological features within the arolium and euplantulae, nor between different legs of the same individual, nor between different euplantulae on the same tarsus. We found nine types of AM among the examined species. Adult females of exemplary species for the corresponding AMs are shown in Figure 2. Smooth euplantular surfaces are the most frequently observed forms (Figure 3A), followed by conical outgrowths of different aspect ratios (nubby, Figures 3F–H). Rare forms include flat pads (found in Necrostica annulipes, Figure 3B), plateaus (present in Epidares nolimetangere and Dajaca monilicornis, Figures 3C,L), coarse (found in Kalokorinnis and Oreophoetes, Figure 3D), maze (found in Leiothasma, Figure 3E), ridges (found in the Lanceocercata taxa Argosarchus horridus and Megacrania phelaus, Figure 3I), acanthae (only found in Timema, Figure 3J), Beutel and Gorb, 2008), and hairs (setae, present only in Dinaphasma saginatum, Figure 3K). The AMs of every of these species is illustrated and described in detail in Büscher et al. (2018).
Phylogenetic Relationships

The DNA sequence alignment consisted of 762 base pairs (bp), 695, 328, and 615 from the COI, COII, H3, and 28S genes respectively. The maximum likelihood relative substitution rates of mitochondrial 1st + 2nd codon positions, mitochondrial 3rd codon positions, H3 and 28S genes were 0.035, 4.732, 0.065, and 0.077 respectively. We observed strong support [Bayesian posterior probability (BPP) = 1] for several monophyletic groups that were also recovered in previous studies (Figure 4), including Necrosciinae, Phylliinae, Aschiphasmatinae, Pseudophasmatidae, Lanceocercata, and Anisacanthidae (Leiophasma + Parectatosoma). However, bootstrap (BS) values were somewhat lower with only Phylliinae, Aschiphasmatinae, and Anisacanthidae (Leiophasma + Parectatosoma) receiving 100% BS support. Clades not supported, but also monophyletic in accordance with previous studies (Bradler et al., 2014, 2015; Goldberg et al., 2015) are Clitumninae (BPP = 0.54, BS < 50%), Diapheromerinae (BPP = 0.53, BS = 72%), and Heteropteryginae (BPP = 0.74, BS < 50%). The three subgroups of the latter, Datamininae (Epidares + Pylaemenes + Orestes), Heteropteryginae (Haaniella + Heteropteryx), and Obriminae (Aretaon + Sungaya) are each highly supported with BPP
values of 1 and BS values of 98%, 100% and 100% respectively. Dataminae and Heteropteryginae are placed as sister taxa (BPP = 0.71, BS < 50%), which is in contrast to previous studies that suggest alternative topologies for these groups (Bradler et al., 2015; Goldberg et al., 2015). Further noteworthy results include a rather robust topology for Lanceocerata that largely corroborates earlier analyses, including a clade of taxa from New Caledonia (Canachus) + New Zealand (Argosarchus + Clitarchus) (BPP = 1, BS = 93%) and a sister group relationship between Dimorphodes and all remaining Lanceocerata, and with Cladomorphinae being sister clade to Stephanacridini (Macrophasma) + Lanceocerata (Buckley et al., 2009, 2010; Bradler et al., 2014, 2015). Necrosciinae also comprises Korinninae (Kalokorinnis) as suggested before (Goldberg et al., 2015) and Conlephasma, which has been discussed as being either related to Necrosciinae or Pseudophasmatidae (Gottardo and Heller, 2012) and is here unambiguously placed as sister group to Neohirasea + Phaenopharos (BPP = 1, BS = 92%) within Necrosciinae. A Malagasy clade consisting of Achrioptera + Anisacanthiade is well supported (BPP = 1, BS = 68%) as recovered before by Bradler et al. (2015). A further noteworthy result is the placement of the enigmatic African Bacillinae taxon Xylia as sister group to Aschiphasmatinae (BPP = 1, BS = 59%), which corroborates the finding of Buckley et al. (2009). One surprising result is our recovery of Phylliinae nested within Lonchodinae, albeit weakly supported. This is at odds with all previous phylogenetic analyses observing monophyletic Lonchodinae (Buckley et al., 2009; Bradler et al., 2014, 2015; Goldberg et al., 2015), and we attribute this result to a shortage of taxon sampling within these two subfamilies. The deeper nodes, e.g., the radiation of major phasmatodean lineages and enigmatic longstanding taxa like Agathemera, Bacillus, and Bactrododema, are poorly resolved and largely unsupported, which is a common observation for phylogenetic studies of Phasmatodea (Buckley et al., 2009; Bradler et al., 2014, 2015; Goldberg et al., 2015).

**DISCUSSION**

**Evolution of Attachment Structures**

The recovered phylogeny was largely consistent with current phylogenetic hypotheses on stick and leaf insects with the exception of the placement of Phylliinae within Lonchodinae, which is not well supported and probably an artifact due to the limited taxon sampling of the present study. The poorly resolved
radiation among the major phasmatodean lineages impedes a reliable ancestral character state reconstruction of euplantular adhesive structures for stick and leaf insects at the moment. Furthermore, the condition for this character complex in *Timema* (acanthae) is unlike in any of the investigated members of its sister group Euphasmatodea, which consequently does not allow...
for an outgroup comparison. We are confident that one of the most frequent forms, smooth and nubby, which are also present in closely related groups like Orthoptera, Blattodea, and Embioptera (Gorb et al., 2000; Perez Goodwyn et al., 2006; Clemente and Federle, 2008; Büscher et al., 2018), represents the ground pattern of AM in Euphasmatodea, and indeed the ancestral state reconstructions favor smooth (Figure 5). We also conclude that the rare AM types are derived for the respective taxa, e.g., coarse for Kalokorinnis and Oreophoetes, plateaus for Epidares, maze for Leioaphasma, and hairs (adhesive setae) for Dinophasma, and this is supported by the ancestral state reconstructions. Moreover, the euplanular structure ridges evolved twice within Lanceocercata, in Arcosarchus and Megacrania. Our reconstructions support independent origins of the nubby types (Figure 5). In general, unambiguously monophyletic groups exhibit a diversity of structures, e.g., in Aschiphasmatinae the smooth (Abroisma), hairy (Dinophasma), and plateaus (Dajaca) forms are found. While some closely related taxa possess the same euplanular types, such as nubby AMs in Neohirasea, Phaenophasaros, and Conlephasma in Necrosciinae, or smooth AMs in Anchialae, Eurycnema, and Tropidoderus in Lanceocercata, the occurrence of specific AMs do not generally reflect phylogenetic relationships. Besides Clitumninae and Phyllinae, none of the well-supported major clades bears a uniform AM type. The type of AM appears to be associated with ecomorphs, e.g., smooth euplanular surfaces are more frequently found in slender tree-dwellers than in stout ground-dwellers, whilst the attachment pads of ground-dwelling species more often bear conical cuticular outgrowths with different aspect ratios. However, this does not entirely explain the distribution of AMs across phasmid taxa. For instance, Heteropterygidae are uniformly ground-dwelling phasmsids, including egg-deposition into the soil, yet they exhibit varying types of adhesive structures: In Obriminae (Aretazon, Sungaya), the euplanular surface is nubby, whereas smooth euplanulae are found in Heteropteryginae (Heteropteryx, Haaniella). Yet, characterization of distinct ecomorphs still needs to be addressed based on substantiated criteria such as morphometrics and niche specialization. One further potential explanation of the presence of certain AMs is body size. Bigger species might exhibit smooth euplanulae more frequently, e.g., the large Bactrododema, Eurycnema, Achrioptera, and Eurycantha. Assumption of this potential trend gains further support by the fact that the smaller juvenile Eurycantha individuals still have nubby euplanulae while those of adults are smooth (Gottardo et al., 2015). Heteropteryginae bearing smooth euplanulae are generally bigger than Obriminae with nubby euplanulae. Then again, the ground-dwelling Oreutes and Epidares are of the same small size and still possess different AMs.

Functional Relevance of Attachment Microstructures

The functional properties of the different AMs in Phasmatodea are only partially known so far. Experimental data are available for Phasmatodea only for the nubby and the smooth type (Busshardt et al., 2012; Labonte and Federle, 2013; Labonte et al., 2014), hence, the other remain hypothetical or are inferred from other taxa. The nubby AM is shown to be load sensitive and less susceptible to different surface roughnesses (Busshardt et al., 2012; Labonte and Federle, 2013; Labonte et al., 2014). Nubby attachment microstructures, if they are not the ground type of AMs, possibly evolved convergently in the different lineages as a response to a broad range of surfaces in the preferred habitats. In contrast, smooth AMs perform better on smooth surfaces (Busshardt et al., 2012). Many species possessing this AM live in trees and often are food plant specialists. These species possibly face rather smooth surfaces, whilst species with nubby structures possibly find a broader range of surface roughnesses on the ground. Other types of AMs might have evolved due to insect-plant interactions (e.g., Friedemann et al., 2015) or specific environmental conditions, e.g., specialized structured surfaces or wet surfaces (Grohmann et al., 2015). On wet surfaces, splitting the contact surface like in the flat pads, plateaus, and maze AMs possibly reduces hydroplaning and stick-slip motions. This effect has been shown for mushroom shaped and hexagonal bioinspired artificial surfaces (Värenberg and Gorb, 2007, 2009). Irregularly shaped microstructures, such as ridges, might have evolved due to food plant specialization. The anisotropy of the AM causes dissimilar adhesive forces with and against the structures (Filippov and Gorb, 2013) and might be used for generating propulsion on structured surfaces (Clemente et al., 2009). Megacrania phelaus feeds exclusively on plants with a parallel leaf venation (Hsiung, 2007). This food plant association possibly initiated the development of the structured AM in this species. The hairy system of attachment pads, herein only represented by D. saginatum, is reported to possess similar adhesion and friction forces to the smooth system (Bullock et al., 2008). The comparison of the adhesive pads of C. morosus and the dock beetle Gastrophysa viridula (Coleoptera: Chrysomelidae) suggested that fibrillar adhesive systems may be more efficient in terms of self-cleaning than smooth ones (Clemente et al., 2010).

CONCLUSION

Stick and leaf insects have more diverse euplanular microstructures than previously reported (Beutel and Gorb, 2008). Nine different types can be distinguished whereby the nubby type can be further divided into three different distinct types based on the specific ratio of each conical outgrowth. Hereby the different types of AMs do not follow a phylogenetic pattern, but rather depend on the ecological niche a species inhabits or its body size. Large canopy-dwellers more frequently appear to exhibit smooth euplanulae while smaller ground-dwellers apparently show nubby AMs. The morphological diversity found in each clade of phasmsids suggests the convergent evolution or reversal of certain euplanular types. A high number of species is already illustrated and described in a comprehensive comparative analysis of the tarsal morphology in Phasmatodea (Büscher et al., 2018). To reliably reconstruct the evolution of these adhesive types, a denser taxon sampling and a better resolved phylogeny of these taxa are necessary.
FIGURE 5 | Phasmatodea phylogeny and ancestral character state reconstruction of euplantular microstructures with pie charts representing relative ML support at ancestral nodes according to color code in figure legend.
DATA AVAILABILITY STATEMENT

All datasets analyzed for this study are included in the manuscript and the Supplementary Material. The DNA sequences used for the phylogenetic reconstruction can be found at GenBank [https://www.ncbi.nlm.nih.gov/genbank/] under the accession numbers listed in Supplementary Table 1. The alignment used for the phylogenetic reconstruction is provided as a Nexus input file as Supplementary Data Sheet 1.

AUTHOR CONTRIBUTIONS

THB, CG, SG, and SB designed research. THB generated SEM data and photographs. TRB obtained and analyzed molecular data. THB and SB wrote manuscript. TRB, CG, and SG contributed to manuscript editing. All authors have approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2018.00069/full#supplementary-material

Supplementary Table 1 | Taxon sampling.

Supplementary Data Sheet | Nexus input file.
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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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