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Dual chemical sequestration: a key mechanism in transitions among ecological specialization

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Platyphora leaf beetles form a vast group of tropical species each feeding on a restricted set of host plants and exhibiting bright coloration warning predators against their chemical protection. These beetles offer an exceptional opportunity for understanding the evolution of phytochemical sequestration. Indeed, qualitative studies of defensive secretions indicate that *Platyphora* species acquire toxicity via sequestration of plant secondary metabolites. All produce pentacyclic triterpene saponins from sequestered plant amyrins, but our analyses also indicate that many *Platyphora* species produce a dual chemical defence, that is, they are additionally protected by lycopsamine-type pyrolyzidine alkaloids that they also sequester from their host. This paper reports on the evolution of chemical defence and host affiliation in *Platyphora* leaf beetles as reconstructed on a molecular phylogeny of mitochondrial and nuclear DNA sequences. The analyses indicate that dual sequestration could be the key mechanistic means by which transitions among ecological specializations (i.e. restricted host-plant affiliations) are made possible.

Keywords: chemical defence; toxin sequestration; ecological specialization; molecular phylogeny

1. INTRODUCTION

Although animals can acquire toxicity by *de novo* synthesis of active compounds, there is increasing evidence that the bioaccumulation of natural poisons can lead to an efficient chemical defence. This latter strategy can be achieved by feeding on chemically protected prey or by entering into a symbiotic association with toxic micro-organisms (Mebs 2001). Sequestered compounds are taken from an exogenous source and used by the sequestering organisms as precursors of, among others, toxins, deterrents and pheromones (Duffey 1980; Eisner & Meinwald 1995). Although both autogenous and exogenous defensive strategies are successful in plant and animal evolution (Duffey 1980; Adler 2000), *de novo* biosynthesis of toxins seems to be much more frequent than sequestration with the possible exception of herbivorous insects. When physiological and ecological parameters are examined, sequestration of pre-existing toxins is not necessarily 'cheaper' or 'simpler' than their endogenous biosynthesis. Indeed, an organism that actively accumulates toxic metabolites from another not only has to develop various adaptations to detoxify, accumulate and use xenobiotics (Duffey 1980), but also has to rely, at least temporarily, on a diet allowing it to reach a sufficient level of toxicity or to accommodate toxin-producing symbionts. Although the source of toxins is usually actively searched for by the sequester, cases of purely passive or opportunistic toxicity acquisitions have been reported: for example, contamination of fishes by toxin-producing micro-organisms (Mebs 2001) or sequestration of man-made pesticides (Eisner *et al.* 1971).

The use of exogenous metabolites for chemical defence requires a complex suite of adaptations. Such a series of characters is unlikely to frequently co-develop, and this is probably the reason that exogenous strategy is not more widespread. Nevertheless, the external acquisition of toxins and their subsequent use in chemical defence have convergently evolved in widely divergent animal lineages including birds (Dumbacher *et al.* 2000), amphibians (Daly *et al.* 1997), molluscs (Cimino & Ghiselin 1998), fishes and various aquatic (Mebs 2001) and terrestrial (Duffey 1980) arthropods.

Chrysomelid leaf beetles (Coleoptera: Chrysomelidae) constitute a very large group of species (more than 30 000), each feeding on a restricted number of plant taxa. Leaf beetles have developed various antipredation mechanisms, some of which resort to chemical defence. As most plants are chemically protected, chrysomelids have developed various detoxification mechanisms. Yet, many chemically protected chrysomelids biosynthesize *de novo*, rather than sequester, defensive compounds (Pasteels *et al.* 1988). Investigations of plant-chemical sequestration in leaf beetles indicate this strategy evolved in several lineages of this family (Hsiao & Pasteels 1999; Termonia *et al.* 2001). Whatever the prerequisites are to sequester host-plant toxins, a possible important corollary is that these adaptations could increase the efficiency with which an ecological niche is used, but could also prevent the species from shifting to new host plants.

Tropical *Platyphora* leaf beetles (subfamily Chrysomelinae, tribe Chrysomelini) constitute an exceptional model for the analysis of both the constraints and the evolvability of ecological affiliations associated with the ability to sequester phytochemicals. Indeed, each known species belonging to this vast genus is chemically protected through the sequestration of plant secondary metabolites

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(Pasteels *et al.* 2001) and feeds on a restricted set of host-plant species from one out of six families: Boraginaceae; Asteraceae; Asclepiadaceae; Convolvulaceae; Solanaceae; or Apocynaceae. Here, the evolution of chemical defence and host affiliation in *Platyphora* leaf beetles as reconstructed on a molecular phylogeny of mitochondrial (mt) and nuclear (nu) DNA data is reported.

2. MATERIAL AND METHODS

Genomic DNA was extracted from ethanol-preserved single adult individuals. We based our phylogenetic analyses on nucleotide sequences from three mt and one nu gene fragments from 21 *Platyphora* specimens (19 species) and three non-ambiguous outgroup species (*Calligrapha fulvipes*, *Stilodes fuscolineata* and *Stilodes* sp.). The polymerase chain reaction (PCR)-amplified (FastStart Taq, Roche, Mannheim, Germany) target DNA fragments were:

(i) a 504–550 bp segment of the mt 12S ribosomal RNA (12S) (primers SR-N-14759 and SR-J-14233 from Simon *et al.* (1994));
(ii) a 536–700 bp segment of the mt cytochrome oxidase I gene (COI) (C-J-1751 and reverse complement of C-J-2441 from Simon *et al.* (1994));
(iii) a 582–641 bp segment of the mt cytochrome oxidase II gene (COII) (modTL2-J-303 and modC2-N-3661 from Termonia *et al.* (2001)); and
(iv) a 430–572 bp segment of the nu phosphoenolpyruvate carboxykinase gene (PEPCK) (primers: at040201F1, 5′-TTTCAGATTTTGGGAATCACC-3′; at040201R1, 5′-GWMCCAATTRTTACCACGCC-3′; at040201F2, 5′-AAGAAAAGATACATCGCTGC-3′; at040201R2, 5′-CATCCCTTCCCAAAACACTC-3′).

For the PEPCK fragment, the outgroup was represented by a fourth species (*Desmogramma subtropica*) because PCR amplifications failed in the three others. Direct sequencing (dRhodamine Cycle Sequencing, electrophoresis on ABI 377; Applied Biosystems, Foster City, CA, USA) of complementary strands was performed on different PCR products. However, only a 284 bp portion of 12S from *P. vespertina*, a 109–175 bp portion of COI from *Platyphora* sp. and *P. decorata*, and a 215/180/197 bp fragment of PEPCK from *P. petulans*, *P. eucosma* and *Platyphora* sp. could be sequenced because of secondary structure problems. These fragments were sequenced twice (but on the same strand) on two different PCR products. Missing data represent less than 3% of the analysed dataset. All sequences reported in this paper were deposited at GenBank under accession numbers AY055489–AY05581. Alignments of the protein-coding genes (COI, COII and PEPCK exons) were trivial and MacClade (v. 3.05) (Maddison & Maddisson 1992) was used to determine their open reading frames (ORFs). The program Soap (Loytynoja & Milinkovitch 2001) was used to produce one 12S alignment for each of the 25 different sets of alignment parameters (weighted matrix, gap-opening penalties from 11 to 19 by steps of two and extension penalties from 3 to 11 by steps of two). Any positions where the alignments differed were excluded (Loytynoja & Milinkovitch 2001). Similarly, the intron portion (69–247 bp) of the PEPCK fragment was excluded from the analysis. A 5% χ^2-test indicated that none of the sequences significantly differed in nucleotide composition from the distribution estimated in the maximum-likelihood (ML) model implemented in Puzzle v. 4.0.2 (Strimmer & Haeseler 1996).

All maximum-parsimony (MP) analyses (unweighted and weighted) were performed with Paup* (Swofford 2000) with heuristic searches. The tree bisection–reconnection (TBR) branch-swapping algorithm was used and the MULPARS and COLLAPSE options were used. We checked whether starting with 'simple' and 'closest' stepwise-addition sequences yielded identical trees. We also performed 10 000 replicates both with random starting trees and with starting trees obtained by random stepwise addition. The stability of the cladograms was tested with the Goloboff fit criterion (Goloboff 1993) ($k = 0$, 2, 4, 6 and 8). The stability of the various inferred clades was estimated by bootstrapping (400 replicates) (Felsenstein 1985) and by computing for all branches the Bremer support (BS) (Bremer 1994) using the program Treerot v. 2 (Sorenson 1999). *A priori* topology-dependent permutation tail probability (T-PTP; Faith 1991) analyses were performed to statistically test monophyly of a 'PA clade' (suggested by biochemical data (Hartmann *et al.* 2001)). We also compared unweighted- versus weighted-parsimony analyses (substitution types for which saturation was obvious from saturation plots were excluded or downweighted). All MP analyses were separately performed with each of the four gene fragments and with the combined dataset (12S + COI + COII + PEPCK). Partition homogeneity tests (1000 replicates) (incongruence length difference; Farris *et al.* 1994) on COI versus COII, 12S versus COI + II and nu versus mt, did not indicate significant incongruence among these datasets ($p = 0.29$, 0.78 and 0.66, respectively).

The ML method of phylogeny inference was also used on the combined (mt + nu) dataset with the following Paup* settings: transition to transversion (Ti : Tv) ratio and proportion of invariable sites (P_{inv}) estimated by the ML Hasegawa–Kishino–Yano (HKY) model (Hasegawa *et al.* 1985) with the rates for the variable sites assumed to follow a four categories γ-distribution with shape parameter estimated by ML, empirical nucleotide frequencies and sub-tree pruning–regrafting branch-swapping. ML bootstrap analyses (400 replicates, simple stepwise addition, TBR swapping) were performed with the HKY model after constraining the Ti : Tv, P_{inv}, and γ-distribution shape parameters from the ML tree of the original non-resampled dataset. Alternative phylogenetic hypotheses were compared statistically by means of Kishino–Hasegawa (KH) ML ratio tests (Kishino & Hasegawa 1989).

Neighbour-joining (NJ) analyses were performed on the combined dataset (including bootstrapping, 400 replicates) with Paup* using LogDet distances (Lockhart *et al.* 1994) to correct for possible differences in the base composition among the lineages. Any sites estimated to be invariable by ML were removed prior to distance calculations.

Pyrrolizidine alkaloids (PAs) and pentacyclic triterpene saponins (referred to as saponins throughout the text) chemical analyses that were not already characterized in Pasteels *et al.* (2001) were carried out as described in that study. The evolution of chemical defensive strategies and host-plant affiliations was parsimoniously reconstructed.

3. RESULTS

The unweighted MP analyses of the combined dataset yield one best tree whose rooted phylogram is shown in figure 1*a*. All species sequestering the PAs form a monophyletic group (hereafter called the 'PA clade' and shown

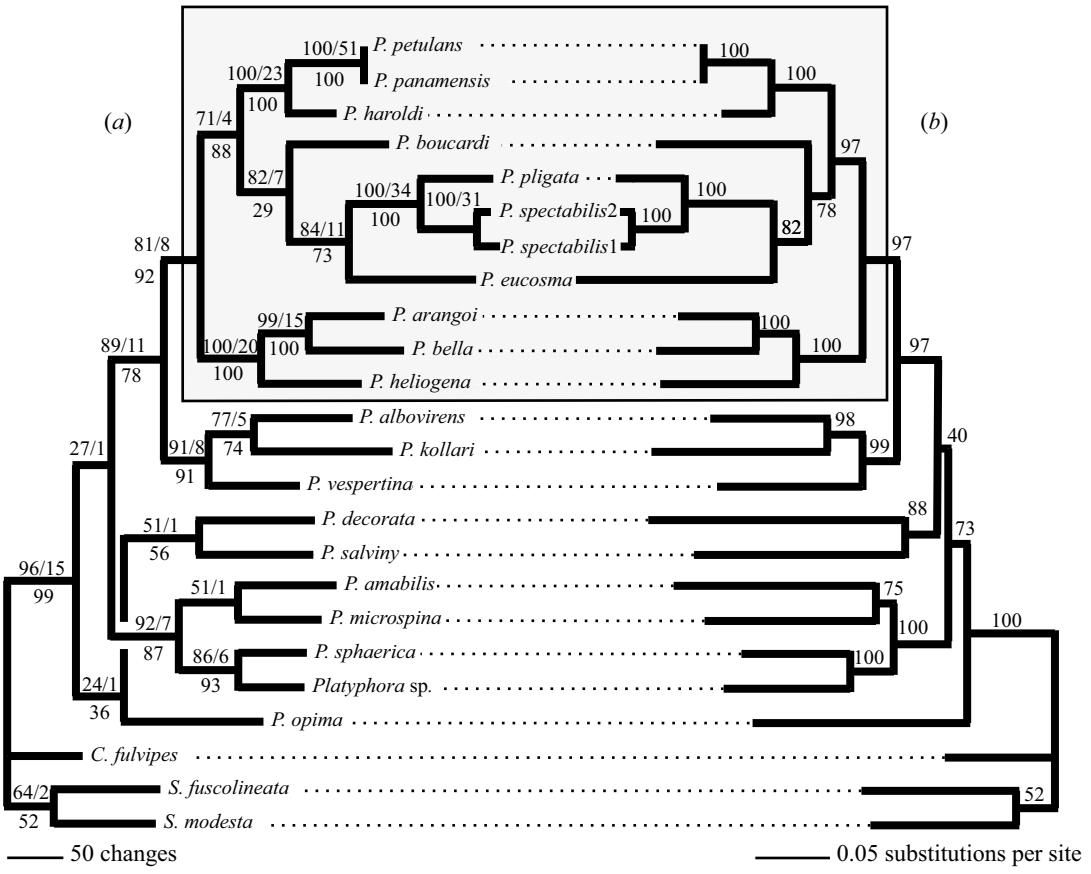


Figure 1. (*a*) MP phylogram (tree length of 2779) among *Platyphora* leaf beetles; numbers indicate MP bootstrap values and BS (above branches) and NJ LogDet bootstrap values (below branches). (*b*) ML phylogram ($-\ln L = 14977.334$, Ti : Tv = 1.778, $P_{inv} = 0.46$, γ-distribution shape parameter = 0.592) with bootstrap values indicated above the branches. Outgroup species belong to the genera *Calligrapha* (*C.*) and *Stilodes* (*S.*). The box indicates the 'PA clade', that is, species sequestering lycopsamine PAs.

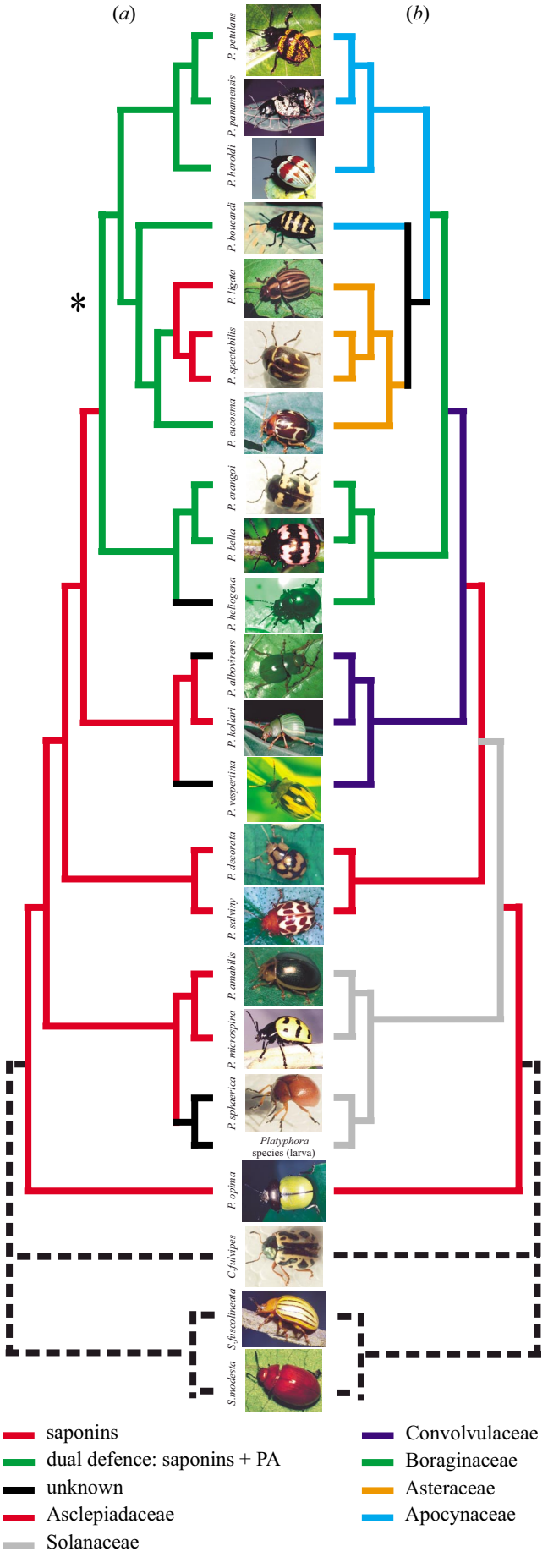
by the shaded box in figure 1) supported by bootstrapping (bootstrap values; BV = 81), a Bremer support (BS) of 8 and significant T-PTP ($p < 0.001$). The strict consensus among the trees obtained under weighted MP analyses (transitions ignored or weighted three times less than transversions) is fully compatible with the MP tree shown in figure 1*a*. All branches are stable to Goloboff weighting ($k = 3-8$) except under strong weighting of noisy characters ($k = 0$ and 2) for which a slightly different topology is obtained (figure 1*b*). This same alternative topology is obtained under ML analysis on the combined (mt + nu) dataset (figure 1*b*, bootstrap values indicated above the branches). NJ analyses of the combined dataset using LogDet distances ($P_{inv} = 0.46$) give topologies largely compatible (i.e. for all nodes supported by a BV of more than 51) with those obtained under MP and ML (BV under NJ are indicated under the branches of figure 1*a*). All analyses strongly support (BV = 97 under ML) the monophyly of the PA clade. Constraining the non-monophyly of the PA clade entails a significant decrease in likelihood ($\delta \ln L = 18.0$; $P_{KH} = 0.05$). All analyses after the exclusion of characters unstable to variations of alignment parameters (Loytynoja & Milinkovitch 2001) yield results very similar to those shown in figure 1. The topology differences observed among the MP, ML and NJ approaches only affect nodes supported by very low BV, that is, lineages associated with Solanaceae and Asclepiadaceae, and have no impact on the following discussion.

4. DISCUSSION

The mass spectrometry analyses indicate (figure 2*a*) that all studied species sequester amyrins that they metabolize into saponins found in their defensive secretions. The beetles occurring on host plants containing lycopsamine PAs (from the families Apocynaceae, Boraginaceae and Asteraceae) additionally sequester these chemicals and exhibit a dual chemical defence based on both saponins and PAs. MP reconstruction of the evolution of *Platyphora* adult chemical defence (figure 2*a*) on any of the MP, ML and distance trees discussed in §3 indicate that

(i) the sequestration of PAs is a derived character that evolved only once, a result compatible with the observation that these species sequester alkaloids through an original biochemical mechanism (Hartmann *et al.* 2001);
(ii) despite the rise of this PA-based strategy, many species combined it with the ancestral saponin-based chemistry into a dual sequestration system; and
(iii) a lineage within the PA clade experienced a reversal in its defence strategy, that is, they only sequester amyrins.

Amyrins are triterpenes found in the epicuticular wax of more than 57 plant families. Phytochemical constraints related to these compounds cannot account for the fact that the *Platyphora* species studied here are restricted to



only six of the amyrin-containing plant families. Moreover, mapping of plant affiliations on the molecular phylogeny of *Platyphora* indicates that host affiliation is very conservative at the plant-family level (figure 2*b*). Lycopsamine PAs have only been detected in five plant families (Hartmann & Witte 1995); three of these act as hosts for *Platyphora* species. MP reconstruction of host-plant affiliations (figure 2*b*) suggests a minimum of six independent host-plant family shifts.

Two previous works on leaf beetles (Hsiao & Pasteels 1999; Termonia *et al.* 2001) demonstrated that the transition from one chemical defence to another occurred through the development of a dual defence. We suggest here that these analyses combined with the present study indicate that the dual-defence system, *de facto*, increases the evolvability of host affiliation and of chemical protection. Indeed, the rise of a dual-defence strategy is a means by which a lineage could either switch to a new phytochemical bridge (involving a new, albeit potentially overlapping, set of host plants) or evolve back to the ancestral metabolism (cf. figure 3). We suggest that such transitions among single-defence metabolisms via a dual chemical strategy is a general pattern in the evolution of chemical defence and host-plant affiliation, as it was observed in three independent lineages characterized by very different chemistries (Hsiao & Pasteels 1999; Termonia *et al.* 2001; this study). Furthermore, quantitative gas chromatography–mass spectrometry analyses (Pasteels *et al.* 1996; Termonia & Pasteels 1999) indicated that some leaf beetles exhibiting a dual defence are able to downregulate one of the two defensive metabolisms according to the relative proportions of plant precursors available in the host. If such regulation is indeed adaptive, the dual defence could be a somewhat unstable state, albeit requisite to shifts among more specialized ecological states (i.e. single-defence metabolisms). This pattern could be a very general mechanism by which shifts, not only among defensive strategies, but also among other physiological specializations requiring detoxification and/or sequestration of exogenous compounds, are taking place. Indeed, host-derived chemical defence is a remarkable solution to a general problem that all phytophagous insects must face: plant-chemical detoxification.

There is an emerging understanding that evolutionary principles have practical applications in many areas, including conservation and management (Rausher 2001). We suggest that the evolutionary sequence of chemical

Figure 2. (*a*) Parsimonious reconstruction of chemical sequestration and defensive strategies on the ML tree from figure 1*b*: red, sequestration of just the plant amyrins used as the precursors of saponin-defensive secretions; green, dual sequestration of both lycopsamine-type alkaloids and amyrins from food plants containing these two secondary metabolites (i.e. within the plant families Boraginaceae, Asteraceae and Apocynaceae). Species from the lineage indicated by an asterisk within the PA clade experienced a reversal in their chemical defence and sequestered amyrins only. However, these taxa are still able to detoxify alkaloids (see § 4 for details). (*b*) MP reconstruction of food plant family affiliations (identification below the family level has not been performed yet). Photographs show an adult specimen of each corresponding species.

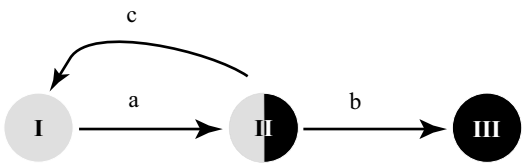


Figure 3. Proposed general evolutionary pattern of chemical defensive strategies in leaf beetles. I is the ancestral defence based on either an autogenous metabolism (Hsiao & Pasteels 1999) or a sequestered plant toxin (Termonia *et al.* 2001; this study). II is a dual-defence strategy based on the coexistence of either sequestration and autogenous synthesis (Hsiao & Pasteels 1999) or two exogenous metabolisms (this study). The dual defence is potentially compatible with a broader range of host-plant affiliations. III is a derived single-defence strategy (independent of I) based on either an autogenous metabolism or a sequestered plant toxin (Termonia *et al.* 2001). Arrows indicate possible state transitions: a, from an ancestral single-defence strategy to dual defence; b, from dual defence to a derived single-defence strategy; c, from dual defence back to the ancestral single-defence metabolism. Direct transition between two single-defence strategies can exceptionally occur when the two corresponding metabolisms are mutually exclusive (Termonia *et al.* 2001).

defence and host affiliation should be considered in the management of natural and cultivated populations. For example, the *Platyphora* leaf beetles that experienced reversal of their defence strategy (from dual to saponin based; cf. figure 2*a*) feed on some of the many species of Asteraceae-lacking lycopsamine PA compounds. However, laboratory experiments indicate that these insects maintained the ancestral ability to tolerate PAs (T. Hartmann and J. M. Pasteels, unpublished data), although they did not retain the capacity to secrete these compounds in their defensive secretions. Furthermore, on the five plant families that contain lycopsamine-type PAs—Apocynaceae, Asteraceae, Boraginaceae, Santalaceae and Sapotaceae—the first three are reported to act as hosts for *Platyphora* beetles that possess the key innovation for the sequestration of PAs. However, the genus *Platyphora* is poorly known (this study presents, for the first time to our knowledge, data on their host-plant affiliations) and certainly one can not exclude the possibility that beetles of the PA clade could feed on at least some of the Santalaceae and Sapotaceae available in their tropical distribution.

We think that investigations of host-plant associations in an evolutionary perspective go far beyond the inference of past events. Indeed, evolutionary patterns, such as the one uncovered here, have the potential to help develop models estimating the host shifts that are most likely to occur. This could help one to take management actions to reduce the risk and impact of shifts, for example towards plants of commercial value.

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