

# Evolutionary diversification of the bean beetle genus *Callosobruchus* (Coleoptera: Bruchidae): traits associated with stored-product pest status

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## Abstract

Despite the fact that many plant-feeding insects are pests, little effort has been made to identify key evolutionary trait transitions that allow taxa to acquire or lose pest status. A large proportion of species in the genus *Callosobruchus* are economically important pests of stored, dry postharvest beans of the tribe Phaseoleae. However, the evolution of this feeding habit is poorly understood. Here, we present a reconstruction of the phylogeny of the Asian and African *Callosobruchus* based on three mitochondrial genes, and assess which traits have been associated with the evolutionary origin or loss of ability to reproduce on dry beans. Our phylogenetic analysis showed that species group into the *chinensis* and the *maculatus* clades, which are also supported by genital morphology, and an additional paraphyletic group. Ancestral ability to use dry beans has been lost in the *chinensis* clade but acquired again in *C. chinensis*. Dry-bean use and host-plant use were both phylogenetically constrained and transitions in the two were significantly correlated. Host shifts from the subtribe Phaseolinae to Cajaninae were more common than the reverse and were more likely in species using young beans. The ability to use dry beans was more likely gained when using Phaseolinae hosts and promoted habitat shifts from tropical to temperate regions. Adaptation to arid climate was also associated with the ability to reproduce on dry beans and on Phaseolinae. Thus, our analysis suggests that physiological adaptations to an arid climate and to Phaseolinae hosts both render beetles predisposed to become pests of cultivated beans.

**Keywords:** Bayesian inference, COI and COII, Fabaceae, host-plant shifts, plant–herbivore interactions, *Wolbachia*

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## Introduction

Comparative research on insect–plant co-evolution has by tradition focused on parallel evolution in insects and their hosts, and has shown that herbivorous insects tend to specialize on specific plant taxa and/or plant tissues (Ehrlich & Raven 1964; Strong *et al.* 1984; Futuyma 1986;

Futuyma & Moreno 1988). Much less is known about the types of traits that allow insects to shift hosts and to utilize particular resources. Our understanding of the evolution of insect pests on agricultural crops is obviously an important issue from an economic point of view, but may also involve key adaptations that can shed general light on the evolutionary ecology of herbivorous insects. Stored product pests are a diverse group of insects that are well adapted to using dry and hardened food as a resource (Robinson 1928; Chapman 1931; Linsley 1944; Ishii 1952; Cotton 1956). While the hardness of dried seeds *per se* can serve as a deterrent against seed predators, including seed beetles of the family Bruchidae (Janzen 1977; Southgate

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1979; Kitch *et al.* 1991; Dongre *et al.* 1993), the loss of toxic chemicals during postmaturity drying processes may also increase survival of such granivores. Bruchid beetles primarily utilize beans from the family Fabaceae as their hosts (Johnson 1981). Most bruchids are oligophagous; their host range is limited to restricted plant taxa, typically tribes and subtribes for species utilizing the legume subfamily Faboideae (Jermy & Szentesi 2003; Tuda *et al.* 2005). The ability to use dry beans as a food resource is widespread in this family. In Bruchinae, a derived group within the Bruchidae, many nonpest species do exhibit the ability to use dry seeds (Watanabe 1985; Shimada 1988; Tuda *et al.*, unpublished) and these may be pre-adapted to becoming pests of stored beans (Watanabe 1985; Shimada 1990; Yoshida 1990).

The genus *Callosobruchus* Pic (Bruchinae) includes approximately 20 species (Borowiec 1987) and an unusually large proportion of these are pests of stored beans. As larvae, *Callosobruchus* species utilize seeds of legumes of the tribe Phaseoleae (Fabaceae), including many beans cultivated for human consumption (Johnson 1981). Recent research on this genus has led to the discovery of several new species that feed on legumes without economic importance (Arora 1977; Kingsolver 1999; Anton 2000; Tuda 2003; Tuda *et al.* 2005). While one might think that it would be natural for taxa that utilize seeds of Phaseoleae to become pests of stored beans, our understanding of why only some species are able to reproduce continuously, without intermission by reproductive diapause, on dry and hardened beans is very limited (see Labeyrie 1981). Climatic factors,

primarily the length of the reproductive season, have been suggested as key correlates of pest status (Cotton 1956; Kurota & Shimada 2002). However, other traits may also play a role, such as the presence of endosymbiotic bacteria (Tsuchida *et al.* 2004).

Here, we seek associations between the evolution of ability to reproduce on dry beans and a suite of other characters in the genus *Callosobruchus*. We first test the extent to which the ability to use dry beans is phylogenetically constrained, and then test for associations with the following variables: (i) host taxa in the wild; (ii) a suite of climatic variables; and (iii) the presence or absence of endosymbiotic bacteria (i.e. *Wolbachia*).

## Materials and methods

### Taxon sampling

Mature leguminous seeds, both native and introduced, were collected from various sites in tropical to temperate Asia and Africa and were brought into the laboratory. Emerging beetles were fixed and preserved in >99% acetone until use for molecular analysis. The sampled bruchids, their host plants, sampling location, and locality of native distribution are listed in Table 1. We used *Zabrotes subfasciatus* (Boheman) of the bruchid subfamily Amblycerinae and *Acanthoscelides obtectus* (Say) of the subfamily Bruchinae as outgroups. A total of 14 *Callosobruchus* species were subjected to the following analyses.

**Table 1** Bruchid species, their host plants and locality of collection. Natural range estimation is shown in parentheses as Asian (A), African (Af) and American (Am)

Bruchid species	Host plant	Collector	Locality (natural range)
<b>Bruchinae</b>			
<i>Callosobruchus analis</i> (Fabricius)	<i>Vigna radiata</i>	To	Myanmar (Af + A)
<i>Callosobruchus chinensis</i> (Linnaeus)	<i>Vigna angularis</i>	U	Japan (A)
<i>Callosobruchus dolichosi</i> (Gyllenhal)	<i>Cajanus scarabaeoides</i>	T	Thailand (A)
<i>Callosobruchus imitator</i> Kingsolver	<i>Vigna umbellata</i>	T	Thailand (A)
<i>Callosobruchus latealbus</i> (Pic)	<i>Rhynchosia acuminatifolia</i>	Ta	China (A)
<i>Callosobruchus maculatus</i> (Fabricius)	<i>Vigna unguiculata</i>	C	India (Af)
<i>Callosobruchus nigripennis</i> (Allard)	<i>Cajanus scarabaeoides</i>	T	Thailand (A)
<i>Callosobruchus phaseoli</i> (Gyllenhal)	<i>Lablab purpureus</i>	C	U.K. (Af)
<i>Callosobruchus pulcher</i> (Pic)	<i>Cajanus cajan</i>	Ne	Hawaii (A)
<i>Callosobruchus rhodesianus</i> (Pic)	<i>Vigna unguiculata</i>	G	Zimbabwe (Af)
<i>Callosobruchus semigriseus</i> (Motsch.)	<i>Dunbaria bella</i>	T	Thailand (A)
<i>Callosobruchus subinnotatus</i> (Pic)	<i>Vigna subterranea</i>	C	Ghana (Af)
<i>Callosobruchus theobromae</i> (Linnaeus)	<i>Cajanus scarabaeoides</i>	T	Thailand (A)
<i>Callosobruchus utidai</i> Tuda	<i>Dunbara podocarpa</i>	T	Thailand (A)
<i>Acanthoscelides obtectus</i> (Say)	<i>Phaseolus vulgaris</i>	J + S	Hungary (Am)
<b>Amblycerinae</b>			
<i>Zabrotes subfasciatus</i> (Boheman)	<i>Phaseolus vulgaris</i>	C	Columbia (Am)

J + S, Jermy and Szentesi; T, Tuda *et al.*; Ta, Tateishi; U, Utida; To, Toquenaga; Ne, Nemoto; G, Giga; C, Credland.

### DNA analysis

DNA was extracted with DNeasy kit (QIAGEN and Machery-Nagel) following the manufacturers' instructions. Partial sequences of mitochondrial genes, encoding cytochrome *c* oxidase subunit I (COI) and subunit II (COII) that are interleaved by tRNA-Leucine (tRNA<sup>Leu</sup>) gene were amplified with the following primer sets: 5'-GGATCACCTGATATAGCATTYCC-3' (modC1J-1751, modified from Simon *et al.* 1994) in combination with 5'-GCTAATCATCTAAAAATTTAATTCCTGTTGG-3' (revC1J-2441, modified from Simon *et al.* 1994), and 5'-CTTTATCAACATTTATTTGATTTTTT-3' (COI2-1) in combination with 5'-TACTCCAATAAATATTATAATAAATTG-3' (COI2-2) for COI (Tuda *et al.* 1995, 2004); 5'-TAATATGGCAGATTAGTGCATTGGA-3' [Gomez-Zurita *et al.* 2000; modified TL2-J-3037 (Simon *et al.* 1994)] and 5'-GAGACCATTACTTGTTCAGTCATCT-3' [Gomez-Zurita *et al.* 2000; modified TK-N-3785 (Simon *et al.* 1994)] for tRNA<sup>Leu</sup> – COII. Polymerase chain reaction and sequencing were done as detailed in Tuda *et al.* (2004). The total size of the sequenced regions was 1651 bp (COI – 952 bp, tRNA<sup>Leu</sup> – 21 bp and COII – 678 bp). The sequence data include no indels and the base composition was AT rich, as is typical for insect mitochondrial sequences. The data are deposited in GenBank, under the accession nos DQ459035–459050 for COI and DQ459020–459034 for tRNA<sup>Leu</sup> and COII.

### Character states of host plants

Hosts were classified into either of the two subtribes of Phaseoleae that were found to harbour *Callosobruchus* beetles: Phaseolinae and Cajaninae. Primary host subtribe of respective beetle species was assigned according to host taxa under nonstorage condition, by excluding all records from stored beans (see Tuda *et al.* 2005). A recent molecular phylogenetic study of the hosts (Kajita *et al.* 2001) showed that these two subtribes form strongly supported groups and that Cajaninae is clearly monophyletic. The ability to use dry mature host beans as larval medium was examined under natural as well as laboratory environments for all bruchid species included here (Tuda *et al.*, unpublished). We also checked for the presence of *Wolbachia* infections in multiple individuals of all species, by using a pair of standard *Wolbachia*-specific primers (Kondo *et al.* in preparation).

Species habitats were classified into climatic zones based on the data gathered from the online climate database World Weather Information Service, provided by the World Meteorological Organization ([www.worldweather.org](http://www.worldweather.org)). Since legumes in tropical and temperate areas bear seeds in arid seasons, which provide bruchids resources to reproduce (Africa, Sanon *et al.* 1998; Delobel *et al.*, unpublished; Asia, Niyomdham, personal communication; Tuda,

personal observation; Central America, Janzen 1967), we focused on climatic variables during arid seasons. First, the number of arid months (rainfall < 50 mm/month) and the mean temperatures during the arid months in the habitats of respective species (Appendix) were subjected to a standard principal component analysis. The first principal component explained a large fraction (93.6%) of variance in the climatic factors tested and the mean temperature during the arid months correlated strongly with this axis ( $r = 1.00$ ) while the number of arid months per year did not ( $r = -0.0029$ ). We hence classified habitats into two groups, tropical and temperate, based on temperature (Table 2; Appendix I). Second, to test the effect of aridity, we also categorized habitats into arid (arid months per year  $\geq 5$  months) or humid (arid months per year < 5 months) (Table 2; Appendix I).

### Phylogenetic reconstruction

**Model choice.** Prior to phylogenetic analyses, a priori selection of a nucleotide substitution model was done based on the Akaike information criterion (Akaike 1973) using the program *MRAIC.PL* version 1.4 (Nylander 2004) in conjunction with *PHYML* version 2.4.4 (Guindon & Gascuel 2003). *MRAIC.PL* compares 24 standard substitution models, including models allowing rate variation, by utilizing likelihood scores calculated by *PHYML* (all parameters estimated during tree search). This analysis showed that a general time reversible model (GTR) (Rodriguez *et al.* 1990), combined with the assumption that a proportion of sites was invariable (I) (Gu *et al.* 1995) and a gamma distribution for modelling rate variation ( $\Gamma$ ) (Yang 1994), minimized the Akaike information criterion for both COI and COII. We hence chose this model (GTR + I +  $\Gamma$ ) for our entire data set.

**Phylogenetic inference.** We modelled our sequence data in a Bayesian framework, using *MRBAYES* version 3.0b4 (see Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003). Exploratory analyses showed that the estimated values for the model parameters were similar for COI and COII, while the estimates differed substantially across the three codon positions. We thus grouped the nucleotides at the first, second, and third codon positions into different partitions, and allowed the overall rate of substitution, the gamma shape parameter, the proportion of invariable sites, and the rate of evolution (i.e. rate multipliers) to differ at the different positions. This aside, we used default priors for all model parameters.

The posterior distribution of trees was approximated using Markov chain Monte Carlo as implemented in *MRBAYES*. We ran four independent runs with four parallel chains (Metropolis coupling; Huelsenbeck & Ronquist 2001), where each run started from a randomly chosen

**Table 2** Host utilization, dry bean use and habitat climate of all species

Species	Natural hosts	Main host subtribe	Dry bean use	Climate
<i>chinensis</i> clade				
<i>C. chinensis</i>	<i>Cajanus</i> , <i>Rhynchosia</i> <i>Dunbaria</i> , <i>Vigna</i>	Cajaninae	Y	Te, arid
<i>C. utidai</i>	<i>Dunbaria</i>	Cajaninae	N	Tr, arid
<i>C. semigriseus</i>	<i>Dunbaria</i>	Cajaninae	N	Tr, humid
<i>C. dolichosi</i>	<i>Cajanus</i>	Cajaninae	N	Tr, humid
<i>C. nigripennis</i>	<i>Cajanus</i>	Cajaninae	N	Tr, humid
<i>C. theobromae</i>	<i>Cajanus</i>	Cajaninae	N	Tr, humid
<i>C. pulcher</i>	<i>Cajanus</i>	Cajaninae	N	Tr, humid
<i>maculatus</i> clade				
<i>C. analis</i>	<i>Vigna</i>	Phaseolinae	Y	Tr, arid
<i>C. rhodesianus</i>	<i>Vigna</i>	Phaseolinae	Y	Te, arid
<i>C. maculatus</i>	<i>Vigna</i>	Phaseolinae	Y	Tr, arid
<i>C. subinnotatus</i>	<i>Vigna</i>	Phaseolinae	Y	Tr, arid
Paraphyletic group				
<i>C. latealbus</i>	<i>Rhynchosia</i>	Cajaninae	Y	Te, humid
<i>C. imitator</i>	<i>Vigna</i>	Phaseolinae	Y	Tr, arid
<i>C. phaseoli</i>	<i>Lablab</i> , <i>Vigna</i>	Phaseolinae	Y	Tr, arid
Outgroup				
<i>A. obtectus</i>	<i>Phaseolus</i>	Phaseolinae	Y	Te, arid
<i>Z. subfasciatus</i>	<i>Phaseolus</i>	Phaseolinae	Y	Tr, arid

Te, temperate; Tr, tropical.

tree, with 10 million generations each and sampled every 1000th generation. The first 1 million generations were discarded in each run as a burn-in phase. A comparison of all parameters from the four runs, using TRACER version 1.2.1 (Rambaut & Drummond 2005), confirmed that the chains had converged on the same target distribution and tree topology in all runs (Huelsenbeck *et al.* 2002). Trees and parameter values from the four runs were then pooled and a majority-rule consensus tree was calculated.

#### Phylogenetic constraints and correlated evolution

We then used our majority-rule consensus molecular phylogeny to test evolutionary scenarios for the use of dry beans in *Callosobruchus*. Congruence of dry bean use, host taxonomy, *Wolbachia* infection and climate with our molecular phylogeny was first tested by a permutation tail probability (PTP) test (Faith & Cranston 1991) as implemented in PAUP\* (Swofford 2002), using 2000 replications. Randomization within characters was applied only to ingroup taxa (i.e. *Callosobruchus* species) (Trueman 1996).

We tested for correlated evolution between all of our variables (including dry bean use) using a continuous-time Markov model in a maximum likelihood framework, as implemented in DISCRETE 4.0 (Pagel 1994). We used likelihood ratio test to compare independent- and dependent-

evolution models and their statistical significance was tested by Monte Carlo simulation with 2000 iterations, again using DISCRETE 4.0. If the dependent evolution model was significantly better than the independent model, transition rates were further tested by setting the transition rate to zero in the dependent model (i.e. a backward procedure). When backward procedure did not detect any significant transition rates, alternatively, by allowing transition rates conditional to the state of the other trait (by adding a single transition rate to the independent model), we tested whether an additional transition rate was significant (i.e. a forward procedure). For both backward and forward procedures, the critical value of the likelihood ratio  $-2\ln(H_0/H_1)$  is approximately  $\chi^2$  distributed with 1 degree of freedom (i.e. the difference in the numbers of parameters between  $H_0$  and  $H_1$ ), where  $H_0$  and  $H_1$  are hierarchical models with the former being the simpler (see Pagel 1994). We also tested whether each transition rate in the independent models was significantly different from zero. We did not employ parsimony analysis because of its tendency to underestimate evolutionary change in long branches (Pagel 1999b).

When estimating ancestral states at internal nodes, we used a continuous time Markov model in a maximum-likelihood framework, using MULTISTATE (Pagel 1999a). For optimization of ancestral states, we used local rather than global optimization (see Pagel 1999b).

Results

Phylogenetic reconstruction

The Bayesian phylogenetic analysis yielded a well-resolved phylogeny, given in Fig. 1, with high clade support, especially for more basal nodes (posterior probabilities 0.95–1.00). The Bayesian posterior probability of this topology (with *Callosobruchus dolichosi* being sister species to the group containing *C. semigriseus*, *C. utidai* and *C. chinensis*) was 0.199, and the 50% most credible set of topologies (cumulative posterior probability < 0.5) contained only three additional topologies with only very minor alterations. In the first of these (posterior probability 0.156), *C. dolichosi* and *C. semigriseus* group together as sister species. In the second (posterior probability 0.055), *C. pulcher* is a sister species to *C. theobromae* and *C. nigripennis*. In the third (posterior probability 0.054), *C. semigriseus* replaces *C. dolichosi* as the basal taxa to the other three species in that clade.

Within the genus, three phylogenetic groups were thus clearly distinguishable: one clade containing *C. chinensis*, another containing *C. maculatus* and a third paraphyletic group of species. This topology is also congruent with morphological character states of male genitalia (see Table 3). Species in the *maculatus* clade are primarily distributed in Africa and commonly feed on *Vigna* of the subtribe Phaseolinae (Tables 1 and 2, Fig. 1). The *chinensis* clade primarily contains species from subtropical and/or temperate Asia. The hosts of these species are *Cajanus*, *Dunbaria* and *Rhynchosia* of the subtribe Cajaninae, with polyphagous species (i.e. *C. chinensis*) feeding on *Vigna* as well (Table 2). The remaining paraphyletic group feed on either of two Phaseoleae subtribes under natural conditions (Table 2).

Phylogenetic constraints and correlated evolution

The evolution of dry bean use and host utilization were both significantly congruent with the *Callosobruchus*

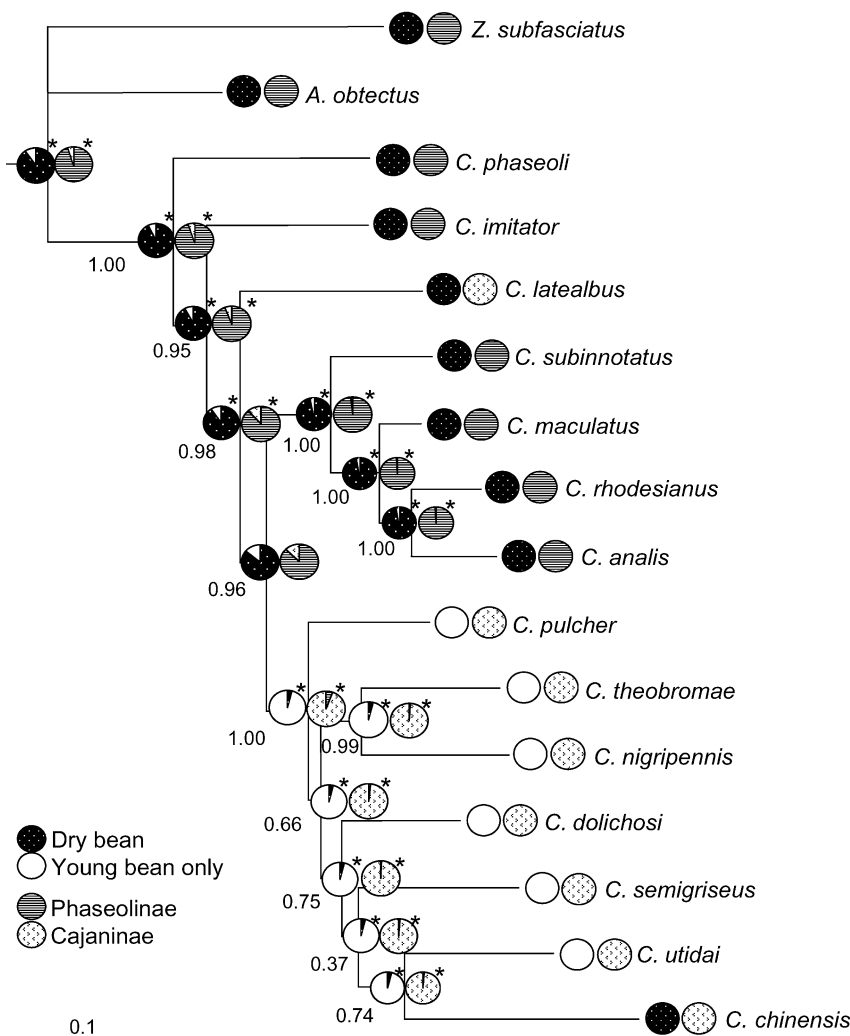


Fig. 1 (a) molecular phylogeny of the genus *Callosobruchus*. Shown is the 50% majority-rule consensus tree from the Bayesian analyses. The numbers below nodes represent Bayesian posterior probabilities. Observed character states are indicated at terminal nodes and the maximum likelihood estimates of ancestral states at internal nodes under local optimization, shown as pie diagrams for probability of each character state (left, the ability to use dry, hardened mature beans; right, host subtribes). An asterisk indicates that the probability of one state at a given internal node is significantly higher than that of the other.

**Table 3** Morphology of male genitalia in *Callosobruchus*

Species	Median lobe	Lateral lobes (parameres)	Endophallic plates	Exophallic valve
<i>chinensis</i> clade				
<i>C. chinensis</i>	long and slender	Y-shape, broad at apex	2	spear-head
<i>C. utidai</i>	long and slender	Y-shape, broad at apex	2	spear-head
<i>C. semigriseus</i>	long and slender	Y-shape, broad at apex	2	spear-head
<i>C. dolichosi</i>	long and slender	Y-shape, broad at apex	2	spear-head
<i>C. nigripennis</i>	long and slender	Y-shape, broad at apex	2	spear-head
<i>C. theobromae</i>	long and slender	Y-shape, broad at apex	2	spear-head
<i>C. pulcher</i>	long and slender	Y-shape, broad at apex	2	spear-head
<i>maculatus</i> clade				
<i>C. analis</i>	short and stout	V-shape, broad at apex	2	triangular
<i>C. rhodesianus</i>	medium	V-shape, narrow at apex	6	triangular
<i>C. maculatus</i>	short and stout	V-shape, broad at apex	0	triangular
<i>C. subinnotatus</i>	short and stout	V-shape, broad at apex	2	triangular
Paraphyletic group				
<i>C. latealbus</i>	medium	V-shape, broad at apex	2	triangular
<i>C. imitator</i>	long and slender	V-shape, narrow at apex	2	spear-head
<i>C. phaseoli</i>	medium	V-shape, broad at apex	6	triangular
Outgroup				
<i>A. obtectus</i>	medium	V-shape, broad at apex	2	triangular

	Dry bean use	Host taxonomy	<i>Wolbachia</i>	
Host taxonomy	3.88 ( <b>0.010</b> )	—	—	
<i>Wolbachia</i>	2.32 (0.047)	0.793 (0.28)	—	
Climate				
Tropical/Temperate	2.92 ( <b>0.014</b> )	0.599 (0.47)	1.62 (0.11)	
	4.25 ( <b>0.005</b> )	5.39 ( <b>0.002</b> )	0.758 (0.39)	Arid/Humid

**Table 4** Likelihood ratio tests of correlated evolution between dry-bean and host taxonomy, climate and *Wolbachia* infection. Log likelihood ratios are followed by *P* values in parentheses, estimated by Monte Carlo simulations. Bolded *P* values indicate significant differences at the critical level (*P* = 0.02)

phylogeny (PTP,  $P = 0.0045$ ,  $P = 0.0045$ , respectively), while neither *Wolbachia* infection nor climatic variables were congruent with the phylogeny (PTP, *Wolbachia*  $P = 1.0$ , tropical/temperate  $P = 1.0$ , arid/humid  $P = 0.064$ ).

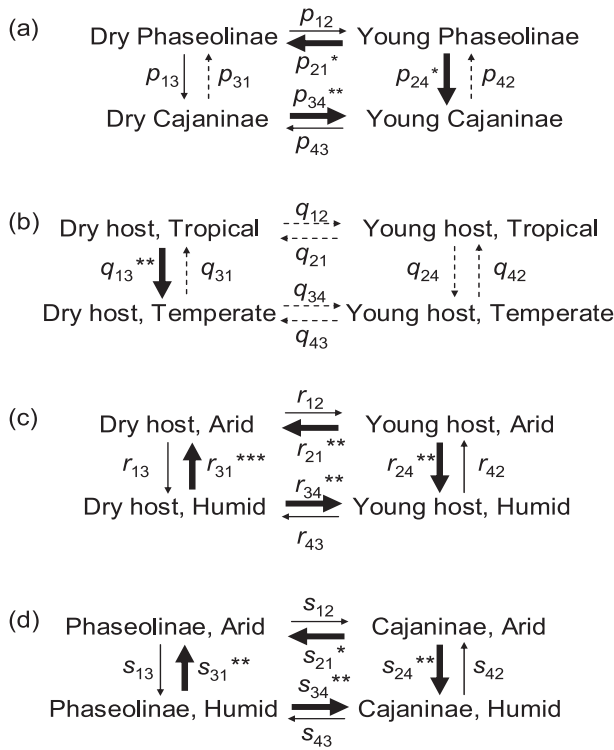
We found a significant correlation in evolution of dry bean use and host utilization (Table 4). The ancestral state of the paraphyletic group and the *maculatus* clade was ability to use dry Phaseolinae seeds and the ancestral state of the *chinensis* clade was utilization of young, fresh Cajaninae seeds (Fig. 1). None of the transition rates were significantly different from zero, when tested by a backward procedure ( $-2 \ln H_0/H_1 < \chi^2_{1,0.05}$ ,  $P > 0.05$ ). When assessed with a forward procedure, however,  $p_{34}$  was significantly higher than  $p_{12}$  ( $-2 \ln H_0/H_1 = 7.95$ ,  $P < 0.01$ ), indicating that a loss of the ability to use dry beans was more likely when the host is Cajaninae compared to when it is Phaseolinae (see Fig. 2a for transitions). The opposite trend was also significant. An ability to use dry beans was more likely to be gained when the host is Phaseolinae ( $p_{21} > p_{43}$ ) ( $-2 \ln H_0/H_1 = 5.82$ ,  $P < 0.025$ ). Host shift was also conditional upon dry bean use, such that a shift from

Phaseolinae to Cajaninae was less likely to occur when using dry beans ( $p_{13} < p_{24}$ ) ( $-2 \ln H_0/H_1 = 6.38$ ,  $P < 0.025$ ).

The independent evolution model of dry bean use showed that both  $\alpha_1$  (transition rate from young to dry bean use) and  $\beta_1$  (transition rate from dry to young bean use) were significantly different from zero and were 0.662 and 0.370, respectively. In the independent evolution model of host subtribes used, the host shift rate from Phaseolinae to Cajaninae,  $\beta_2$ , was significant (0.716) but that from Cajaninae to Phaseolinae,  $\alpha_2$ , was not significantly different from zero, indicating asymmetric host shifts between the two host subtribes.

Transitions in dry bean use and tropical/temperate climate were also correlated (Table 4) and the transition rate  $q_{13}$  was significantly different from zero ( $-2 \ln H_0/H_1 = 7.60$ ,  $P < 0.01$ , Fig. 2b), indicating that habitat shifts from tropical regions to temperate regions are more likely when using dry beans. No other transition rates  $q_{ij}$  were significantly different from zero (see Fig. 2b).

Transitions in dry bean use and the length of the arid season were strongly correlated (Table 4). None of the transition



**Fig. 2** Transition rates from models of correlated evolution of characters in *Callosobruchus*. (a) Correlated evolution between dry bean use and host subtribes. (b) Correlated evolution between dry bean use and tropical/temperate climate. (c) Correlated evolution between dry bean use and arid/humid climate. (d) Correlated evolution between host subtribes and arid/humid climate. Thick arrows indicate transition rates that were significantly higher than either zero (by a backward procedure) or the rates indicated by thin arrows (by a forward procedure). Broken-line arrows are transition rates that were nonsignificant from zero. \*:  $P < 0.05$ , \*\*:  $P < 0.01$ , \*\*\*:  $P < 0.001$ .

rates were significantly different from zero, when tested with a backward procedure ( $-2 \ln H_0/H_1 < \chi^2_{1,0.05}$ ,  $P > 0.05$ ). However, with a forward procedure, beetles using dry bean were more likely to shift from humid to arid areas than were beetles using young beans ( $r_{31} > r_{42}$ ), whereas beetles using young beans were more likely to shift from arid to humid areas compared to those using dry beans ( $r_{24} > r_{13}$ ) (Fig. 2c). Arid habitat also promoted an evolutionary shift to the use of dry beans ( $r_{21} > r_{43}$ ) whereas humid habitats was associated with the reverse shift ( $r_{34} > r_{12}$ ) (Fig. 2c).

Transitions in host utilization and shifts between arid/humid habitats were significantly correlated (Table 4). Forward tests showed that the use of Phaseolinae was more likely in arid climate and use of Cajaninae was more likely in humid areas (see Fig. 2d).

Infections by *Wolbachia* was only marginally correlated with dry bean use and uncorrelated with the other traits (Table 4).

## Discussion

In general, our results suggest that there are two stable adaptive zones of evolutionary attraction within the genus *Callosobruchus* and that transitions between these are unlikely. One is to occupy humid areas with short arid season and use young and fresh beans of Cajaninae hosts. The other is to inhabit arid environments with longer arid season and use Phaseolinae hosts. It is species exhibiting the latter characteristics that show the ability to use dry and hardened beans as a food resource and thus tend to become pests of stored products. Below, we will discuss some of the implications of our findings.

Our analyses suggest that the use of Cajaninae as host plants may in effect be an evolutionary trap in *Callosobruchus*. Species using Cajaninae (with the exception of *C. chinensis*) do not use Phaseolinae even though *Vigna* are abundant in many of the areas occupied by these species (Tuda *et al.*, unpublished). In contrast, *C. maculatus* and other Phaseolinae-using species are capable of utilizing some Cajaninae species as well, at least in the laboratory (Janzen 1977; Dongre *et al.* 1993). The dry seeds of Cajaninae may have a thicker coat (Dongre *et al.* 1993) and generally seem harder to penetrate than those of Phaseolinae, and this may prevent species in the *chinensis* clade from evolving an ability to use the dry seed stages of their hosts. Similarly, the hardness of dry Cajaninae beans may limit the natural host range of species utilizing dry or mature stages of Phaseolinae beans.

The use of dry beans may also have promoted a shift from tropical habitats to temperate regions. Adaptation to dry food, either through use of metabolic water stored in bound form or through diapause, can indirectly enhance cold hardiness (Robinson 1927; Chapman 1931; Block 1996) and may thereby be associated with an ability to tolerate lower temperatures. Adaptation by bruchids to low humidity in arid areas may have further served as pre-adaptation to the use of seeds with low water content (i.e. dry beans). Conversely, adaptation to a humid climate may prevent bruchids from developing physiological tolerance against low water content of dry seeds. Alternatively, long arid seasons and the associated availability of legume seeds may tend to favour repeated reproduction, or multivoltinism, in bruchids. In the process of losing reproductive diapause, difficulty in lengthening adult lifespan under aridity may have contributed to gaining reproductive ability without feeding (Watanabe 1990). There is, however, at least one exception to this generalization. *C. latealbus* inhabits humid areas but is able to feed on dry host seeds. This may be due to its basal position in the genus or, alternatively, to infections by *Wolbachia*. For the latter, careful experimental studies are needed to elucidate the potential importance of such symbionts.

Recent comparative studies on bruchid beetles have concluded that this group shows fairly high taxonomic

conservatism in host use, but that host shifts to distant taxa do occur during their evolution (Kergoat *et al.* 2004, 2005a, b). The results of our study agree well with this general conclusion. Major *Callosobruchus* clades specialize on particular host subtribes and while host shifts to different taxa are rare they do occur. A host-selection experiment using the two subtribal hosts (*Vigna* vs. *Cajanus*) shows evolutionary lability of adult preference (behavioural adaptation) and evolutionary conservatism in larval performance (physiological adaptation) in *C. maculatus* (Wasserman & Futuyma 1981), and the latter could help maintain host specificity (see also Credland 1987; Tucic *et al.* 1995; for intrageneric host selection, see Kawecki & Mery 2003; Messina 2004). Our study also highlights the uniqueness of *C. chinensis* within the *chinensis* clade. This species apparently shows adaptations that allow it to utilize a wide range of maturing stages of host beans and to have a wide natural host range and a large geographical distribution (Tuda *et al.* 2005). The nature of the adaptations that provide *C. chinensis* these capabilities requires further investigation, including its interaction with *Wolbachia* and the factors discussed below.

For bruchids pre-adapted to utilize dry seeds, proximity to human stores of dried seeds (Cotton 1956) and polymorphism in dispersal and reproductive traits (Utida 1954, 1967, 1972, 1981; Kiritani 1955; Caswell 1960; Nakamura 1966; Sano 1967; Ouedraogo & Huignard 1981; Messina 1984; Sano-Fujii 1984, 1986; Umeya 1987) should have further promoted evolution towards gaining pest status. The level of secondary metabolites in beans decreases during the process of drying and the stores of many potential host beans in close proximity may have contributed to a decreased level of host selectivity by adults, thus resulting in the widening of host range seen in, for example, *C. chinensis* and *C. maculatus*.

Janzen (1969; 1981) stressed morphology, phenology and the chemistry of seeds as the most important factors determining interactions between bruchids and their host plants. Secondary metabolites, such as nonprotein amino acids, can certainly serve as chemical defence against herbivores including granivores (Birch *et al.* 1989; Gatehouse *et al.* 1990). We note that Phaseoleae species lack a toxic secondary compound, L-canavanine, observed in other Faboideae (Rosenthal 1977; Bell *et al.* 1978; Bisby *et al.* 1994), which could help explain the fact that the host range of *Callosobruchus* is limited to Phaseoleae. In support of this suggestion, bioassay tests have shown lethal effects of canavanine on the larvae of *C. maculatus* (Janzen *et al.* 1977; Oliveira *et al.* 1999). Likewise, it is possible that chemical defence substances may limit the host range of the seed predators of monophyletic Cajaninae (cf. Dongre *et al.* 1993). Furthermore, morphological traits of hosts, such as the size and texture of beans, can play an important role as proximate cues for host recognition by adult beetles (e.g. in *C. chinensis*, Ishii

1952; in *C. maculatus*, Sulehrie *et al.* 2003; in other bruchine, Szentesi 2003).

In summary, our study indicates that adaptation to a long arid climate tends to make potential pest species more likely to gain stored-bean pest status. However, our results also show that idiosyncrasies of particular taxa, such as specific adaptations to their natural hosts, play an important role.

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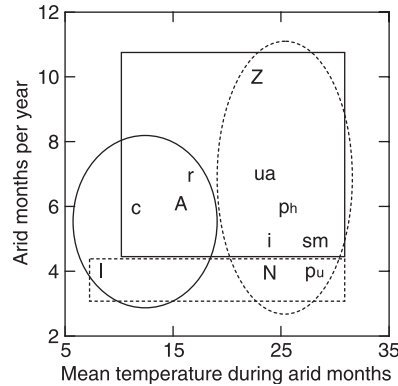
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MT pursues evolutionary ecology of plant-herbivore and herbivore-predator (or parasitoid) interactions, using pest insects and their natural enemy. JR and GA are interested in sexual selection and male-female coevolution. SB's interest lies in agricultural entomology in general. NW is working on microbial control of insect pests by using *Bacillus thuringiensis*.

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## Appendix I



Clustering of species' habitats according to the mean temperature ( $^{\circ}\text{C}$ ) and the number of arid months (rainfall  $< 50$  mm/month) per year. Solid line circle: temperate; dashed line circle: tropical; solid line square: arid; dashed line square: humid. Habitat codes for the different species are; c, *chinensis*; pu, *pulcher*; a, *analis*; r, *rhodesianus*; m, *maculatus*; s, *subinnotatus*; l, *latealbus*; ph, *phaseoli*; i, *imitator*; u, *utidai*; A, *A. obtectus*; Z, *Z. subfasciatus*; and N, Northern Thailand (*semigriseus*, *dolichosi*, *nigripennis*, *theobromae*).

## Appendix II

Aridity and temperature of the habitats of *Callosobruchus* species. As a typical habitat, we considered a location in which a species was abundant and frequently observed or, alternatively, where the type specimen was collected. Aridity was measured as the number of months with low rainfall ( $< 50$  mm/month) per year

Species	Habitat location	Months with low rainfall	Mean temperature ( $^{\circ}\text{C}$ ) during the arid months
<i>chinensis</i> clade			
<i>C. chinensis</i>	Kunming, China	6	11.4
<i>C. utidai</i>	Patna, India	7	23.0
<i>C. semigriseus</i>	Chiang Mai, Thailand	4	23.6
<i>C. dolichosi</i>	Chiang Mai, Thailand	4	23.6
<i>C. nigripennis</i>	Chiang Mai, Thailand	4	23.6
<i>C. theobromae</i>	Chiang Mai, Thailand	4	23.6
<i>C. pulcher</i>	Manila, Philippines	4	28.0
<i>maculatus</i> clade			
<i>C. analis</i>	Port Louis, Mauritius	7	24.2
<i>C. rhodesianus</i>	Harare, Zimbabwe	7	16.7
<i>C. maculatus</i>	Abuja, Nigeria	5	27.9
<i>C. subinnotatus</i>	Kumasi, Ghana	5	27.3
Paraphyletic group			
<i>C. latealbus</i>	Guiyang, China	4	8.2
<i>C. imitator</i>	Mae Hong Son, Thailand	5	24.0
<i>C. phaseoli</i>	Mahajanga, Madagascar	6	25.6
Outgroup			
<i>A. obtectus</i>	Mexico City, Mexico	6	15.5
<i>Z. subfasciatus</i>	Los Cabos, Mexico	10	22.7