Adaptive evolution of cryptic coloration: the shape of host plants and dorsal stripes in *Timema* walking-sticks

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The adaptive significance of cryptic colour patterns has seldom been analysed in a phylogenetic context. We mapped data on the presence vs. absence of dorsal stripes, and the use of needle-like vs. broad foliage, onto a recent phylogeny of *Timema* walking-sticks, in order to infer the evolutionary history of these traits and test the hypothesis that the dorsal stripe is an adaptation for crypsis on needle-like leaves. By maximum parsimony optimization, the dorsal stripe has evolved five or six times in this clade, each time in association with the use of vegetation with needle-like leaves. Maddison's concentrated changes test showed that this association between morphology and habitat was statistically significant. By contrast, results based on Pagel's maximum likelihood analyses did not reach significance, probably because the large number of origins of dorsal stripe introduces statistical uncertainty. These results suggest that the adaptations for crypsis can arise readily and in parallel, in the appropriate selective environment. However, they may also constrain the evolution of host-plant use, as there is no unambiguous case of *Timema* species with dorsal stripes shifting to broad-leaved plants. © 2008 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2008, **94**, 1–5.

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INTRODUCTION

Crypsis, or the adaptation of reducing visibility to predators, is one of the most common defensive strategies in insects and other animals that are heavily preyed upon (Poulton, 1909; Thayer, 1909; Cott, 1940; Edmunds, 1974; Endler, 1978). This survival strategy has been demonstrated in some species to be subject to strong selection (e.g. Steward, 1977; Endler, 1984; Brakefield, 1987; Brodie, 1989; Sandoval, 1994a; Majerus, Brunton & Stalker, 2000), sometimes with a simple genetic basis (e.g. Sandoval, 1994a; Hoekstra & Nachman, 2003). However, there is little information about how adaptations to crypsis evolve over evolutionary time in a phylogenetic context (e.g. Descimon, 1986; Armbruster & Page, 1996). The phylogenetic analysis of crypsis is important because it is primarily via phylogenetics that we can understand the evolutionary lability of cryptic colour patterns, how they evolve in relation to long-term change in environments, and how among-species diversity in adaptations related to crypsis is generated and maintained.

Cryptic insects such as caterpillars and grasshoppers often have a dorsal stripe. Such stripes are thought to mimic specific leaf patterns or shapes (Thayer, 1919), or to modify the appearance of the shape of the prey through disruptive coloration (Poulton, 1909; Cott, 1940; Rienks, 1992). The effectiveness of a stripe in concealing its bearer, as for most colour patterns, depends on the environment. For example, a dorsal stripe may be highly visible on a uniform background, but effective for crypsis on grasses, which are rich in linear shapes (e.g. Rienks, 1992).

In this paper we used a molecular phylogeny to infer how dorsal stripes have evolved, and test for an adaptive association between dorsal stripes and the background (the morphology of host-plant leaves) in *Timema* walking-sticks (Insecta: Phasmatoptera). In particular, we evaluate the hypothesis that needle-shaped leaves have convergently selected for dorsal stripes among diverse lineages of *Timema*, and that

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this convergence has been driven by selection for crypsis.

MATERIAL AND METHODS

Walking-sticks of the family Timemidae comprise 21 described species, all found in the western USA and in Baja California, Mexico. They feed on the leaves of their host plants at night and rest on the leaves or twigs during the day. *Timema* are typically green, brown, or grey, and may exhibit patterns such as stripes, scales, or dots, which help them to closely resemble their host plants (Sandoval, 1994a, b).

A *Timema* species may feed on one or several host plant species. The host plants upon which polyphagous *Timema* species live are not closely related chemically or taxonomically (Crespi & Sandoval, 2000). The hosts can also vary strikingly in appearance, in both the colour and shape of leaves. For example, in *Timema cristinae*, one host, chamise (*Adenostoma fasciculatum*), has short dark-green needle-like leaves and brown bark, whereas another host, *Ceanothus spinosus*, has broad oval light-green leaves and green stems. A green striped morph is associated with chamise, whereas a plain green morph is associated with *Ceanothus* (Sandoval, 1994a, b; Nosil, Crespi & Sandoval, 2002). Birds and lizards prey differentially on these morphs, with the striped morph surviving predation more effectively on *Adenostoma*, and the unstriped morph surviving better on *Ceanothus* (Sandoval, 1994a, b; Nosil, 2007). Other species of *Timema* have a dorsal stripe, whereas most do not bear a stripe.

We collected and photographed individuals of all known species of *Timema* walking-sticks, and recorded the presence or absence of dorsal stripes. We also recorded whether the host plant had needle-like or flattened broad leaves. For most species, we also recorded whether the insects rested on the leaves, twigs, or trunks of their host plants. We classified leaves as needle-like if they were cylindrical and several times longer than wide. The surface of broad leaves was oval shaped, so that they were less than two times longer along the axis than they were wide. In addition, broad leaves were thin in cross section.

The phylogeny used here (Fig. 1) was derived from the cytochrome oxidase I data in Crespi & Sandoval (2000) and Law & Crespi (2002), with the addition of new sequences for *Timema* sp. nov. on Limber pine (*Pinus flexilis*) and *Timema coffmani* on juniper. This data set includes 416 base pairs of the cytochrome oxidase I gene for 165 specimens. For the Law & Crespi (2002) data, species in the 'northern clade' (*Timema douglasi*, *Timema poppensis*, *Timema californicum*, *Timema shepardi*, *Timema knulli*, *Timema petita*, and *Timema landelsensis*) were each constrained to be sister taxa to their morphologically indistinguishable sexual relative (i.e. *T. douglasi* with *T. poppensis*, *T. shepardi* with *T. californicum*, *Timema genevieve* with *Timema podura*, and *Timema tahoe* with *Timema bartmani*); these constraints were necessitated by the extensive paraphyly and polyphyly among the recently evolved more-northerly species of *Timema* (Law & Crespi, 2002). Neighbour-joining bootstrap values (200 replicates) are shown on Figure 1, for values above 50%. We evaluated the robustness of the statistical results to uncertainty in the phylogeny by moving the relevant branches and repeating the analyses, as noted below.

We used two analytic methods to test our main hypothesis: that the use of plants with elongate, needle-like leaves select for dorsal stripes. First, we used Maddison's (1990) concentrated changes test to determine whether the origins of the dorsal stripe are more frequent along lineages of *Timema* that use one or more host plants with needle-like leaves, than along lineages that use broad leaves. By this method, which is essentially a phylogenetically based randomization test, ancestral states are inferred using maximum parsimony. Second, we used Pagel's (1994) maximum likelihood test for association among discrete variables. This method does not assume a particular set of ancestral states for the analysis, but instead estimates transition rates from the data, and uses a probabilistic model for inferring the likelihood of joint changes in foliage form and stripe presence, vs. a model of independent evolution for the two traits. As such, Pagel's test is considerably more conservative, especially with multiple origins of the two traits. For both methods, we first analysed our full data set, and then conducted an analysis after removing the four species (*Timema ritensis*, *T. podura*, *T. genevieve*, and *T. coffmani*) that are known to rest on trunks or stems, rather than foliage. Branch lengths were set to one (all equal) for both forms of analysis.

RESULTS

Of the 21 known *Timema* species, eight species bear a dorsal stripe and 13 species lack a stripe (Table 1). Of those bearing a stripe, all were associated with at least one host plant species that had needle-like leaves. Six species did not have a dorsal stripe, but used host plants with needle-like leaves. Of those six species, four (*T. podura*, *T. genevieve*, *T. ritensis*, and *T. coffmani*) were brown, grey, or beige, and rested on the trunk instead of the leaves of their host plants. Only two species, *T. nakipa* and *T. boharti*, had green morphs without a stripe, and used host plants with needle-like leaves. Both species are generalists, with chamise being one of several hosts.

By the concentrated changes test on the full data set, there were either six origins and no losses of the dorsal
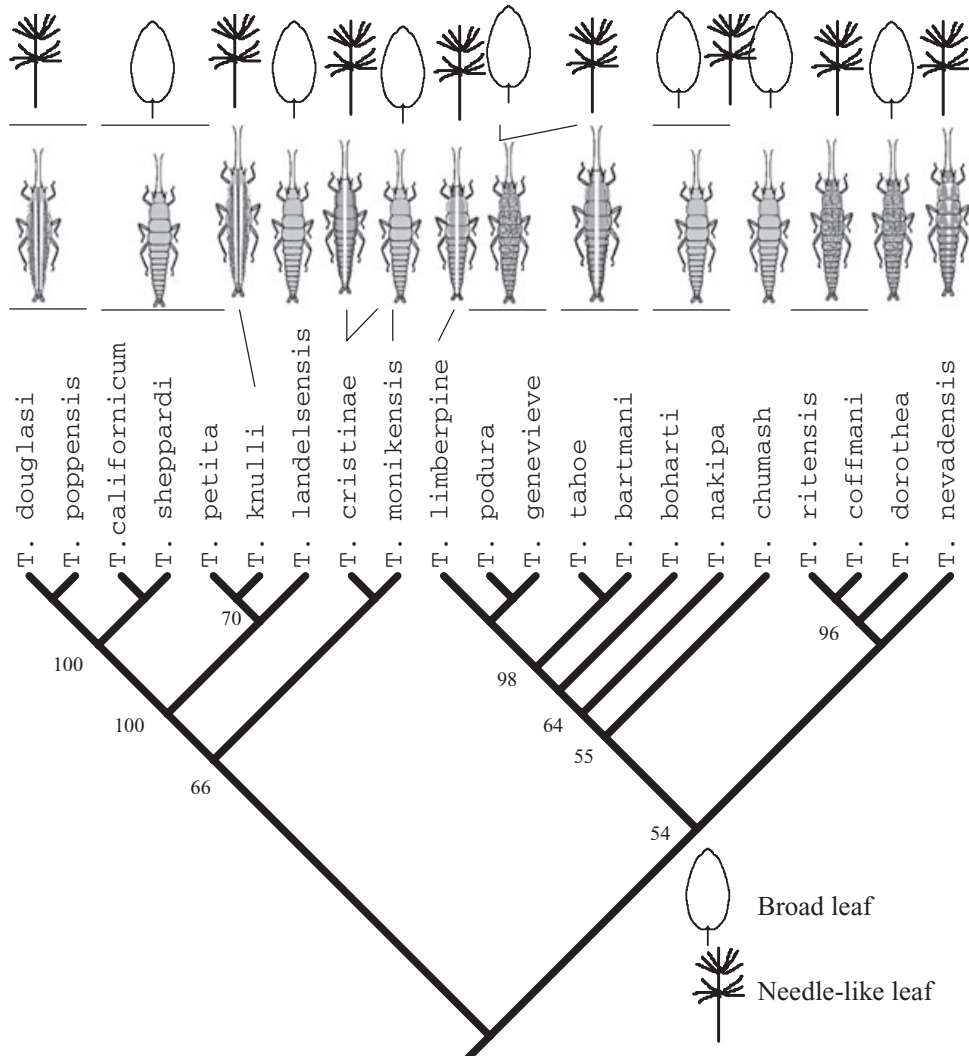
Figure 1. Molecular phylogeny of *Timema* species, with the colour pattern of each *Timema* species and the shape of the leaf of the host plants illustrated above the branch tips. The horizontal lines below the insects indicate that the *Timema* species listed below the line are of the same colour pattern (plain or striped). The horizontal line below the plant symbols indicate that the *Timema* species listed below the line feed on the host plants above the line. *Timema cristinae* has both striped and plain morphs, so it has vertical lines leading to each morph. *Timema boharti* and *Timema nakipa* each feed on broad and needle-like leaves. The statistics for the association between colour pattern and type of leaf is described in the text.

Table 1. Counts of species of *Timema* walking-sticks that are either green, with or without a dorsal stripe, or grey without a stripe, and the morphology of the plant on which they rest

Resting place	Walking-stick		
	Green striped	Green unstriped	Gray unstriped
Leaves – needle like	8	2	0
Leaves – broad	0	7	0
Trunk	0	0	4

stripe, or five origins and one loss, in the common ancestor (*T. podura*, *T. genevieve*) (Fig. 1). Under both scenarios, all of the origins of the stripe were inferred to have taken place on lineages where the *Timema* used plants with needle-like leaves. The probability of six origins on branches with needle-like leaves occurring by chance is 0.013, and the probability of five origins and one loss is 0.011. The statistical significance of these results was robust to alternative phylogenetic placements (e.g. of the species in the clade on juniper, the clade *T.* sp. nov. on limber pine + *T. podura* + *T. genevieve* + *T. tahoe* + *T. bartmani*, and the northern clade). Similar results were obtained from analysis of the data set excluding the four species known to rest on trunks or stems. For this data set, we inferred five origins (and no losses) of the stripe, all on branches inferred to have needle-shaped leaves (*P* = 0.0068). By Pagel's test for correlated evolution, analysis of the full data set, comparing a model of correlated evolution vs. independent evolution for the two traits, gave a log likelihood ratio of 5.0 (d.f. = 4, *P* = 0.29), and analysis of the reduced data set (excluding the four species known to rest on trunks or stems) yielded a ratio of 7.31, which approached statistical significance (d.f. = 4, *P* = 0.12).

DISCUSSION

This is the first phylogenetic, statistically based study on the adaptive significance of cryptic coloration in herbivorous insects. Our analyses show that the dorsal stripe of *Timema* walking sticks has evolved five or six times, in each case being associated with use of a host-plant species with elongate foliage. This pattern of parallel evolution is also supported by the morphological differences between the six striped species, as the stripe varies among species in width, colour, and number (one or two). Thus, dorsal stripes apparently exhibit considerable evolutionary mutability, evolving de novo in association with the adoption of novel, needle-leaved host plants such as chamise, douglas fir, redwood, white fir, and limber pine.

The variation in stripedness in *T. cristinae* is genetically controlled by one loci with partial dominance of alleles (C. P. Sandoval, unpubl. data). Natural selection by birds and host preference maintain a correlation between morph frequency and host-plant frequency observed in the field (Sandoval, 1994a; Sandoval & Nosil, 2005), whereas migration between host-plant patches swamps this pattern. It is unknown if apostatic selection (frequency dependent selection) by preference for the more familiar morph (Raymond, 1984; Raymond & Allen, 1990) contributes to the maintenance of the polymorphism in areas of a single host plant.

The association between needle-like foliage and the dorsal stripe among species was statistically significant using Maddison's (1990) concentrated changes for both the full data set and the data set excluding the four species that rest on bark. However, using Pagel's (1994) likelihood-based methods, only the reduced data set yields results that approach statistical significance. This difference in results arises because under Maddison's parsimony-based approach, more origins of needle-like leaf use and stripes engender greater statistical power, but under the likelihood-based approach, more origins result in higher inferred transition rates and greater statistical uncertainty in reconstructions (e.g. Pagel, 1999), even when the associations between the two variables are virtually perfect (match completely) at the tips of the tree. Enhanced confidence in inferences from Pagel's test could derive from larger sample sizes, stronger assumptions concerning ancestral states, or analyses that take account of polymorphism in stripes, host-plant use, or in both (e.g. in *T. cristinae* and *T. boharti*).

Although all species of *Timema* with a dorsal stripe rest on needle-leaved vegetation, not all species that eat needle-leaved vegetation have evolved a dorsal stripe. In some of these cases, the species rests on other parts of the plant. For example, individuals of the unstriped species *T. podura*, *T. genevieve*, *T. ritensis* and *T. coffmani* rest on the trunks or stems of their needle-leaved host plants, and are thus not expected to exhibit a stripe in this microhabitat. By contrast, the unstriped species *T. boharti* and *T. nakipa* use a needle-leaved plant, *Adenostoma*, as one of their hosts, and utilize other plant species without needle-like leaves, which may lead to selection against a stripe because of negative fitness consequences for striped individuals on these plants. Further analyses of this and other species with multiple host plants and polymorphic colour patterns should lead to insights on how the local frequency of needle-leaved host plants influences the origin and maintenance of the dorsal stripe in heterogeneous backgrounds.

Stripes have been hypothesized to mimic leaf needles (Thayer, 1919) or to prevent recognition of the body by drawing attention to an unrecognizable shape (i.e. disruptive coloration; Cott, 1940; Merilaita, 1998). In disruptive coloration the body outline is disrupted because one or more of the colours at the margin of the body matches the background, whereas in crypsis and mimicry the colour and pattern of the body represent a random sample of the colour and pattern of the background. The colour and stripes in *Timema* appear to be more cryptic than disruptive.

The high evolutionary lability of the dorsal stripe in *Timema* suggests that cryptic colour patterns can evolve readily under appropriate environmental cir-

cumstances. However, the evolution of stripes may also constrain future evolutionary transitions: for example, no entirely striped *Timema* species has host-shifted to use host plants with broad foliage. Such plants apparently offer no safe microhabitats for striped *Timema*, although unstriped *Timema* may relatively easily make the reverse transition (to plants with needle-like foliage) via resting on trunks or twigs. These considerations suggest that the dorsal stripes of *Timema*, and cryptic colour patterns in other animals, may direct the future ecological potential of populations and species.

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