

Phylogenetic evidence for the evolution of ecological specialization in *Timema* walking-sticks

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Abstract

We used phylogenetic and ecological information to study the evolution of host-plant specialization and colour polymorphism in the genus *Timema*, which comprises 14 species of walking-sticks that are subject to strong selection for cryptic coloration on their host-plants. Phylogenetic analysis indicated that this genus consists of three main lineages. Two of the lineages include highly generalized basal species and relatively specialized distal species, and one of the lineages comprises four specialized species. We tested for phylogenetic conservatism in the traits studied via randomizing host-plant use, and the four basic *Timema* colour patterns, across the tips of the phylogeny, and determining if the observed number of inferred changes was significantly low compared to the distribution of numbers of inferred changes expected under the null model. This analysis showed that (1) host-plant use has evolved nonrandomly, such that more closely related species tend to use similar sets of hosts and (2) colour pattern evolution exhibits considerable lability. Inference of ancestral states using maximum parsimony, under four models for the relative ease of gain and loss of plant hosts or colour morphs, showed that (1) for all models with gains of host-plants even marginally more difficult than losses, and for most optimizations with gains and losses equally difficult, the ancestral *Timema* were generalized, feeding on the chaparral plants *Ceanothus* and *Adenostoma* and possibly other taxa, and (2) for all models with gains of colour morphs more difficult than losses, the ancestral *Timema* were polymorphic for colour pattern. Generation of null distributions of inferred ancestral states showed that the maximum-parsimony inference of host-plant generalization was most robust for the most speciose of the three main *Timema* lineages. Ancestral states were also inferred using maximum likelihood, after recoding host-plant use and colour polymorphism as dichotomous characters. Likelihood analyses provided some support for inference of generalization in host-plant use at ancestral nodes of the two lineages exhibiting mixtures of generalists and specialists, although levels of uncertainty were high. By contrast, likelihood analysis did not estimate ancestral colour morph patterns with any confidence, due to inferred rates of change that were high with respect to speciation rates. Information from biogeography, floristic history and the timing of diversification of the genus are compatible with patterns of inferred ancestral host-plant use.

Diversification in the genus *Timema* appears to engender three main processes: (1) increased specialization via loss of host-plants, (2) retention of the same, single, host-plant and (3) shifts to novel hosts to which lineages were 'preadapted' in colour pattern. Our evidence suggests that the radiation

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of this genus has involved multiple evolutionary transitions from individual-level specialization (multiple-niche polymorphism) to population-level and species-level specialization. Ecological studies of *Timema* suggest that such transitions are driven by diversifying selection for crypsis. This paper provides the first phylogeny-based evidence for the macroevolutionary importance of predation by generalist natural enemies in the evolution of specialization.

Introduction

Understanding the evolution of ecological specialization and generalization is one of the major unresolved questions in evolutionary biology (Mayr, 1942; Simpson, 1953; Levins & MacArthur, 1969; Futuyma, 1976, 1983, 1991; Futuyma & Moreno, 1988; Jaenike, 1990; Thompson, 1994). Analyses of this question remain challenging because they must seek to connect ecological processes with historical patterns, in relation to the genetic and phenotypic underpinnings of ecological adaptation unique to a given lineage (e.g. Via, 1984; Futuyma & McCafferty, 1990). Many studies have used comparative approaches to infer correlates of specialization and generalization (e.g. Otte & Joern, 1977; Bernays & Chapman, 1994), quantified trade-offs in performance on different resources (e.g. Rausher, 1984; Fry, 1990; Mackenzie, 1996), measured diversifying selection (e.g. Craig *et al.*, 1997), or inferred broad-scale patterns of evolutionary transition between specialist and generalist habits (Benson, 1950; Holloway & Hebert, 1979; Mitter & Brooks, 1983; Strong *et al.*, 1984; Futuyma & Moreno, 1988; Thompson, 1994; Kelley & Farrell, 1998). However, few analyses have combined species-level or population-level phylogenetic information with data on ecological causes, in species for which aspects of the genetic and phenotypic bases of ecological adaptation are known (e.g. Lanyon, 1992; Losos *et al.*, 1994; Armbruster & Baldwin, 1998).

In this paper, we use a combination of phylogenetic (Sandoval *et al.*, 1998) and ecological information to analyse the evolution of specialization in herbivorous walking-sticks in the genus *Timema*. In particular, we ask whether host-plant specialization has evolved from generalization, or vice versa, and if evolutionary changes in colour patterns are associated with changes in host-plant use. We use these macroevolutionary inferences, and information on ecological processes from intraspecific studies (Sandoval, 1994a,b), to evaluate alternative hypotheses for how host-plant use and colour polymorphism have evolved.

Methods

Biology of *Timema*

The walking-stick genus *Timema* (Insecta: Phasmatoptera: Timemidae) is useful for the study of how specialization

evolves because: (1) it is a small clade of 14 described species that exhibits both highly specialized species, feeding on one host-plant, and generalized species, feeding on up to four unrelated hosts (Vickery, 1993; Sandoval, 1994a,b; Sandoval & Vickery, 1996); (2) the mechanism of adaptation to different host-plant species, selection by vertebrates for crypticity of colour patterns via matching of foliage and stems, has been studied in detail (Sandoval, 1993; 1994b); (3) several *Timema* species show intraspecific, among-population variation in host-plant use, which may provide clues to causes of interspecific divergence; (4) some species are polymorphic for colour pattern, while others are monomorphic (Sandoval, 1993, 1994a; Sandoval & Vickery, 1996), which allows analysis of the role of colour polymorphism in the evolution of ecological adaptation; and (5) the genetic basis of colour pattern variation is understood in one species, and has been inferred in others (Sandoval, 1993).

Timema are restricted to the mountains in western North America between 30 and 42°N (Vickery, 1993). The genus apparently originated about 20 million years ago in southern California or Arizona, in conjunction with the origin and spread of the chaparral biome to which most species are adapted (Axelrod, 1980, 1989; Sandoval *et al.*, 1998). Much of the diversification of the genus took place about 1.5–3 million years ago, coincident with the primary periods of uplift of the Sierra Nevada, Coastal and Transverse ranges (Wahrhaftig & Birman, 1965; Dibblee, 1966; Norris & Webb, 1990). Three species, *T. tahoe*, *T. genevieve* and *T. douglasi*, are parthenogenetic, and phylogenetic evidence suggests that they are of tytoparthenogenetic rather than of hybrid origin (Sandoval *et al.*, 1998).

Timema are herbivores and primarily inhabit host-plants in chaparral vegetation (Vickery, 1993). They rest on branches or leaves of vegetation during the day and feed at night, relying on crypsis for protection against predators. Their colour patterns are fine-tuned to match the colour pattern of their host-plants, and at least three species exhibit host-associated colour polymorphisms (Sandoval, 1994a,b; unpublished data). In *T. cristinae*, one locus and four alleles control colour and a separate locus and several alleles control the presence of a stripe (Sandoval, 1993). Colour and stripe pattern are likely to be genetically determined in other species as well since no species appears able to change colour in relation to its host-plant background when raised in the laboratory.

Timema cristinae (Sandoval, 1994a,b) and *T. podura* (Sandoval, unpublished data) have different host-associated morphs maintained locally via differential predation by visually orientated predators (birds and lizards). In both species, transplant experiments in the field with predators excluded have shown that, for two of the colour morphs, feeding on *Ceanothus* resulted in higher or similar fecundity than feeding on *Adenostoma*. Thus, there was no evidence for a physiological trade-off between colour morphs with respect to host-plant quality. However, in both species, each colour morph preferred to rest on the host-plant on which it is most cryptic and safe from predators, and predation experiments using wild scrub jays showed that predation rates were much higher for morphs resting on the host-plant on which they were less cryptic (Sandoval, 1994a; unpublished data). Thus, in both of these colour-polymorphic species, there are strong trade-offs with respect to crypsis, and individuals prefer relatively safe hosts. These studies indicate that the *Timema* species polymorphic for colour pattern provide excellent examples of multiple-niche-polymorphisms (Levene, 1953; Hedrick *et al.*, 1976; Maynard Smith & Hoekstra, 1980; Hedrick, 1986).

Sampling and ecological data

We collected specimens from 17 populations, comprising all 14 described species, throughout the range of *Timema*, from Northern Baja California to Southern Oregon. At each collection site (Sandoval *et al.*, 1998), we recorded the relative proportion of walking-sticks on each plant species. We sampled by shaking branches of the host-plants inside of a sweep net and counting how many walking-sticks were collected from each branch. We brought the walking-sticks to the laboratory, and raised them to maturity for identification and photographing. We deposited voucher specimens in the California Academy of Sciences and the personal collection of C. Sandoval. W. Ferren (University of California, Santa Barbara) or C.S. identified the plants. To simplify discussion of host-plant ranges, we gave letter codes to host-plant species (Appendix 1).

For the analysis of colour pattern evolution, we grouped colour morphs in four categories: green (G), grey (Y), red (reddish-brown) (R), or beige (B) (Appendix 1). Species that exhibit the same colour morph by this categorization may vary in the hue of their colour, or they may have another pattern such as a stripe. Based on breeding experiments, we assume that it is easier for a basic colour pattern such as green to evolve to a different shade of green than for green to evolve into a different colour category.

To determine the number of host-plants used by each species and population, we analysed the frequency of walking-sticks on each host-plant from field collections and chose the ones on which at least 5% of walking-sticks were present as hosts. To confirm that a plant

species was used as a host, the walking-sticks were fed and raised to maturity in the laboratory with each plant species. Sampling of walking-sticks from the plants during the day is a reliable method to estimate host use because these walking-sticks remain on their host-plants. Laboratory preference tests provide results concordant with field distribution data (Sandoval, unpublished data). Host-plant use in the field (i.e. ecological breadth, Fox & Morrow, 1981) is not synonymous with host-plant availability (Colwell & Futuyma, 1971), since in most populations potential host-plants (i.e. plants used by other *Timema* species) are present but not utilized (Sandoval, unpublished data).

Phylogenetics

To infer a phylogeny of *Timema* species, we used the 660 base pairs of cytochrome oxidase I, mitochondrial DNA sequence data from Sandoval *et al.* (1998), and added to the data matrix partial COI sequence from the recently described species *T. coffmani*. We analysed the data using maximum parsimony (branch-and-bound searching), maximum likelihood (using the HKY model, empirical base frequencies, and a transition/transversion ratio of two) and neighbour joining (using the Kimura 2-parameter model), in PAUP* version d64 (written by David L. Swofford). These multiple forms of analysis allow for assessment of phylogeny robustness with respect to phylogenetic assumptions. We used as out-groups two species of Phasmatidae, *Baculum extradentatum* and *Anisomorpha buprestoides*, and the cockroach *Blattella germanica* (Sandoval *et al.*, 1998). The degree of support for nodes was evaluated using bootstrapping with maximum parsimony and neighbour joining (500 replicates).

We evaluated the influence of topological uncertainty on our phylogeny-based analyses by repeating several analyses using several of the most plausible topologies, with alternative topologies chosen on the basis of relative strength of bootstrap values (Losos, 1995).

Analyses of host-plant use and colour morph evolution

Evolutionary conservatism of host-plant use and colour morphs

To assess the degree of evolutionary conservatism in host-plant use, we counted, for each model of character gains and losses (described below), the number of inferred changes from character optimizations on the phylogeny. We then evaluated the degree of nonrandomness of our data by randomly permuting the character data across the tips of the phylogeny 200 times using the 'shuffle' algorithm in MacClade 3.0 (Maddison & Maddison, 1992), and comparing the distribution of numbers of inferred changes from this randomization to the observed number of inferred changes (Maddison & Slatkin, 1991; Winkler & Sheldon, 1993; Stone & Cook,

1998). Our hypothesis under test is that the actual data set exhibits significantly fewer inferred changes than the simulated, random data sets, which would indicate that the trait is conserved phylogenetically (Maddison & Slatkin, 1991).

Inference of ancestral states of host-plant use and colour polymorphism

We inferred ancestral patterns of host-plant use and colour morphs using maximum parsimony and maximum likelihood. With maximum parsimony, we used four alternative models of change: (1) gains and losses weighted equally, (2) gains 1% more difficult than losses, (3) gains twice as difficult as losses and (4) gains four times as difficult as losses. The assumption of greater relative difficulty of gain vs. loss of host-plants is supported by experiments and observations showing that morphs that are not well adapted for their host-plant background are locally eliminated due to predation from vertebrates (Sandoval, 1993, 1994b), and by evidence from many other phytophagous insects that gains of new host-plants appear highly constrained (e.g. Farrell & Mitter, 1994; Futuyma & McCafferty, 1990). We used this wide range of magnitudes of increasing relative difficulty of gains vs. losses to explore the influence of this range upon our optimization results.

We conducted our character optimizations in MacClade (Maddison & Maddison, 1992) using step-matrices that contained cells representing the minimum number of steps required to transform one monomorphic or polymorphic taxon into another (Mardulyn & Pasteels, 1994; Ronquist, 1994). This use of character optimization allows for the presence in ancestors of polymorphic character-state combinations that are not present in terminal taxa.

Timema species use one to four host-plants, and exhibit one to four colour morphs. In our discussion of specialization and generalization, we consider species with one host-plant or colour morph as being specialized, and species with more than one host-plant or colour morph are considered relatively generalized. Maximum parsimony, and other methods (e.g. Schultz *et al.*, 1996; Martins & Hansen, 1997), infer ancestral states as weighted means of the species values, which in our data may tend to result in the inference of generalization. For example, we might tend to infer ancestral host-plant generalization regardless of the phylogenetic positions of specialists and generalists, especially under models with gains more difficult than losses. To address this bias in the method, we produced empirical null distributions of the number of ancestral inferred hosts or colour morphs, and compared the actual inferred number of hosts or colour morphs to the null expectation. We generated the null distributions by randomizing species values across the tips of the phylogeny (Maddison & Slatkin, 1991; Maddison & Maddison, 1992). For each of 200 such

randomizations, using each of our four models of gains and losses, we inferred ancestral states using parsimony, and counted the number of inferred host-plants or colour morphs at the nodes of interest. When more than one equally parsimonious ancestral state was inferred after a randomization, we averaged across them (e.g. with two equally parsimonious reconstructions of colour morphs at a node, one reconstruction with four colours, and one with three colours, we used 3.5 as the inferred value). For each node of interest, for each of the models of gains and losses, we thus determined whether or not the actual number of inferred ancestral states was in the 5% tail of the distribution of numbers of ancestral states generated via randomization of species values across the tips of the tree. This randomization test allows us to determine the extent to which the numbers of inferred ancestral host-plants or colour morphs depend on the phylogenetic positions of the species values.

Maximum likelihood models for inference of ancestral states have yet to be developed for situations involving polymorphism in ancestors and descendants, or for situations involving more than two character states (Schluter *et al.*, 1997). To apply the likelihood approach to ancestor reconstruction to our data, we therefore coded species or populations as generalized (using two or more species) for host-plant use or specialized (using only one host), and polymorphic (having more than one colour morph) or monomorphic (having one colour morph) for colour pattern. We then used the algorithm developed by Schluter *et al.* (1997) (see also Garland *et al.*, 1999) to infer the relative likelihoods of ancestral alternative states at different nodes of the tree, bearing in mind that considerable information on the degree of host-plant generalization and colour polymorphism, and the identities of host-plants and colour morphs, is lost as a result of the simplified coding. Thus, this maximum-likelihood analysis is expected to be more conservative as regards the detection of evolutionary patterns.

Compatibility of inferred ancestral host-plants and inferred ancestral colour patterns

We assessed the degree of compatibility between inferred ancestral host-plants, and inferred ancestral colour morphs, by using the associations of host-plants with colour morphs in extant taxa as predictors of which colour morph and host-plants should be associated in the inferred ancestral *Timema*. Thus, we used the host-plant – colour morph associations in extant taxa to derive expected colour morph patterns from the set of possible inferred ancestral host-plants, and we compared these expected colour morph patterns to the set of ancestral colour morph patterns inferred using parsimony. This analysis was conducted (1) using all four models of trait evolution and (2) using only the three models in which gains were more costly than losses.

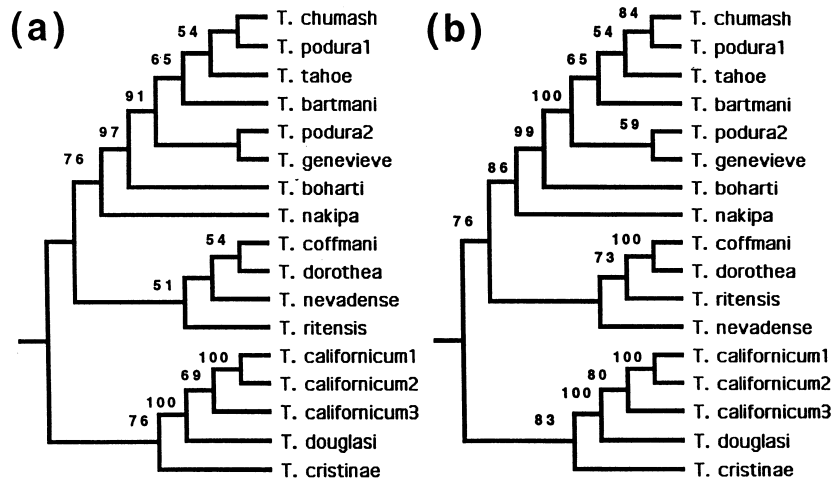


Fig. 1 Results of phylogenetic analyses of COI data. (a) One of the nine shortest maximum-parsimony trees, which is identical in topology to the maximum-likelihood tree. Maximum-parsimony bootstrap values over 50% are shown above the branches. (b) Neighbour-joining tree, with bootstrap values shown above the branches.

Results

Phylogeny of *Timema*

The addition of *T. coffmani* to the *Timema* data set used in Sandoval *et al.* (1998) resulted in topologies that were unchanged in branch order and similarly well resolved and well supported, and *T. coffmani* was sister-taxon to *T. dorothea* in all analyses. Maximum parsimony analysis yielded nine shortest trees of length 813, which differed only with regard to two nodes within the clade (*T. genevieve* + *T. podura* 1 and 2 + *T. chumash* + *T. tahoe* + *T. bartmani*). One of the nine shortest trees from maximum parsimony analysis was identical with the maximum likelihood tree, and the maximum likelihood and neighbour joining trees (Fig. 1) were identical except for transposed placements of *T. ritensis* and *T. nevadense*. Bootstrap analyses using neighbour-joining and maximum parsimony demonstrated that uncertainty in topology was concentrated in the clade (*T. genevieve* + *T. podura* 1 and 2 + *T. chumash* + *T. bartmani*), the putative clade (*T. nevadense* + *T. dorothea* + *T. coffmani* + *T. ritensis*), and the node connecting this clade with its putative sister-taxon. The former clade appears to have evolved in a burst of diversification about 1.5–3 million years ago (Figure 3 in Sandoval *et al.*, 1998), such that speciation events may have been temporally clustered. To infer the evolutionary history of *Timema* host-plant use and colour patterns, we used the maximum likelihood tree as the primary topology analysed.

Evolution of host-plant use

Three main lineages can be identified in the phylogeny of *Timema* (Figs 1 and 2). In two of the lineages, the most basal species, *T. cristinae* and *T. nakipa*, are the most generalized species for host-plant use in the genus (Fig. 2). The basal positions of these two species are strongly supported by high bootstrap values (Fig. 1), as is

the relatively basal position of *T. boharti*, which is also highly generalized and polymorphic. In contrast to these two lineages, the third main *Timema* lineage consists of four specialized species, three of which use juniper as their host-plant.

We focused our inferences of ancestral states on the three lineages in our phylogeny that are relevant to the hypothesis that generalism was ancestral: (1) the root of the lineage for which *T. cristinae* is basal (clade 2), (2) the root of the lineage for which *T. nakipa* is basal (clade 1) and (3) the root of the entire tree (Fig. 2). The latter two cases include substantially more lineages and taxa than the former case, and as such these two cases will provide the most powerful analyses.

Evolution of colour patterns

The most basal lineages in two of the three main *Timema* lineages, *T. cristinae* and *T. nakipa*, each exhibits three of the four main colour morphs found in *Timema*, and the species *T. boharti*, which has all four colour morphs, is also relatively basal within its lineage (Fig. 2). The more distal lineages in these clades each contains species with one, two or three morphs. In the third main lineage, which includes *T. ritensis*, *T. nevadense*, *T. dorothea* and *T. coffmani*, the putatively most basal taxon, *T. ritensis*, is monomorphic. We note, however, that the positions of *T. ritensis* and *T. nevadense* are among the most uncertain in our phylogeny, and if *T. nevadense* was basal, then this clade would also include a polymorphic most basal lineage.

Evolutionary conservatism of host-plant use and colour morphs

For each of the four models of host-plant evolution, the number of changes inferred for the actual data was significantly lower than the number of changes inferred from the randomized data (gains equally difficult as

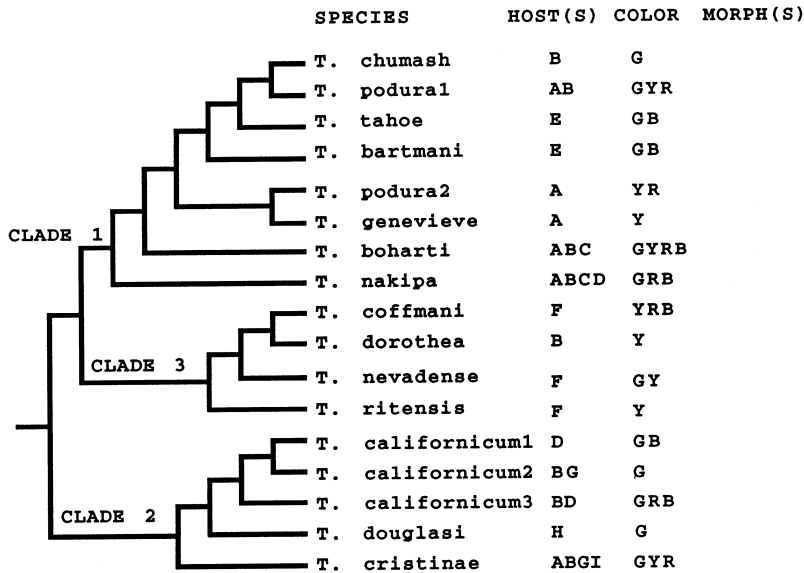


Fig. 2 Host-plants and colour morphs in *Timema*. Host-plants: A = *Adenostoma fasciculatum* (chamize), B = *Ceanothus* species (ceanothus), C = *Archytostaphylos* species (manzanita), D = *Quercus* species (oak), E = *Abies concolor* (white fir), F = *Juniperus* species, G = *Heteromeles arbutifolia* (heteromeles), H = *Pseudotsuga mensizii* (douglas fir), I = *Pickeringia montana* (pickeringia), P = *Pinus* species (pine), S = *Sequoia sempervirens* (coastal redwood), T = *Cercocarpus betuloides* (cercocarpus). Colour morphs: G = green, Y = grey, R = red, B = beige.

losses, $P < 0.04$; gains 1% more difficult, $P < 0.02$; gains twice as difficult, $P < 0.05$; gains four times as difficult, $P < 0.02$). These results demonstrate that host-plant use is evolutionarily conserved across our phylogeny. The lineage with *T. cristinae* basal has a disproportionate representation of *Ceanothus* (B), oak (D) and *Heteromeles* (G) as hosts, the lineage with *T. nakipa* basal includes mainly species with *Adenostoma* (A), *Ceanothus* and white fir (E) as hosts, and the lineage with *T. ritensis* basal has three of four species on juniper (Fig. 2).

For colour morphs, the number of changes inferred for the actual data was not significantly lower than the number of changes inferred for the randomized data for any model ($P > 0.10$ for all). Use of parsimony to infer specific ancestral colour patterns for specific lineages is therefore not necessarily expected to yield accurate results.

Inference of ancestral states

Maximum-parsimony analysis of host-plant use

Inferred ancestral host-plant ranges for the genus *Timema* (i.e. the inferred state at the root of the *Timema* phylogeny) included two or more hosts under all equally parsimonious reconstructions when gains of hosts were more costly than losses (Table 1). The most striking pattern in all reconstructions is that the chaparral plants *Ceanothus* and *Adenostoma* were among the inferred ancestral hosts for all cases of inferred host-plant use involving two or more hosts.

The number of inferred ancestral host-plants was significantly higher than expected under the null model for the base of the largest of the three main clades of *Timema* (clade 1), when gains were equally probable as losses ($P < 0.02$), 1% more difficult ($P < 0.02$) or twice as difficult ($P < 0.04$). By contrast, when host-plant gains

were four times as difficult as losses, the number of inferred ancestral host-plants was not significantly higher than expected by chance ($P > 0.15$), because four host-plants were inferred for this node after many of the randomizations. For the root of clade 2, and for the root of the entire tree, the numbers of ancestral host-plants inferred were not significantly greater than expected under the null model. Thus, host-plant generalization is also usually inferred for these nodes regardless of the phylogenetic distributions of generalists and specialists across the tips of the tree.

Maximum-likelihood analysis of host-plant use

Under the simplest possible likelihood model, with forward and backward transition rates set equal and estimated as 0.47 (Schluter *et al.*, 1997), generalization was the inferred ancestral state for the most basal nodes in both of the *Timema* lineages exhibiting a mixture of generalists and specialists, and specialization was inferred for most of the most distal nodes in these lineages (Fig. 3). The degree of uncertainty in the inferred states, as indicated in the pie diagrams as the relative likelihoods of the alternative reconstructions, was generally high, and only one of the likelihood ratios (for the node (*T. dorothea* + *T. coffmani*)) reached the 95% statistical significance value of 7.4 discussed by Schluter *et al.* (1997). The nodes with highest inferred relative likelihood of generalization (about 2/3) were located at the bases of the two main *Timema* lineages containing mixes of specialist and generalist species.

Maximum-parsimony analysis of colour polymorphism

With gains and losses of colour morphs equally difficult, polymorphism was inferred for the root of the tree in 32 of 36 equally parsimonious reconstructions (Table 1). With gains more difficult than losses, one most parsimonious

Table 1 Maximum-parsimony reconstructions of ancestral states for host-plants and colour morphs of *Timema* walking-sticks. Shown are total numbers of most-parsimonious reconstructions inferred using MacClade, and the state or states inferred for the root of the *Timema* phylogeny, the root of clade 1, and the root of clade 2 (Fig. 2). For host-plants, A = *Adenostoma*, B = *Ceanothus*, C = *Archytostaphylos*, D = *Quercus*, G = *Heteromeles*, and I = *Pickeringia*, and for colour morphs, G = green, Y = grey, R = red, and B = beige.

	Model of character evolution			
	Gains and losses equally likely	Losses 1% more likely	Losses twice as likely	Losses four times more likely
Root of entire tree:				
Host-plant use	8 reconstructions AB (4), B (4)	1 reconstruction AB	7 reconstructions ABGI, ABCD, ABI, ABG, ABC, ABD, AB	1 reconstruction ABCD
Colour morphs	36 reconstructions GYR (16), GY (16), Y (4)	1 reconstruction GYR	16 reconstructions All GYRB	1 reconstruction GYRB
Root of clade 1:				
Host-plant use	8 reconstructions ABC (4), AB (4)	7 reconstructions ABCD (5), ABC (2)	1 reconstruction ABCD	1 reconstruction ABC
Colour morphs	36 reconstructions GYRB (24), YB (8), GYB (4)	1 reconstruction GYRB	1 reconstruction GYRB	1 reconstruction GYRB
Root of clade 2:				
Host-plant use	8 reconstructions AB (4), B (4)	7 reconstructions ABGI, ABG, ABI, ABD (2), AB (2)	1 reconstruction ABD	1 reconstruction AB
Colour morphs	36 reconstructions GYR (16), GY (20)	1 reconstruction GYRB	1 reconstruction GYRB	1 reconstruction GYR

reconstruction was found for each model: for gains 1% more difficult than losses, green, grey and red were the inferred ancestral states, and for gains twice or four times

as difficult as losses, all four colour morphs were inferred as present in the ancestor (Table 1). Colour polymorphism was also inferred for the roots of clades 1 and 2, under all models of relative cost of gains and losses (Table 1).

The number of inferred ancestral colour morphs was significantly higher than expected under the null model only for the base of clade 1, the largest of the three main clades, and only with gains equally likely as losses ($P < 0.05$). When gains were more difficult than losses, three or four colour morphs were usually inferred for the roots of clades 1 and 2, and for the root of the entire tree, regardless of the phylogenetic distributions of the species values.

Maximum-likelihood analysis of colour polymorphism

Using likelihood analysis of colour polymorphism, with states coded as monomorphic and polymorphic and equal assumed rates of change forward and backward, all nodes were inferred as exhibiting equal likelihoods for each of the two states, so ancestral states could not be inferred

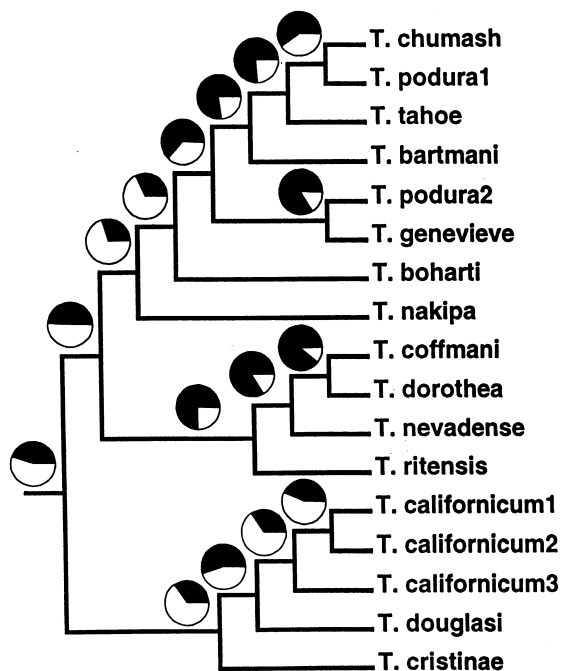


Fig. 3 Results of maximum-likelihood inference of ancestral states for host-plant use, with the filled proportion of the Schluter pies representing the relative likelihood for an ancestral state of specialization (one host-plant), and the unfilled proportion representing the relative likelihood of an ancestral state of generalization (more than one host-plant). For the equivalent analysis of colour morph evolution, all pies are half-filled. See text for details.

with any degree of confidence. This high degree of uncertainty is associated with a high inferred rate of change relative to speciation events, with forward and backward instantaneous transition rates (Schluter *et al.*, 1997) estimated at 11.4.

Compatibility of inferred ancestral host-plants and inferred ancestral colour patterns

When the results from all four models of host-plant character optimization using maximum parsimony are combined, we have eight possibilities for ancestral host-plant ranges at the root of the tree, B, AB, ABC, ABD, ABG, ABI, ABCD and ABGI (Table 1). Five of these host-plant ranges are present within one or more extant species, such that the colour morphs associated with these host-plant ranges can be predicted to be Y or B (with plant B), GYR (with plants AB and ABGI), GRB (with plants ABCD) and GYRB (with plants ABC) (Fig. 2). This prediction is well supported by ecological studies showing that *Timema* species colour patterns are well adapted for crypsis on their host-plant backgrounds (Sandoval, 1993; 1994a,b). Under our four models of colour morph evolution, we have inferred four possible ancestral colour morphs or sets of morphs, Y, GY, GYR and GYRB, to compare with the predicted ancestral colour morphs Y or B, GYR, GRB and GYRB. Three of these colour morphs or sets of morphs, Y, GYR and GYRB, match between the inferred and the predicted.

We can evaluate the statistical significance of the degree of compatibility described above in two ways. First, statistically significant compatibility would be indicated by the improbability of obtaining three matches under a random model, in this case a model of choosing without replacement a set of five, and a set of four, from the nine observed colour morphs or colour-morph combinations found among the extant species. The probability of obtaining three matches by chance under this scheme can be shown by simple calculation of probabilities to be 960/3024, or 0.317. Second, statistically significant incompatibility would be indicated by obtaining a sufficiently small number of matches between the predicted and inferred that it was statistically unlikely. The probability of a single match by this scheme is 180/3024, or 0.06, which suggests that significant incompatibility would be indicated by obtaining only 1 or 0 matches. We interpret these results to mean that, considering all models of character evolution, we can neither accept nor reject a hypothesis of incompatibility between inferred ancestral host-plants and inferred ancestral colour morphs.

When the results from the three models of host-plant and colour morph character optimization with gains more difficult than losses are combined, we have seven possibilities for ancestral host-plant ranges, AB, ABC, ABCD, ABGI, ABI, ABG and ABD. The former four host-plant ranges are present among one or more extant taxa,

such that the colour morphs associated with these host-plant ranges can be predicted to be GYR (with plants AB or ABGI), GRB (with plants ABCD) and GYRB (with plants ABC). Under these three models of colour morph evolution, we have inferred only two possible ancestral sets of colour morphs, GYR and GYRB, to compare with the ancestral colour morphs predicted from host-plant data, GYR, GRB and GYRB. Two of these sets of colour morphs, GYR and GYRB, match between the inferred and the predicted. Given random draws of three, and two, colour morphs or combinations of morphs from the observed set of nine, the odds of two matching is 6/72, or 0.083, which is the lowest probability that can be obtained with this set of data. By contrast, the probability of no matches is 30/72, or 0.42. Thus, when gains in host-plants and colour morphs are assumed to be more difficult than losses, our data exhibit the highest possible degree of compatibility between inferred ancestral host-plants and inferred ancestral colour morphs.

Host-shifting and the evolution of colour patterns

Our maximum-parsimony character optimizations suggest the occurrence of three major host-plant shifts, each to host-plants that are taxonomically distant, from gymnosperm to angiosperm or vice versa: (1) the ancestor of *T. douglasi* shifting to douglas fir from *Ceanothus* or *Ceanothus* and oak, with this shift accompanied by the acquisition of a longitudinal stripe and longer body, providing a closer match to the needles of Douglas fir; (2) the ancestor of *T. bartmani* and *T. tahoe* shifting to white fir from *Adenostoma* or *Adenostoma* and *Ceanothus*, with the beige morph of these two species found on dead leaves, and the green morph on living leaves, and the shift also involving the acquisition of a longitudinal stripe; and (3) the ancestor of *T. dorothea* shifting to *Ceanothus* from juniper, and in association with the shift gaining white specks that provide a close match to *Ceanothus* bark. We evaluated the hypothesis that pre-adaptations in colour pattern (i.e. moderately close initial matches to a host-plant, which are later improved by selection) facilitated these shifts by determining, from our optimizations, whether or not the colour pattern of each of these taxa was inferred to have also been present in its closest inferred ancestor that inhabited the host-plant or plants from which the shift would have occurred. For example, we asked whether or not the most recent inferred ancestor (with a different colour or set of colours) of the green species *T. douglasi* was inferred to have exhibited a green morph. The colour pattern of the host-shifted species was present in the inferred ancestor under all optimization models for *T. douglasi* and *T. dorothea*. For *T. bartmani* and *T. tahoe*, only one inferred ancestor, under one of the optimization models, had a set of inferred ancestral colours (beige and grey) that was partially incompatible with the colour morphs of these two extant species (beige and green).

These analyses are consistent with the hypothesis that the possession of particular colour morphs facilitated shifts to host-plants that were taxonomically distant but similar in background colour to the ancestral host or hosts.

Discussion

Our results suggest that the ancestral *Timema* walking-sticks were most likely generalists in host-plant use, feeding on chaparral plants in the genera *Ceanothus* and *Adenostoma* and possibly other hosts, and that they probably exhibited colour polymorphism. The evidence for ancestral generalization in host-plant use is strongest for the most speciose of the three main *Timema* lineages, within which the two most basal species are highly generalized. From a relatively generalized ancestor, at the root of this lineage or for the genus as a whole, the evolution of host-plant use and colour pattern apparently involved three main types of events: (1) descendant lineages evolving to use a subset of the hosts of ancestors, such that generalists evolve towards relative specialization; (2) specialists giving rise to other specialists (e.g. juniper-feeding species); and (3) host-switching, which involved adoption of a novel host to which a lineage was preadapted in colour pattern, whereby a specialist evolves from either a generalist or a specialist.

What are the implications of our results for models of the evolution of specialization, and how do our findings compare with those from other taxonomic groups? The first pattern found in *Timema*, generalization and polymorphism spawning specialization, does not concord closely with traditional models of adaptive radiation whereby a generalist ancestor yields specialist descendants (Mayr, 1942, pp. 294–295; Simpson, 1953, pp. 222–230; Grant & Grant, 1989), because in *Timema* the polymorphic, generalized ancestral lineage would have been host-plant and colour-morph specialized at the level of individuals, rather than exhibiting a single, generalized multipurpose phenotype. Instead, this pattern fits well with West-Eberhard's (1986) model of adaptive diversification via ancestral polymorphic species undergoing divergent selection, accompanied by speciation, to yield monomorphic descendant lineages. The second and third observed patterns observed, which primarily involve specialized species that give rise to specialized descendants, are common in phytophagous insects (Futuyma & Moreno, 1988). Our parsimony analyses suggest that shifting between host-plant taxa has involved movement onto plants to which a population was preadapted in its colour pattern (i.e. moderately well adapted due to a fortuitous initial degree of crypsis).

Previous phylogenetic or taxonomically based studies of the evolution of specialization have uncovered cases of transitions from both specialization to generalization

(Lanyon, 1992; Muller, 1996; Armbruster & Baldwin, 1998; Crespi & Abbot, 1999), and generalization to specialization (Courtney, 1986; Losos *et al.*, 1994; Funk *et al.*, 1995; Kelley & Farrell, 1998). These shifts may be driven by selective pressures at the trophic level above, below or at the level of the taxon of interest (Levins & MacArthur, 1969; Futuyma, 1976; Lawton, 1986; Bernays & Graham, 1988; Jermy, 1988; Bernays & Chapman, 1994; Pellmyr *et al.*, 1997). To understand how ecological range evolves, we must assess what selective pressures are involved in each direction of ecological transition. The evolution and maintenance of specialization should be favoured by the presence of physiological, morphological or life-history trade-offs (Futuyma & Peterson, 1985; Futuyma & Moreno, 1988; Bernays & Chapman, 1994; Joshi & Thompson, 1995; Feder *et al.*, 1997), although empirical evidence for such trade-offs remains limited (Via, 1984; Fry, 1990; Jaenike, 1990; Mackenzie, 1996). In herbivorous insects, most studies of specialization have focused on the effects of selection on physiological traits such as the ability to detoxify or sequester plant toxins (e.g. Ehrlich & Raven, 1964; Bernays & Graham, 1988; Jermy, 1988; Tauber & Tauber, 1989; Berenbaum, 1990; Denno *et al.*, 1990). Our study indicates that trade-offs in crypsis on different host-plants can also be an important mechanism of defence that selects for specialization (see also Brower, 1958; Otte & Joern, 1977; Bernays & Chapman, 1994), and it represents the first phylogenetic evidence for the macroevolutionary importance of predation by generalist natural enemies (Sandoval, 1993, 1994a) in the evolution of resource breadth. We predict that selection for crypsis by generalist enemies will be found to be an important mechanism for the evolution of specialization in many other animals, because it normally involves strong trade-offs and a high intensity of selection (Endler, 1978, 1984).

We believe that evidence beyond our ancestor reconstructions themselves, bearing on assumptions underlying the inferences and the accuracy of inferred ancestral states, is critical to evaluating the plausibility of our inferences. For *Timema*, this ancillary evidence comes from three main sources: (1) knowledge of the selective processes involved in the matching between host-plants and colour morphs (Sandoval, 1993; 1994a,b); (2) predictions concerning associations between inferred ancestral host-plants, and inferred ancestral colour morphs; and (3) the biogeography and timing of diversification of *Timema*, in relation to the floristic history of their host-plants (Sandoval *et al.*, 1998).

The selective pressures influencing crypsis on host-plants have been analysed in detail for colour-polymorphic, generalist populations of *T. cristinae* (Sandoval, 1993) and *T. podura* (Sandoval, unpublished data). These studies have shown that there are strong trade-offs with regard to crypsis on different host-plants, which are mediated by vertebrate predators, but that no such

trade-offs exist for fecundity on different plants. Strong selection for matching the host-plant should make evolutionary gains of new plants more difficult than losses, although such gains may be facilitated by the presence in a polymorphic species of a morph that matches a new plant reasonably well at the outset. Indeed, for each of the cases of inferred host-plant shift, involving *T. douglasi*, *T. bartmani* and *T. tahoe*, and *T. dorothea*, most character optimizations of colour morph suggested that the morph exhibited by these putatively host-shifting species was present in their most recent common ancestor with another *Timema* lineage. Moreover, in each of these three cases, enhancement of the degree of crypsis evolved via the addition of details of pattern to the colour morph.

If ancestral *Timema* were colour-polymorphic and generalist, and exhibited trade-offs for crypsis across host-plants as found in extant species, then the diversification of the genus has apparently involved evolutionary transitions from such intraspecific trade-offs (multiple-niche polymorphisms) to trade-offs expressed at the levels of populations and species. The mechanism for this process, suggested by within-population studies in two species, is the split of generalist species into specialized ones due to strong differential predation, driven by predators selecting for colour morphs that are most cryptic on a subset of possible hosts.

A second line of evidence bearing on the plausibility of our inferred ancestral states is the degree of concordance between inferred host-plants and inferred colour morphs. We found that, for models with gains of host-plants or colour morphs more costly than losses, the data exhibited the highest possible degree of matching between inferred ancestral host-plants and inferred ancestral colour morphs, although the low power of our randomization test precluded statistical significance of this result at $P < 0.05$. By contrast, using all four models of character evolution (i.e. including the model with gains and losses of equal difficulty), we could realistically reject a hypothesis of neither compatibility nor incompatibility, although three of four inferred colour morphs or sets of colour morphs matched the predictions. We interpret these results as consistent with a reasonable degree of internal consistency between our optimizations of host-plants and colour morphs.

The third line of evidence challenging the plausibility of our inferences of ancestral states is information on past events from biogeography, the timing of diversification of the genus, and the floristic history of the host-plants. Since the seven most basal species on our phylogeny (*T. cristinae*, *T. boharti*, *T. nakipa*, *T. nevadense*, *T. dorothea*, *T. coffmani* and *T. ritensis*) are currently distributed in southern California, southern Nevada, Arizona or northern Mexico, while five of the seven most distal species are found to the north, in central or northern California and Nevada, we believe that the genus probably originated in the southern part of its current distribution (Sandoval

et al., 1998). Maximum-likelihood analysis of the COI data, under clock-constrained vs. unconstrained models, did not lead to rejection of the validity of a molecular clock, and application of an insect COI-sequence molecular clock yielded estimates of time of origin of the genus of about 20 million years ago (Sandoval *et al.*, 1998). According to the maximum-likelihood tree under the molecular clock model, the earliest divergences in *Timema* produced the lineages giving rise to the species *T. nevadense*, *T. dorothea*, *T. ritensis* (and, by inference, *T. coffmani*), and the genus underwent a burst of diversification between about 1.5 and 3 million years ago, roughly coincident with the main periods of uplift of the Sierra Nevada, Transverse, and Coastal ranges of California and Nevada (Wahrhaftig & Birman, 1965; Dibblee, 1966; Norris & Webb, 1990; Sandoval *et al.*, 1998).

If the chaparral plant genera *Ceanothus* and *Adenostoma* were among the ancestral hosts of *Timema*, then they should have been present in the south-west United States or northern Mexico at least about 20 million years ago. The sclerophyllous chaparral vegetation zone first appeared in the south-western US in the mid-Eocene, about 40–45 million years ago, as an understory component in woodland, and expanded its range, from Arizona, Colorado and Nevada into southern California, over the last 20 million years (Axelrod, 1989). Within this region, pollen of *Ceanothus* and *Heteromeles* has been recorded from deposits 17–18 million years old, *Abies* and *Pseudotsuga* pollen dates back to about 26 million years ago, and pollen of *Cercocarpus* and *Quercus* has been dated to 46 and 34 million years ago, respectively. The age of *Adenostoma*, the other inferred ancestral host-plant of *Timema*, is unknown, at least in part because it lives in relatively dry areas and has a very small seed that fossilizes poorly. Thus, except for one host-plant that does not have a fossil record, all inferred ancestral host-plants were about as old or older than the *Timema* lineages inferred to feed upon them.

A final line of evidence relevant to the ancestral states of *Timema* host-plant use and colour pattern is the putative sister-taxon to the genus; if the sister-taxon was generalized and colour-polymorphic, then the case for this ancestral condition would be considerably strengthened. Sellick (1998) provides evidence from egg morphology that *Timema* are sister-group to the rest of the phasmids, and Vickery (personal communication) suggests that *Diapheromera* may be the genus most-closely related to *Timema*. Within *Diapheromera*, host-range data are available only for *D. femorata*, which is generalized, its hosts including *Quercus*, *Vitis*, *Prunus* and *Robinia*. Moreover, this species exhibits all four of the same general colour morphs, green, beige, brown (similar to reddish-brown) and grey, as *Timema* (Essig, 1926). *D. femorata* is distributed east of the Rocky mountains, although it approaches *Timema* in distribution in New Mexico. Another candidate for the closest relative to *Timema* is

the genus *Pseudosermyle*, which is found throughout the south-western US within the range of *Timema*, but for which host data are not yet available (Caudell, 1903; Essig, 1926). Identification of the sister-group to *Timema* requires a robust phylogeny for North American phasmids in conjunction with data on host ranges, but the available information is not incompatible with our inferences.

We believe that the concordance of information from the three sources described above provides good evidence that the evolutionary history of *Timema* has involved an increase in ecological specialization at the species and population levels. However, there are at least two other hypotheses that warrant consideration. First, the ancestral *Timema* may have been specialized, and descendant lineages may have gained hosts sequentially over time via phyletic change, leading to a pattern of more basal, older lineages exhibiting more generalized host-plant use. This hypothesis appears implausible because it makes the *ad hoc* assumption of a loss of all but one or a few host-plants during or soon after speciation events, and it is contradicted by the host-plant specialization of *T. nevadense* and *T. ritensis*, which are among the oldest lineages in the genus (Sandoval *et al.*, 1998).

A second alternative hypothesis is that the ancestral *Timema* used juniper as its host-plant, as do three of the four species in the relatively ancient clade (*T. nevadense* + *T. ritensis* + *T. dorothea* + *T. coffmani*). By this hypothesis, an ancestor specialized on juniper would have given rise to one or both of the other two main lineages of *Timema*. But whether or not the ancestral *Timema* was specialized on juniper, its descendant sister-lineage or lineages are still inferred to have been generalized on *Ceanothus*, *Adenostoma*, and possibly other plants under all models with gains more difficult than losses, such that the main ecological trajectories in the genus are still from generalization to specialization, and specialization to specialization. A hypothesis of directionality of host-plant shift from juniper to chaparral plants such as *Ceanothus* and *Adenostoma* could be tested experimentally by determining whether or not species that live on juniper can survive and breed on chaparral plants (e.g. Keese, 1998), and vice versa, or via elucidation of patterns of genetic variation for the use of different host-plants (e.g. Futuyma *et al.*, 1993, 1994, 1995).

Further studies of the evolution of ecological specialization in *Timema* require the joint phylogenetic and ecological analysis of among-population variation in host-plant use and colour patterns, to assess the roles of local resource distribution and abundance and population history in specialization, speciation and diversification. Such analyses will allow us to connect within-population selection pressures to among-population divergence patterns, and directly test models postulating that ecological divergence drives

the evolution of reproductive isolation (Funk, 1998; Schluter, 1998; Thompson, 1998).

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Species	Hosts	Host code	Colour morphs	Morph code
<i>T. chumash</i>	<i>Ceanothus</i> spp.	B	Green	G
<i>T. podura 1</i>	<i>Ceanothus</i> spp. <i>Adenostoma fasciculatum</i>	AB	Green, grey, red	GYR
<i>T. tahoe</i>	<i>Abies concolor</i>	E	Green, beige	GB
<i>T. bartmani</i>	<i>Abies concolor</i>	E	Green, beige	GB
<i>T. podura 2</i>	<i>Adenostoma fasciculatum</i>	A	Grey, red	YR
<i>T. genevieve</i>	<i>Adenostoma fasciculatum</i>	A	Grey	Y
<i>T. boharti</i>	<i>Adenostoma fasciculatum</i> <i>Ceanothus</i> spp. <i>Archytostaphylos</i> spp.	ABC	Green, grey, red, beige	GYRB
<i>T. nakipa</i>	<i>Adenostoma fasciculatum</i> <i>Ceanothus</i> spp. <i>Archytostaphylos</i> spp. <i>Quercus</i> spp.	ABCD	Green, red, beige	GRB
<i>T. coffmani</i>	<i>Juniperus</i> spp.	F	Grey, red, beige	YRB
<i>T. dorothea</i>	<i>Ceanothus</i> spp.	B	Grey	Y
<i>T. navadense</i>	<i>Juniperus</i> spp.	F	Green, grey	GY
<i>T. ritensis</i>	<i>Juniperus</i> spp.	F	Grey	Y
<i>T. californicum 1</i>	<i>Quercus</i> spp.	D	Green, beige	GB
<i>T. californicum 2</i>	<i>Ceanothus</i> spp. <i>Heteromeles arbutifolia</i>	BG	Green	G
<i>T. californicum 3</i>	<i>Ceanothus</i> spp., <i>Quercus</i> spp.	BD	Green, red, beige	GRB
<i>T. douglasi</i>	<i>Pseudotsuga mensizii</i>	H	Green	G
<i>T. cristinae</i>	<i>Adenostoma fasciculatum</i> <i>Ceanothus</i> spp. <i>Heteromeles arbutifolia</i> <i>Pickeringia montana</i>	ABGI	Green, grey, red	GYR

Appendix 1 List of *Timema* species and corresponding character states (host-plants and colour morphs).