Plant lock and ant key: pairwise coevolution of an exclusion filter in an ant–plant mutualism

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Although observations suggest pairwise coevolution in specific ant–plant symbioses, coevolutionary processes have rarely been demonstrated. We report on, what is to the authors' knowledge, the strongest evidence yet for reciprocal adaptation of morphological characters in a species-specific ant–plant mutualism. The plant character is the prostoma, which is a small unligified organ at the apex of the domatia in which symbiotic ants excavate an entrance hole. Each myrmecophyte in the genus *Leonardoxa* has evolved a prostoma with a different shape. By performing precise measurements on the prostomata of three related myrmecophytes, on their specific associated ants and on the entrance holes excavated by symbiotic ants at the prostomata, we showed that correspondence of the plant and ant traits forms a morphological and behavioural filter. We have strong evidence for coevolution between the dimensions and shape of the symbiotic ants and the prostoma in one of the three ant–*Leonardoxa* associations.

Keywords: ant–plant interaction; coevolution; mutualism; myrmecophytism; prostoma

1. INTRODUCTION

Tropical ant–plant symbioses are often considered as classical examples of coevolved mutualisms. However, strong pairwise coevolution appears to be rare in these systems (Davidson & McKey 1993a,b). Species specificity is usually limited (e.g. Ward 1993; Yu & Davidson 1997), although exceptions occur (Yu & Davidson 1997; Fiala *et al.* 1999). Guilds of ants often interact with guilds of plants and each guild may be taxonomically diverse (Davidson & McKey 1993a,b). Taxonomic patterns (Longino 1989) and phylogenetic analyses (Ward 1991, 1993; Ayala *et al.* 1996; Chenuil & McKey 1996) suggest numerous cases of *de novo* colonization and host shifting rather than long associations between lineages. Even in cases where a number of congeneric ant species are associated with a number of congeneric plants, close examination has revealed that, in both the ant and plant lineages, symbiotic associations have independently arisen two or more times (e.g. Ward 1993; Ayala *et al.* 1996; Fiala *et al.* 1999). 'Currently there is no known case of long-term, parallel cladogenesis of ants and plants' (Ayala *et al.* 1996, p. 428). Such observations have led to the view that ongoing coevolution in these systems is rare or that coevolution is 'diffuse', thereby limiting the potential for strong pairwise evolution of morphological characters. When the characters of a plant and its associated ants do appear to be coadapted, comparative analysis suggests that this is often due to 'ecological fitting' (Janzen 1985) of pre-adapted partners (Davidson & McKey 1993a,b; Yu & Davidson 1997). Similar ecological processes shape the patterns of association, with ant species in competitive hierarchies being sorted out along ecological gradients (e.g. in resource supply rates), producing ant–plant associations that are predictable if not completely specific (Davidson *et al.* 1991).

However, some ant–plant symbioses are species specific, at least in part of the geographic range of one of the partners (McKey 1984, 1991; Chenuil & McKey 1996; Yu & Davidson 1997; Fiala *et al.* 1999). Despite some suggestive observations (Yu & Davidson 1997), no clear evidence demonstrates the reciprocal evolution of characters in such systems. We report here on, what is to the authors' knowledge, the strongest evidence yet for reciprocal adaptation of morphological characters in a species-specific ant–plant mutualism.

Our study concerns three myrmecophytic taxa of *Leonardoxa* (Leguminosae; Caesalpinioideae) and their specific ant associates. The plant trait we studied is the prostoma of *Leonardoxa*, which is a highly localized small spot on the swollen twigs, which are specialized as myrmecodomatia (figure 1). The prostoma remains unligified as the twig matures, thereby facilitating entry into the domatium by symbiotic ants that excavate an entrance hole at the prostoma. A true prostoma appears to have been reported in only two genera of myrmecophytes, namely *Cecropia* (Cecropiaceae) (Müller 1880; Schimper 1888; Bailey 1922; Janzen 1973; Yu & Davidson 1997) and *Leonardoxa* (McKey 1991, 2000). However, comparable structures, namely self-opening slits, occur in diverse myrmecophytes with caulinary domatia (table 1). (The narrow entrances of leaf-pouch domatia (Davidson & McKey 1993a) are closely analogous, but these anatomically very different traits are not discussed here.) Several Asian myrmecophytes with self-opening slits are occupied by a diversity of ants, leading Maschwitz *et al.* (1994) to regard them as more open to interlopers than plants with a true prostoma or plants that have specific ant associates (e.g. the host plants of *Cladomyrma* spp. (Moog *et al.* 1998) or *Macaranga* associated with *Crematogaster* (Fiala & Maschwitz 1992)) that are capable of drilling into woody internodes lacking any such specializations. Federle *et al.* (2001) have recently described in stems of some myrmecophytic *Macaranga* a prostoma that has the

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Figure 1. Myrmecodomatium and prostoma of a myrmecophytic subspecies of *L. africana*. The arrow indicates the prostoma, in which symbiotic ants excavate an entrance hole.

form of a straight line running the entire length of the internode, and that also functions quite differently from the structures described here. It guides ants already inside hollow stems to places where they can easily chew supplementary entrance holes, but is undetectable to ants trying to enter the domatia from outside. In contrast to self-opening slits, which could be morphological exclusion filters that prevent the entrance of large insects into the domatia, true prostomata could also be behavioural exclusion filters that keep out opportunistic twig-nesting ants that lack the behaviour of finding the prostoma and digging an entrance hole. However, some ant plants with self-opening slits are specifically associated with one or a few ants. The internodes of *Triplaris americana* (Polygonaceae) have a very narrow self-opening slit and its specific associate *Pseudomyrmex triplarinus* bores its round entrance hole at the slit (Schremmer 1984).

Repeated independent evolution of a prostoma or self-opening slit indicates selection pressures favouring such structures. What adaptive advantages are conveyed by the presence of a prostoma? First, it facilitates the entry of mutualistic ants that recognize it and can drill their entrance holes at this spot. Facilitating quick entry may be particularly important in reducing the risks of predation or parasitism on founding queens (Müller 1880; Yu & Davidson 1997) or in mature colonies. The presence of a prostoma may also greatly extend the window in time that is available for colonization of a domatium. Even when the internode is fully mature and lignified, adapted ants can still enter through the prostoma. This may increase the probability of colonization of a plant by a

founding queen. Later, in established colonies, workers may be able to delay entry into a newly matured domatium, thereby minimizing any disruption of growth that is occasioned by the drilling of an entrance hole. Finally, by simultaneously facilitating the entry of some ants and rendering entry difficult for others, the prostoma (and, perhaps, a self-opening slit) may serve as a selective morphological filter that helps exclude interlopers (McKey 1984, 1991; Yu & Davidson 1997). This last presumed function may be of great importance in maintaining ant-plant mutualisms. The abundance of the resources produced for ants (food and nest sites) in some myrmecophytes is likely to attract interlopers, which could invade the population (Thompson 1994) in the absence of plant structures that favour mutualistic ants.

Despite their presumed importance in ant-plant mutualisms, prostomata and related structures have only been described in general terms. The only detailed studies were made on the prostomata of some *Cecropia* species in the late nineteenth and early twentieth centuries (Müller 1880; Schimper 1888; Bailey 1922). The histological studies of these authors permitted a precise description of the prostoma as a 'diaphragm devoid of hard and tough elements' (Bailey 1922, p. 383).

These early authors varied in their interpretation of the evolutionary significance of the prostoma. Müller's (1880) interpretation was quite subtle. He felt that the origin of the prostoma could be traced to a feature of anatomical organization that is widespread in plants. However, he considered the prostoma itself as an adaptation that is shaped by natural selection in order to facilitate entry by the plant's mutualistic ants. Schimper (1888) extended Müller's (1880) observations and concurred with his interpretation of the prostoma as an adaptation to mutualism with ants. His study includes a precise description and drawing of a cross-section of the prostoma of a *Cecropia* species. The views of Müller (1880) and Schimper (1888) would fit comfortably today in concepts such as 'pre-aptation', 'exaptation' and 'selection following the path of least resistance'.

Bailey (1922, 1924), whose work on the prostoma and similar structures was the most extensive of any author, also saw the prostoma as one expression of a widespread feature of plants. He pointed out that vascular tissues are concentrated on the side of the leaf insertion in the apical part of the newly matured internodes of many plants. Thus, prior to secondary growth, the apex of the internode is thinner and less lignified on the side of the stem opposite the leaf insertion or on sides between leaf insertions in the case of opposite-leaved plants. However, while Bailey (1922) proposed a clear explanation for the origin of the prostoma and for its common localization on all myrmecophytes with prostomata or self-opening slits, his stance was anti-adaptationist: the ant associates of myrmecophytes simply exploited a widespread feature of plants, which was in no way adapted to ants. Bailey's (1922, 1924) opinion carried the day for several decades and, although myrmecophytic symbioses have been the subject of renewed interest since the 1960s, there has been no re-examination of the questions left open by Müller's (1880) and Bailey's (1922) divergent treatments of the prostoma. In particular, no quantitative comparative studies have been conducted on prostomata.

Table 1. Myrmecophytic species having a prostoma (P) or a self-opening slit (S) on their caulinary domatia.

family	taxon	symbiotic ants	structure types	references
Capparaceae	<i>Capparis buwaldae</i> Jacobs	?	S	Maschwitz <i>et al.</i> (1996)
Euphorbiaceae	<i>Macaranga caladiifolia</i> Becc.	<i>Crematogaster</i> / <i>Camponotus</i>	S	Fiala <i>et al.</i> (1996)
Leguminosae	<i>Leonardoxa africana</i> (Baill.) Aubrév. subspecies <i>letouzeyi</i> McKey	<i>A. afer</i>	P	McKey (1991, 2000)
	<i>L. africana</i> (Baill.) Aubrév. subspecies <i>africana</i>	<i>P. phylax</i>	P	McKey (1984, 1991, 2000)
	<i>L. africana</i> (Baill.) Aubrév. subspecies <i>rumpiensis</i> McKey	diverse	P	Chenuil & McKey (1996) and McKey (2000)
	<i>Humboldtia laurifolia</i> Vahl	diverse	S	Bower (1887) and Krombein <i>et al.</i> (1999)
	<i>Humboldtia brunonis</i> Wall.	diverse	S	D. McKey (personal observation) and L. Gaume (personal communication)
Cecropiaceae	<i>Cecropia</i> spp.	<i>Azteca</i> / <i>Pachycondyla</i> <i>luteola</i> / <i>Camponotus</i>	P	Müller (1880), Schimper (1888), Bailey (1922), Janzen (1973), Longino (1989) and Davidson <i>et al.</i> (1991)
Moraceae	<i>Ficus obscura</i> var. <i>borneensis</i> (Miq.) Corner	diverse	S	Maschwitz <i>et al.</i> (1994)
Polygonaceae	<i>Triplaris americana</i> L.	<i>P. triplarinus</i>	S	Schremmer (1984) and Benson (1985)
	<i>Triplaris caracasana</i> Chamisso	<i>Pseudomyrmex</i> sp.	S? and P?	Schimper (1888)
Rubiaceae	<i>Duroia hirsuta</i> Pöppig & Endlicher	?	S	Wheeler (1942)
Rubiaceae	<i>Myrmeconuclea strigosa</i> (Korth.) Merrill	diverse	S	Maschwitz <i>et al.</i> (1989) and C. Brouat (personal observation)
Rutaceae	<i>Zanthoxylum myriacanthum</i> Wall. ex Hook. F.	diverse	S	Maschwitz <i>et al.</i> (1992)
Verbenaceae	<i>Clerodendrum fistulosum</i> Becc.	diverse	S	Maschwitz <i>et al.</i> (1994)

Qualitative observations on *Leonardoxa* (McKey 1984, 1991, 2000) have suggested that the sizes and shapes of prostomata may be different among *Leonardoxa* taxa with different ant associations. The prostoma on almost mature internodes is different in colour (pale red) from the rest of the domatium (pale green). The size and shape of the pale red spot marking the prostoma varies among populations. Furthermore, the prostomata on mature internodes of greenhouse-grown plants that had never been opened by ants spontaneously dried up and produced a recessed scar. These structures also varied in size and shape (McKey 2000). We thus decided to investigate this character in detail. We first define the prostoma in anatomical/histological terms. We show that this structure can be measured with precision in *Leonardoxa*. We then address the following questions. Do the dimensions of the prostoma differ in taxa associated with different ants? Is the variability in prostoma dimensions related to the diversity of ants associated with the plant taxon? Is there a match between the size and shape of the prostoma in a taxon and the characters of mutualistic ants, both (i) behavioural (the size and shape of the entrance holes they drill) and (ii) morphological (the size and shape of the ants themselves)? If matches occur, have they been produced by pairwise coevolution?

2. MATERIAL AND METHODS

Leonardoxa africana (Baill.) Aubrév. (Leguminosae: Caesalpinioideae) is a polytypic species complex of four allopatric subspecies of rainforest understory tree that ranges from extreme southeastern Nigeria through the coastal forests of Cameroon into Equatorial Guinea and northern Gabon (McKey 2000). Three of these subspecies are myrmecophytic, with swollen, hollow internodes that are occupied by ants. The apex of each newly produced ant domatium develops a prostoma in all three, in which symbiotic ants open a hole in order to enter into the domatium. The three myrmecophytes differ in their diversity of associated ants. Each tree of the subspecies *rumpiensis* McKey (= '*L. africana* T2') (Chenuil & McKey 1996) is occupied by a colony of one of *ca.* 15 different species of myrmicine and dolichoderine ants (D. McKey and R. Snelling, unpublished data). Juvenile trees in populations of the subspecies *letouzeyi* McKey (= '*L. africana* T3') (Chenuil & McKey 1996; Gaume & McKey 1998; Gaume *et al.* 1998) may be occupied by any one of *ca.* 10 ant species, whereas mature trees are exclusively occupied by the formicine *Aphomyrmex afer* Emery. This ant has at least one other host, namely *Vitex grandifolia* (Verbenaceae), but *Leonardoxa* is its principal host in our study areas. *Aphomyrmex* tends homopteran trophobionts in the domatia of its hosts, and foliar nectaries of *Leonardoxa africana letouzeyi* are

few and small (Gaume *et al.* 1998). The trees of *Leonardoxa africana africana* McKey (= '*L. africana* T4') (Chenuil & McKey 1996) are associated with the formicine *Petalomyrmex phylax* Snelling, which is a host-specific protective mutualist of this plant (McKey 1984; Gaume *et al.* 1997). Morphologically, this subspecies is the most specialized as a myrmecophyte, with a very early onset of domatia in ontogeny and with numerous large foliar nectaries. The abundant nectar they produce is the principal or sole food source for symbiotic ants but, despite the presence of this attractive and seemingly promiscuous resource, only one other ant has colonized this host, the myrmicine *Catantolus mckeyi* Snelling. This ant, which is also host specific, is a parasite of the mutualism in most populations in the sense that it occupies some trees, thereby exploiting the food and nest sites they produce while providing no protection to the plant and preventing or slowing the establishment of *Petalomyrmex* in these trees (McKey 1984; Gaume & McKey 1999).

The samples used for measurements of prostomata, entrance holes and ants themselves were collected from three sites in Cameroon. Domatia of *L. a. letouzeyi* and its mutualistic ants (*A. afer*) were collected in Korup National Park (5°00' N, 8°45' E), Southwest Province. Material of *L. a. africana* and its ant associates (*P. phylax* and *C. mckeyi*) was collected near Ebodié (2°35' N, 9°50' E), Southern Province. Domatia of *Leonardoxa africana rumpiensis* were collected near Dikome Balue (4°55' N, 9°15' E), Southwest Province. The diverse ant associates of this population were not measured. All plant material was preserved in formalin-acetic acid-alcohol and ants were preserved in ethanol. The samples were obtained from mature trees, but there is no indication that prostoma size or shape change over plant ontogeny.

We collected internodes that had recently completed their expansion and were beginning to lignify but had not yet been opened by ants for measurements of prostomata. In this study, our sample included 5–11 prostomata (each from a different individual tree) for each of the three populations. We collected young actively occupied domatia for measurements of ant entrance holes in order to minimize any variation due to partial closure of the holes (callus growth closes them if they are not maintained by ants). We measured entrance holes on 4–6 domatia on each of five trees occupied by different ant species for *L. a. rumpiensis*. Our sample for *L. a. letouzeyi* included entrance holes on 3–5 domatia on each of three trees occupied by *A. afer*. We measured entrance holes on five domatia on each of six trees occupied by *P. phylax* for *L. a. africana* and on each of six trees occupied by *C. mckeyi*. The ants used for measurements were taken from colonies collected in Korup (*A. afer*) or Ebodié (*P. phylax* and *C. mckeyi*). The dimensions of the largest size class of individuals should determine the dimensions of the filter. We thus used five alate females per species, each belonging to a different colony, for measurements. Alate females must be able to leave domatia (dispersal flight) and enter them (at colony foundation).

The domatia were first hydrated then frozen. Serial cross-sections, each of 50 µm thickness, were performed using a cryotome (Jung Frigocut 2800 E; Leica Microsystems, Nussloch, Germany). The sections were studied by light microscopy using either the autofluorescence of lignin observed under UV light or a lignin-specific red coloration (5 min in a 2% solution of phloroglucinol in absolute ethanol, then 1 min in 50% HCl). We performed serial cross-sections from the base to the apex of the entire internode for *ca.* 20 domatia from each population. Observations of these sections allowed us to define the histological

distinction between the prostoma and the remainder of the domatium.

Transverse sections at the prostoma were very fragile and frequent tearing precluded complete measurements on numerous domatia. We were able to obtain precise measurements of five prostomata of *L. a. letouzeyi*, nine prostomata of *L. a. africana* and 11 prostomata of *L. a. rumpiensis*. The length of each prostoma was calculated by multiplying the number of sections in which the prostoma was present by the thickness of each section (50 µm). The width of each prostoma was measured every 10 sections using a micrometer.

The length and maximum width of each entrance hole were measured for each ant species, again using a micrometer. Alate female ants were removed from alcohol and dissected, the head of each being removed and mounted for measurements. The gaster and alitrunk in ants are usually dorsiventrally compressible, but not the head. The dimensions of the head may thus be critical in determining an ant's ability to pass through an entrance hole. The head of each ant was mounted on a pin so as to allow orientation of the specimen for precise measurement in any plane. We measured the maximum width of the head of each ant (Hölldobler & Wilson 1990) as well as its maximum depth. This last measure corresponds to the maximum distance between the dorsal and ventral surfaces of the ant head. Based on observations of how ants enter these holes, we predicted a correspondence between head width and the length of the prostoma or entrance hole and between head depth and the width of the prostoma or entrance hole. Measurements of ants were made using an electronically assisted monocular lens (Nikon Measuroscope 10; Nikon, Inc., Tokyo, Japan) under $\times 30$ and $\times 50$ magnification.

We performed Mann-Whitney *U*-tests in order to compare the length, width and length:width ratio (which is used as an estimator of the shape) of the prostomata, and the head width, head depth and head width:head depth ratio of the ants. The effects of each treatment (individual tree and occupant ant species) on the measurements were analysed for entrance holes using a general linear model procedure (PROC GLM of SAS v. 6.12 (SAS 1996), type III SS), with tree treatment being nested in the occupant ant species treatment. The means obtained for the length, width and length:width ratio of the entrance holes were compared using Tukey-Kramer tests. In order to compare variability in the prostoma dimensions among the populations, we conducted variance ratio tests (Dagnélie 1975) on the data divided by the mean for each species in order to control for differences in the means.

3. RESULTS

The anatomical structure of the domatia was similar in all three subspecies (figure 2). The inner wall of the central cavity of this cylindrical structure is lined by a continuous ring of sclerenchyma, which is a sclerified and lignified tissue (figure 2a) that appears blue under UV light and red in sections stained with phloroglucinol. In all domatia examined, throughout the entire length of the cylinder this sheath of sclerenchyma is only interrupted at the apex of the internode on the side of the twig opposite the leaf insertion (figure 2b). This sharp, localized interruption, where the sclerenchyma is replaced by parenchyma, defines the prostoma. The distance measured between the two arms of sclerenchyma is the width of the prostoma.

Figure 2. Cross-section of a domatium of *L. a. africana* coloured using phloroglucinol-HCl. The sclerenchyma, which is highly lignified, appears in dark. (a) The ring of sclerenchyma is continuous proximal to the level of the prostoma. (b) The ring of sclerenchyma at the prostoma is sharply interrupted by parenchymatous tissue. e, epidermis; v, vascular bundles; d, cavity of the domatium; s, sclerenchyma; Pr, prostoma.

The size and shape of the prostomata (table 2 and figure 3) differed among the three subspecies studied. The prostomata of *L. a. rumpiensis* were shorter than those of the other two subspecies (*L. a. africana*/*L. a. rumpiensis*, $U_{\min}=1.0$ and $p=0.003$ and *L. a. letouzeyi*/*L. a. rumpiensis*, $U_{\min}=3.0$ and $p=0.006$), the prostomata of *L. a. letouzeyi* were broader than those of the other two subspecies

(*L. a. africana*/*L. a. letouzeyi*, $U_{\min}=0.0$ and $p=0.003$ and *L. a. rumpiensis*/*L. a. letouzeyi*, $U_{\min}=10.0$ and $p=0.054$) and the prostomata of *L. a. africana* differed in shape from those of the other two subspecies (*L. a. letouzeyi*/*L. a. africana*, $U_{\min}=1.0$ and $p=0.005$ and *L. a. rumpiensis*/*L. a. africana*, $U_{\min}=1.0$ and $p=0.0003$), being elongate-elliptic while those of *L. a. letouzeyi* and *L. a. rumpiensis*

Table 2. Sizes and shapes of the prostomata, heads of alate female ants and excavated entrance holes for different ant and plant species.

(The largest dimension is the length for prostoma and entrance hole, and depth for ant head. Means and standard deviations are given for each measurement. For each dimension, different letters indicate significant differences ($p < 0.05$) between species for the mean values of each trait treated independently.)

species	largest dimension (mm)	smallest dimension (mm)	shape coefficient
prostoma			
<i>L. a. africana</i>	1.92 ± 0.30a	0.64 ± 0.06a	0.34 ± 0.07a
<i>L. a. letouzeyi</i>	1.59 ± 0.29a	0.95 ± 0.09b	0.61 ± 0.10b
<i>L. a. rumpiensis</i>	1.07 ± 0.22b	0.72 ± 0.21a	0.67 ± 0.16b
ant head (alate females)			
<i>P. phylax</i>	1.16 ± 0.006a	0.49 ± 0.02a	0.43 ± 0.02a
<i>C. mckeyi</i>	0.96 ± 0.04c	0.51 ± 0.02a	0.53 ± 0.02b
<i>A. afer</i>	1.03 ± 0.03b	0.73 ± 0.06b	0.71 ± 0.06b
entrance hole			
<i>P. phylax</i> on <i>L. a. africana</i>	2.43 ± 0.43b	0.43 ± 0.05a	0.18 ± 0.02a
<i>C. mckeyi</i> on <i>L. a. africana</i>	1.10 ± 0.14a	0.93 ± 0.05c	0.86 ± 0.09b
<i>A. afer</i> on <i>L. a. letouzeyi</i>	0.98 ± 0.03a	0.86 ± 0.05c	0.88 ± 0.04b
diverse ants on <i>L. a. rumpiensis</i>	0.95 ± 0.31a	0.77 ± 0.30b	0.82 ± 0.08b

were more circular. The variance ratio test showed that the length of the prostomata was equally variable in all three subspecies, but that the width of the prostomata was significantly less variable in *L. a. letouzeyi* and *L. a. africana* than in *L. a. rumpiensis* (table 3 and figure 3).

The size and shape of the heads of the alate ants (table 2 and figure 3) associated with each subspecies were also different. Interestingly, the mutualist and parasitic associates of *L. a. africana* (*P. phylax* and *C. mckeyi*, respectively) presented striking contrasts in head dimensions. The maximal head width was different in each pair of species (*P. phylax*/*C. mckeyi* or *A. afer*, $U_{\min} = 0.0$ and $p = 0.012$ and *A. afer*/*C. mckeyi*, $U_{\min} = 2.0$ and $p = 0.04$), with *P. phylax* having the broadest head and *C. mckeyi* the narrowest. The head depths of *P. phylax* and *C. mckeyi* were smaller (*A. afer*/*P. phylax* or *C. mckeyi*, $U_{\min} = 0.0$ and $p = 0.012$) than that of *A. afer*. The head of *P. phylax* differed in shape from those of the other two species (*P. phylax*/*A. afer* or *C. mckeyi*, $U_{\min} = 0.0$ and $p = 0.012$), being dorsiventrally flattened, while those of *C. mckeyi* and (to an even greater degree) of *A. afer* more closely approached a round shape in cross-section. Despite the small sample size, these results appear robust. The distributions were completely non-overlapping in all cases where the differences between species were significant. Alates of the many species associated with *L. a. rumpiensis* were not available for study but, judging from the variation in worker size among these species (M. Poissonnet, unpublished data), it is clear that the range of variation in size among all these potential occupants was much greater than the narrow size range within either of the mutualistic ant species specifically associated with either *L. a. letouzeyi* (*A. afer*) or *L. a. africana* (*P. phylax*).

The dimensions of the entrance holes varied between trees for each ant species (*A. afer*, *P. phylax* and *C. mckeyi* and the set of ants associated with *L. a. rumpiensis* considered together) (length, $F_{16,100} = 6.37$ and $p = 0.0001$, width, $F_{16,100} = 14.58$ and $p = 0.0001$ and shape coefficient, $F_{16,100} = 2.41$ and $p = 0.0052$). However, there were significant differences between the ant species (figure 3) in the length, width and shape of the entrance holes (length,

$F_{3,100} = 194.38$ and $p = 0.0001$, width, $F_{3,100} = 141.49$ and $p = 0.0001$ and shape coefficient, $F_{3,100} = 323.07$ and $p = 0.0001$). The entrance holes of *P. phylax* were significantly longer, narrower and more elongate in shape than those of the other ant species (Tukey–Kramer tests, $p = 0.0001$ for each pairwise comparison), *A. afer*, *C. mckeyi* and the diverse ants associated with *L. a. rumpiensis* (table 2). As a group, the entrance holes excavated by the diverse ants associated with *L. a. rumpiensis* were very variable in width (table 2) in comparison with those excavated by each of the specific mutualistic or parasitic associates of the other two subspecies. These holes were generally narrower than those excavated by *A. afer* (Tukey–Kramer test, $p = 0.04$) or *C. mckeyi* (Tukey–Kramer test, $p = 0.0001$), but not as narrow as those of *P. phylax* (Tukey–Kramer test, $p = 0.0001$).

Figure 3 presents the dimensions of the prostomata for each myrmecophyte in parallel with corresponding dimensions for the ants and entrance holes. It is important to note that symbiotic ants always have head depths that are small enough to enable their passage through the width of a prostoma (figure 3a). Most importantly, figure 3 underlines the strong correspondence between the shapes of the prostoma, of the head of the mutualistic ant (figure 3a) and of the entrance hole (figure 3b) in each of the two systems *L. a. africana* × *P. phylax* (elliptic or flattened shapes) and *L. a. letouzeyi* × *A. afer* (round shapes).

4. DISCUSSION

Our data demonstrate that the prostomata of *L. africana* can be measured with precision and that the prostomata of *Leonardoxa* differed in size, shape and variability among three myrmecophytic subspecies. The prostomata of *L. a. rumpiensis*, which is associated with numerous ant species that chew entrance holes varying in size, were more variable in width than in the other two subspecies. The dimensions of the prostomata in these other two subspecies, each of which is associated with a single mutualistic ant, were relatively constant and matched

Figure 3. Means and standard errors for the dimensions of the prostomata (at the same scale), of the symbiotic ants and of the entrance holes excavated in domatia for each of the four different relationships between symbiotic ants and the three myrmecophytic *L. africana*. (a) Mean (continuous lines) and extreme (dotted lines) dimensions of prostomata and mean dimensions of associated ants. (b) Mean dimensions of prostomata (continuous line) and of entrance holes excavated by ants in the prostomata.

those of the associated mutualistic ant and its entrance holes in size and shape. While prostomata were measured in only a single population of each subspecies, preliminary observations have shown that elongate prostomata characterized all the eight populations of *L. a. africana* we have studied in the field and round prostomata characterized all eight populations of *L. a. letouzeyi* and all five populations of *L. a. rumpiensis* (McKey 2000).

(a) **Were these matches between the dimensions of ant and plant characters driven by coevolution?**

Phylogenetic analysis offers one approach to answering this question. Coevolution is supported if functionally matching characters are apomorphic traits in each of a pair of associated species. Mapping of shape characters onto a phylogenetic analysis of the ants (Chenuil & McKey 1996) confirms the obvious, namely that the dorsiventral flattening of the alates of *P. phylax* is a derived trait, which is autapomorphic in this species. *Petalomyrmex phylax* is the sister taxon of *A. afer* (both genera are monotypic). Neither *Aphomyrmex* nor other related genera of formicines have notably flattened alates, and the round to oblong (in transverse section) head of *Aphomyrmex* is the usual condition in ants placed in the tribes Myrmelachistini (McKey (1991) following Hölldobler & Wilson (1990)) or Brachymyrmecini (Bolton 1995).

Conclusions about the polarity of the character states in prostoma traits based purely on phylogenetic patterns are more difficult to reach for the plants. Although the phylogeny and phylogeography of *L. africana* are still not completely resolved, it is clear on both morphological (McKey 2000) and molecular grounds (Brouat 1999; Brouat *et al.* 2001) that *L. a. letouzeyi* and *L. a. rumpiensis* are very closely related. Together they form a 'transitional' group of myrmecophytes with many traits, both those related to ants and others, having states intermediate between the non-myrmecophyte *Leonardoxa africana gracili-caulis* McKey and the highly specialized myrmecophyte *L. a. africana* (McKey 2000). If ant-related traits evolve in concerted fashion, then the size and shape of the prostomata of the two transitional subspecies might be considered as reflecting intermediate states of this character as well. Following this reasoning, the prostoma of *L. a. letouzeyi* may have evolved at least two adaptations to its associate *A. afer*, namely increased size and decreased variation in width, but there is no evidence that the ant has evolved corresponding adaptations to the plant. The rounded prostoma may have been produced simply by plant adaptation to its mutualistic associate. In contrast, the elongate shape and low variation in width of the prostoma of *L. a. africana* may have evolved in correspondence with the dorsiventral flattening of its specific associate *P. phylax*. These functionally matching apomorphic traits strongly suggest reciprocal adaptation, which is coevolution in the strictest sense, of the partners in this host-specific association.

(b) **The prostoma as a morphological and behavioural filter**

The most general role of the prostoma seems to be to permit colonization of a domatium by ants of appropriate size, even after lignification of the domatium. *Petalomyrmex phylax*, for example, is perfectly able to excavate prostomata in even very lignified twigs. We bagged and

Table 3. Comparison between myrmecophytes of the variability in the prostoma dimensions.

(Variance ratio tests (Dagnélie 1975, vol. 2, pp. 50–51) on the length, width and shape coefficient, with each measurement being divided by the mean of the variable considered for the subspecies. F_{obs} = maximum variance/minimum variance. When $F_{\text{obs}} < F(\text{Snedecor})$ the differences are not significant. Critical values of $F(\text{Snedecor})$ were taken from Table D of Dagnélie (1975, vol. 2, p. 413). An S in parentheses represents a taxon with a specific ant associate and a D in parentheses represents a taxon associated with diverse ants in one host population. n.s. indicates that the differences are not significant and an asterisk indicates that the differences are significant at a level of $p < 0.05$.)

	length		width		shape coefficient	
	$F(\text{Snedecor})$	F_{obs}	$F(\text{Snedecor})$	F_{obs}	$F(\text{Snedecor})$	F_{obs}
<i>L. a. africana</i> (S)/ <i>L. a. rumpiensis</i> (D)	4.30	1.73 ^{n.s.}	4.30	9.98*	4.30	1.37 ^{n.s.}
<i>L. a. africana</i> (S)/ <i>L. a. letouzeyi</i> (S)	5.05	1.39 ^{n.s.}	8.98	1.04 ^{n.s.}	8.98	1.52 ^{n.s.}
<i>L. a. letouzeyi</i> (S)/ <i>L. a. rumpiensis</i> (D)	8.84	1.24 ^{n.s.}	8.84	10.37*	8.84	2.07 ^{n.s.}

tanglefooted young domatia of plants occupied by *P. phylax* in the population of Ebodjé and then removed these barriers after the domatia were fully lignified. We observed the timing of the chewing of entrance holes on control domatia of the same plants. These experiments showed that *P. phylax* workers open entrance holes on young domatia only after they are fully expanded and that they easily open entrance holes of normal size and shape even on fully lignified mature internodes.

The entrance holes opened by *P. phylax* are longer than permitted by the length of the prostoma (figure 3b). However, the potential suitability of the entrance hole as a filter against ants with conventionally shaped bodies is determined by its smallest dimension, whereas the larger dimension is free to vary. Thus, the length of the entrance hole excavated by *P. phylax* does not seem to augment the risk that the plant will be invaded by other ants or parasites. However, it is surprising that the length of the entrance holes made by *Petalomyrmex* is so much greater than the head width of this ant. Our hypothesis for explaining this observation is that the width of the dorsiventrally constricted gaster of alate females sets the minimum length required for the entrance holes of *Petalomyrmex*. Although the gaster of alates is not rigid and thus, unlike the head, cannot be measured with high repeatability, its width, particularly when the gaster is dorsiventrally constricted (as during passage through the entrance hole), is much greater than that of the head.

The prostomata in all the myrmecophytic *Leonardoxa*, while permitting colonization of domatia by ants that are similar in size to the mutualist, exclude ants and other potential interlopers larger than the mutualist associate. Earlier studies (McKey 1984, 1991) have emphasized the possible role of the flattened prostoma in helping exclude the species-specific parasite of the *L. a. africana*/*P. phylax* mutualism, namely the myrmicine ant *C. mckeyi*. In disagreement with this hypothesis, measurements of alate females of *C. mckeyi* show that they should be able to pass through a slit with the dimensions of the prostoma of *L. a. africana* (and through the entrance holes of *P. phylax*, which are of very similar size). Our study and the existence and frequency of this species-specific parasite show that the prostoma is by no means an absolute barrier against this species. Has the parasite *C. mckeyi* evolved counter-adaptations to the exclusion filter of its host? Van Noort & Compton (1996) found evidence in a system

analogous to the one studied here that parasites, like mutualists, have evolved adaptations to the traits of their specific hosts that may function as exclusion filters. They found a strong correlation across species of figs between the head shape of pollinating fig wasps and that of a group of parasitic fig wasps that, like the pollinators, enter the ostiole (a bract-lined pore at the apex of the fig) in order to oviposit from inside the fig. It is perhaps significant that the head depth of *C. mckeyi* is intermediate between those of *A. afer* and *P. phylax*. Does this reflect (imperfect) convergent adaptation of *C. mckeyi* and *P. phylax* to the elongate exclusion filter of *L. a. africana*? Alternatively, did the ancestral *Cataulacus* already possess this head shape, thus being pre-adapted to this plant? Snelling (1979) considered *C. mckeyi* to have affinities with *Cataulacus pygmaeus*. Unfortunately, the head depth of this possibly related species is unknown, so no grounds exist for evaluating these two alternatives. The smaller overall size of the head (and other body parts) of *C. mckeyi* in relation to that of *C. pygmaeus* (Snelling 1979) would facilitate colonization of the domatia of this plant, but many other selective forces could have driven size reduction.

However, whereas *C. mckeyi* is able to pass through the prostoma, our study shows that the entrance holes excavated by *C. mckeyi* are broader than permitted by the dimensions of the prostoma (figure 3b). *Cataulacus mckeyi* defends nest sites by phragmosis (Baroni Urbani 1989), as the entrance hole of each domatium occupied by *C. mckeyi* is virtually constantly plugged by the round shield-shaped dorsal surface of the head of a single worker. For this reason, the width of the entrance holes excavated by *C. mckeyi* (0.93 ± 0.05 mm) seems not to be set by the head depth of alate ants (0.51 ± 0.02 mm) but by the head length (between 0.95 and 0.97 mm for seven workers belonging to different colonies of *C. mckeyi*) (Snelling 1979) of workers that defend domatia. *Cataulacus mckeyi* cannot thus simply chew through the prostoma in order to excavate its entrance hole, but must also chew through some surrounding areas that are heavily lignified in mature internodes. In this sense, the elliptical prostoma can constitute a partial barrier that renders the colonization of mature internodes of *L. a. africana* by this parasitic ant more difficult. In disagreement with earlier hypotheses (McKey 1984, 1991), our study shows that the prostoma is by no means an absolute filter against *C. mckeyi* and that *C. mckeyi* is disadvantaged in colonizing the plant

not solely by its morphological characters, but by a combination of morphology and behaviour.

Exactly how *C. mckeyi* is disadvantaged in colonizing domatia is still under study. In order to test the hypothesis that flattened prostomata restrict colonization by *C. mckeyi* to immature, unligified internodes, we bagged and tangle-footed young domatia of plants occupied by this ant in the Ebodié population and then removed these barriers after the domatia were fully lignified. We also observed the timing of the chewing of entrance holes on control domatia of the same plants. Unfortunately, the activity levels on the *Cataulacus*-occupied plants were low and no entrance holes were opened on either the control or experimental domatia. Whether the window in time for colonization by *C. mckeyi* (for workers and for founding queens) is reduced is thus still an open question.

(c) A major open question: what organisms drove the evolution of the exclusion filter?

Precisely which organisms provided the selective pressure leading to the origin of the coevolved exclusion filter (and which organisms maintain the traits in contemporary populations of plants and ants) is not known. *Cataulacus mckeyi*, the specialist parasite of the mutualism, is one obvious candidate. However, as we have shown, the ability of *C. mckeyi* to chew round entrance holes and not be restricted to prostoma tissue shows that the filter is at most of limited effectiveness against this specialized parasite. What other enemies of the mutualism could have driven coevolution of the filter? One possibility is that the function of the filter is to exclude other plant ants that are less effective mutualists of this plant. For example, *A. afer*, the mutualistic ant of *L. a. letouzeyi*, can also be found in sympatry with *L. a. africana* and *P. phylax* in the same sites, but here it is always associated with another plant, i.e. *V. grandifolia* and has never been observed in domatia of *L. a. africana* (D. McKey, unpublished field observations). Another possibility is that the filter functions in order to exclude predators rather than competitors of *Petalomyrmex*. The predictable resource represented by plant ant nests attracts an important associated fauna (for examples, see Letourneau & Dyer 1998; Krombein *et al.* 1999). This and other open questions could be resolved by field experiments in which the entrance holes of *Petalomyrmex* are artificially enlarged, and by measurements of a large set of ants (and enemies of ants) in habitats where *L. africana* occurs.

(d) The importance of exclusion filters in facilitating pairwise coevolution in ant-plant mutualisms

Yu & Davidson (1997) suggested that pairwise coevolution is generally rare in horizontally transmitted symbioses, and that exclusion filters may be prerequisites for coevolution in these systems by restricting the range of possible associations and by enforcing specificity. Once exclusion filters are in place coevolutionary interactions can be intense and spectacular, even in systems with horizontal transmission (e.g. fig pollination mutualisms) (Anstett *et al.* 1997). A diversity of ant and plant structures appear to function as exclusion filters in ant-plant symbioses, for example prostomata, self-opening slits, long dense trichomes that exclude large ants (Davidson & McKey 1993) and waxy plant surfaces that exclude ants lacking specific adaptations for holding on (Federle *et al.*

1997). Whether resulting from ecological fitting or one-sided adaptation, as appears to be the usual case, or by pairwise coevolution, as argued for the system studied here, these filters put into place the conditions for pairwise coevolution. We suggest that such reciprocal adaptation may be more frequent in ant-plant symbioses than is usually thought. At least one other pair of traits appears to have coevolved in the specific association between *L. a. africana* and *P. phylax*. *Petalomyrmex* is the only genus of the tribe Myrmelachistini in which an association with homopteran trophobionts is known to be absent, thereby suggesting the loss of this trait (Meunier *et al.* 1999), while the foliar nectaries of its host are larger, more numerous and more constant in number than in all other subspecies (McKey 2000), including *L. a. letouzeyi* associated with the homopteran-tending *A. afer* (Gaume *et al.* 1998).

(e) The prostoma of *L. a. africana*: a good model for studying ongoing coevolution

The traits we have focused on in this study are interesting, not so much because of their intrinsic importance, but because they are now relatively easy to study. For example, based on our results we now know that the functionally crucial dimension is the maximum width of the prostoma. Each prostoma can be efficiently characterized with a few measures, i.e. the width of a section taken at its broadest point, so that large sample sizes should be obtainable in future studies. General questions about coevolutionary processes can thus be attacked. For example, do these traits provide evidence for a geographic mosaic of coevolution (Thompson 1999) between ants and plants, not only at the level of the allopatric subspecies considered here, but also among closely related populations? Ant associations among populations of *L. a. africana* vary and in one population *P. phylax* is completely absent (McKey 2000). Furthermore, the outcome of the interaction between the plant and each of its two principal ant associates appears to vary among sites (G. Debout, unpublished data). Do the prostoma of *L. a. africana* or the heads of *P. phylax* alates vary among populations in size, shape or variability, thereby suggesting 'coevolutionary meanderings' in response to spatially and temporally varying interactions or are these traits invariant among individuals and populations, thereby suggesting fixation?

Coevolution may often be very difficult to demonstrate for numerous ant and plant characters. Important coevolutionary processes might involve pairs of characters that are much less obvious upon superficial examination than those considered here (e.g. traits related to host recognition by founding queens) or traits that are more difficult to quantify and that cannot simply be matched up on a trait-for-trait basis. For example, it is likely that ant colony life-history traits and those of their host plants coevolve in myriad ways, but for these complex and inter-related characters an atomistic trait-for-trait analysis, which is possible for the simple characters examined here, is probably doomed to failure. Furthermore, if the characters involved vary in a geographic mosaic, phyletic patterns may fail to provide evidence of coevolution. Studies at finer levels of space and time, i.e. phylogeography and analyses of the processes in populations and metapopulations, will be required. The prostoma of *Leonardoxa* and

the dimensions of its associated ants are a character couple that should allow application of this approach.

We thank the Government of Cameroon for permission to carry out this research, which was funded by the Centre National de la Recherche Scientifique programme 'Environnement, Vie et Sociétés'. Ambroise Dalecky, Gabriel Debout, Laurence Gaume and Alain Ngomi are thanked for their help in collecting ant and plant material. Luc and Marie Moliko are thanked for their hospitality in Ebodjé. We thank Lilian Ceballos, Chantal Debain, Elisabeth Motte-Florac and Mikael Poissonnet for their help with the histological studies. J.-C. Auffray (Institut des Sciences de l'Evolution de Montpellier) is thanked for letting us use the measuroscope in his laboratory. The manuscript was improved by discussions with Ambroise Dalecky, Laurence Gaume, Finn Kjellberg and Michel Raymond and by the comments of three reviewers.

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As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.