



## The timing of insect/plant diversification: might *Tetraopes* (Coleoptera: Cerambycidae) and *Asclepias* (Asclepiadaceae) have co-evolved?

B. D. FARRELL\*

*Museum of Comparative Zoology, Harvard University, Cambridge, MA 02138, U.S.A.*

C. MITTER

*Department of Entomology, University of Maryland, College Park, MD 20742, U.S.A.*

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Ehrlich and Raven's essay on coevolution has stimulated voluminous work on the mechanisms of insect/plant interaction, but few explicit tests of their model's prediction that the evolutionary success of entire insect and plant clades is governed by their putative reciprocal adaptations. This paper begins an inquiry into possible coevolutionary diversification for North American milkweeds of the genus *Asclepias* and one of their few major herbivores, the longhorn beetle genus *Tetraopes*, focusing first on the historical duration and continuity of the interaction. A phylogeny for *Tetraopes* and relatives, estimated from morphology and allozymes, shows evident similarity to a morphology based hostplant cladogram synthesized from the literature, though the significance of the correspondence under heuristic statistical tests depends on the treatment of one beetle species reported (without certainty) from multiple host species. Fossils and biogeography support the interpretation that cladogram correspondence reflects synchronous diversification of these two clades, hence opportunity for coevolution, rather than beetle 'host-tracking' of previously-diversified plants. Cladogram correspondence is more evident at higher than at lower levels, as expected under Ehrlich and Raven's model. An apparent phylogenetic progression in the potency and location of milkweed cardenolides, seemingly related to species diversity of both *Asclepias* and *Tetraopes* subclades, provides further suggestive evidence for that model. The phylogeography of the *Tetraopes/Asclepias* assemblage suggests that extant species evolved largely in their current, often quite localized biomes, facilitating potential experimental tests for hypotheses of adaptation and counteradaptation and their importance to diversification.

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### CONTENTS

Introduction . . . . . 554

\* Correspondence to: B. D. Farrell. Email: bfarrell@oeb.harvard.edu.

Natural history of <i>Tetraopes</i> and allies . . . . .	555
Relationships among hostplants . . . . .	559
Material and methods . . . . .	560
Morphological analysis . . . . .	560
Allozyme electrophoresis . . . . .	561
Phylogenetic analyses . . . . .	563
Comparisons of phylogeny estimates . . . . .	563
Results . . . . .	564
Allozyme data . . . . .	564
Phylogenetic analyses . . . . .	564
Concordance of insect and plant cladograms . . . . .	566
Discussion . . . . .	567
Ages of associated beetles and hosts . . . . .	568
Coevolution? . . . . .	569
Concluding observations . . . . .	572
Acknowledgements . . . . .	573
References . . . . .	573
Appendix . . . . .	577

## INTRODUCTION

Among explanations offered for the great diversity of phytophagous insects and their hostplants, none has been more influential than Ehrlich & Raven's (1964) theory of coevolution. For three decades this proposal has stimulated work on the physiological, genetic and ecological mechanisms of insect/plant interactions, focused especially on the role of plant secondary chemistry (Futuyma & Keese, 1992). Only recently, however, has there begun comparably rigorous investigation of the model's macroevolutionary implication, namely that these interactions have promoted diversification of associated insect and plant groups.

This study opens an inquiry into the possibility of coevolutionary diversification for the North American longhorn beetle genus *Tetraopes* and its primary hosts, the milkweed genus *Asclepias*. We first present a phylogenetic analysis of *Tetraopes*, based on morphology and allozymes. In conjunction with published information on the phylogeny, biogeography and secondary chemistry of *Asclepias*, we then use these results to examine several questions raised by Ehrlich & Raven's theory.

The main focus of this paper is on how long and continuously these beetle and plant species, and their ancestors, have interacted: what has been the opportunity for coevolution? One expectation, if insect and plant lineages have diversified in association, is that the phylogenetic order of divergence among extant host taxa should correspond in some way to that among their associated herbivores. For example, if the insects never switched hosts, the association could be continuous over time, yielding an essentially perfect match of speciation sequences. In contrast, the 'escape and radiation' process envisioned by Ehrlich and Raven should produce an only imperfect correlation of speciation sequences, but a closer match of insect phylogeny to the evolutionary succession of plant defences.

The few explicit studies so far suggest great heterogeneity in the correspondence between insect and hostplant phylogenies. In a review of 14 assemblages for which at least partial cladogram comparison can be made (Mitter & Farrell, 1991; Farrell, Mitter & Futuyma, 1992), the mean fraction of groupings on which insects and hostplants agreed was about 50%, suggesting that some degree of parallel diversification might be common. However, in only about a quarter of the comparisons

was the match significant or nearly so under heuristic statistical tests, and in about the same number there was no correspondence at all.

A biological pattern to this variation was suggested by a study of *Phyllobrotica* leaf beetles, which show the most detailed match to host phylogeny of any herbivorous insect group yet reported (Farrell & Mitter, 1990). These beetles exhibit unusually intimate dependence on their hosts, mostly herbaceous mints in the genus *Scutellaria*. Each beetle species is restricted to a single host species; the larvae are internal feeders, in the roots, and the adults both feed and mate on the larval host. The adults, moreover, appear aposematically coloured, suggesting that like other herbivores of plants that contain iridoid glycosides (Bowers, 1988), they might rely on host-derived toxins for protection from their predators. All these traits have been argued to enforce long-term host fidelity (Feeny, 1987), and may make parallel diversification especially likely.

*Tetraopes* and its hosts provide a test of this conjecture, because the life history of these beetles is strikingly similar to that of *Phyllobrotica*. We will present evidence that phylogenetic relationships within *Tetraopes* are also strongly concordant with those of their hosts. Phylogeny concordance by itself, however, is not sufficient to demonstrate parallel phylogenesis: the entire insect clade could instead be younger than any of the host species, but have undergone colonization and speciation in a sequence dictated by features that mirror host phylogeny. For example, derived plant species might often bear unusual secondary chemistries, causing them to be colonized later than their less distinctive, more primitive relatives.

We will present initial evidence consistent with such 'escape and radiation': *Asclepias* shows a phylogenetic progression in the potency and location of cardenolides, suggesting escalation of defence, which appears related to species diversity of both milkweeds and beetles.

#### *Natural history of Tetraopes and allies*

*Tetraopes* and its apparent nearest relatives, the genus *Phaea*, belong to the monophyletic tribe Tetraopini, subfamily Lamiinae, of the Cerambycidae. The tribes Tetraopini, Hemilophini and Phytoecini together form the apparently monophyletic 'Phytoeciides vrais' of Chapuis (1872), distinguished by bifid tarsal claws, and constitute nearly 25% of the 50 000 described cerambycid species. Larvae of these tribes invariably bore in stems or roots of their hostplants, which are typically herbaceous, while the adults consume the foliage and flowers. A preliminary morphological phylogenetic study treating all 40 genera of Tetraopini plus outgroups confirmed that *Tetraopes* and *Phaea* together form an entirely New World clade, defined by appendiculate tarsal claws. This clade in turn appears most closely related to the Philippine genus *Eustathes*, with which it shares a pronotal umbone. *Tetraopes* is distributed from Guatemala to Canada, and is most diverse in the Sonoran region (Chemsak, 1963; Chemsak & Linsley, 1979). *Phaea*, currently under revision (Chemsak, 1977; J. Chemsak, pers. comm.), appears to contain about 30 species distributed from northern South America (Colombia and Venezuela) to the central United States, and is most diverse in Central America.

The following account of life histories in *Tetraopes* is based largely on the revision

TABLE 1. Distributions and host affiliations of *Tetraopes* species, plus the subset of species in the sister genus *Phaea* included in this study. An asterisk follows the name of each species sampled for allozymes

Taxa	Hosts	Distribution
<i>Phaea jucunda</i>	<i>Ipomoea pandurata</i>	Southeastern U.S.
<i>P. canescens</i>	<i>I. leptophylla</i>	Midwestern U.S.
<i>P. mirabilis</i> *	<i>Thevetia ovata</i>	S. Mexico
<i>P. maryannae</i> *	<i>Stemmadenia palmeri</i>	S. Mexico Nicaragua
<i>P. bipagiata</i>	<i>Stemmadenia palmeri</i>	S. Mexico to Guatemala
<i>Tetraopes mankinsi</i>		Honduras, Guatemala
<i>T. melanurus</i> *	<i>Asclepias tuberosa</i>	Eastern U.S.
<i>T. cleroides</i>		Central Mexico
<i>T. paracomis</i>	<i>Matelea quirsii</i>	Guatemala to Costa Rica
<i>T. comes</i>		S. Mexico to Costa Rica
<i>T. elegans</i>		Baja California
<i>T. ineditus</i>	<i>Marsdenia lanata</i>	Western Mexico
<i>T. batesi</i>		Western Mexico
<i>T. umbonatus</i> *	<i>A. glaucescens</i>	S. Mexico to Nicaragua
<i>T. linsleyi</i>	<i>A. linaria</i>	AZ, TX, NM
<i>T. discoideus</i> *	<i>A. subverticillata</i>	AZ, TX, NM
	<i>A. curassavica</i>	Mexico to El Salvador
<i>T. annulatus</i> *	<i>A. sullivantii</i> (MO)	TX, NM, AZ, North to Canada
	<i>A. subverticillata</i> (AZ)	
	<i>A. speciosa</i> (UT)	
<i>T. pilosus</i> *	<i>A. arenaria</i>	TX, KS, NE, OK
<i>T. tetraphthalmus</i> *	<i>A. syriaca</i>	Northeastern U.S.
<i>T. varicornis</i> *	<i>A. notha</i>	S. Mexico
<i>T. texanus</i> *		TX, OK
<i>T. mandibularis</i> *	<i>A. latifolia</i>	TX, OK
<i>T. quinquemaculatus</i> *	<i>A. amplexicaulis</i>	Midwestern U.S., to S. Ontario
<i>T. subfasciatus</i>		Central Mexico
<i>T. thoreyi</i>		Central Mexico
<i>T. sublaevis</i> *	<i>A. erosa</i>	S. California
<i>T. thermophilus</i>		SE TX, S to El Salvador
<i>T. basalis</i> *	<i>A. eriocarpa</i>	CA, S. Oregon
<i>T. femoratus</i> *	<i>A. speciosa</i>	Central and western North America

by Chemsak (1963), supplemented by observations from fieldwork by the senior author across North and Central America (Tables 1, 2).

*Tetraopes* larvae attack roots, while the adults, whose emergence coincides with hostplant flowering, feed on upper foliage and flowers. The adult females of *Tetraopes tetraphthalmus* (Forster) oviposit 8–20 eggs inside grass stems within a few centimeters of a milkweed plant, gaining access via a hole chewed for the purpose. About 10 days later, the hatchling larvae emerge from the oviposition access, fall to the ground and immediately burrow downward, in presumed search for host roots. Hartman (1977) documented extensive damage to the roots of *Asclepias syriaca* by larvae of *Tetraopes tetraphthalmus*, while Chemsak (1963) presented evidence of similar damage to the roots of *A. erosa* by its herbivore, *Tetraopes sublaevis* Casey; to *A. eriocarpa* by *T. basalis* LeConte; and to *A. speciosa* by *T. femoratus* LeConte. Similar damage is caused by other species of *Tetraopes*. *Tetraopes* larvae feed both inside and outside of the root system and there seems to be a correlation of adult body size with the size of the larval resource (Price & Wilson, 1976; Hespenheide, 1973). Larval root feeding is unique to *Tetraopes* in the subfamily Lamiinae; *Phaea* and other genera mine stems (Linsley, 1961).

With few confirmed exceptions, individual *Tetraopes* species or their subspecies are

TABLE 2. Localities from which population samples of *Tetraopes* and *Phaea* species were obtained for protein electrophoresis

Taxa	Localities sampled
<i>Phaea mirabilis</i>	MEXICO: Estado Morelos, Zumpango del Rio
<i>P. biplogiata</i>	MEXICO: Estado Guerrero, Iguala
<i>P. maryannae</i>	MEXICO: Estado Guerrero, Iguala
<i>Tetraopes discoideus</i>	(1) AZ: Portal MEXICO: (2) Taxco (3) Puebla, Atlixco
<i>T. umbonatus</i>	MEXICO: (1) Guerrero, Xochichalca Ruins (2) Oculixtlhuacan (3) Iguala (4) Puebla, Atlixco
<i>T. melanurus</i>	MD: Prince Georges Co., Patuxent Wildlife Research Center
<i>T. quinque maculatus</i>	MO: (1) Clay Co., Flemington; (2) Le Petite Grande Prairie; (3) Neiwahe Prairie; (4) KS: Reno Co., Hutchinson
<i>T. texanus</i>	(1) MO: Clay Co., Flemington (2) TX: Cooke Co., Gainesville
<i>T. annulatus</i>	NM: (1) McKinley Co. Mesita [16] (2) Gallup [19] (3) AZ: Apache Co., Houck
<i>T. pilosus</i>	(1) KS: Reno Co., Sandhill State Park, Hutchinson (2) TX: Ward Co., Monahans
<i>T. tetrophthalmus</i>	MO: Clay Co.: (1) Independence; (2) Flemington; (3) VT: Chittenden Co.: Colchester; (4) MD: Prince Georges Co. Patuxent Wildlife Research Center
<i>T. mandibularis</i>	TX: Dickens Co., Dickens
<i>T. varicornis</i>	MEXICO: Puebla, Rio Frio
<i>T. femoratus</i>	(1) NV: Reno (2) CA: Graeagle
<i>T. basalis</i>	CA: Plumas Co.; Quincy
<i>T. sublaevis</i>	CA: Riverside Co., Blythe

affiliated with single, differing species of milkweeds in the subgenus *Asclepias* (*Asclepias*). The following brief account is summarized in Table 1. *Tetraopes umbonatus* LeConte is affiliated with *A. glaucescens* throughout southern Mexico (Chemsak, 1963; Farrell, 1991). *Tetraopes paracomis* Chemsak and *Tetraopes ineditus* Chemsak are associated with *Marsdenia* and *Matelea*, respectively, vining milkweeds in the lowland forests of Central America and Mexico (B. Farrell, unpublished). *Tetraopes discoideus* LeConte is the only species confirmed to use two hostplant species (Chemsak, 1963; Farrell, 1991). It ranges from southern Mexico, where it feeds on *A. curassavica* (Chemsak, 1963; Farrell, 1991), to the southwestern United States, where it attacks *A. subverticillata* (Chemsak, 1963; Farrell, 1991), and is absent from the area north of Mexico City to approximately the U.S. border. Both of these hostplant species are in Woodson's (1954) series Incarnatae and their cardenolide profiles are similar (Roeske *et al.*, 1976). *Tetraopes melanurus* Schonherr attacks *A. tuberosa* throughout the eastern United States (Chemsak, 1963; Farrell, 1991). *Tetraopes tetrophthalmus* feeds on *Asclepias syriaca* throughout its range (Farrell, 1991; Hartman, 1977), though an isolated population in a disturbed site in Illinois was reported on *A. verticillata*, where the adults may suffer reduced fitness (Price & Willson, 1976). *Tetraopes femoratus* is affiliated with *A. speciosa* throughout the western United States (Chemsak, 1963; Farrell, 1991), but has been reported in very low numbers from *A. syriaca* at the eastern edge of its range (Lawrence, 1982). *Tetraopes pilosus* Chemsak and its host, *A. arenaria*, are restricted to the Quaternary sandhills of the midwestern U.S. (Farrell, 1991). Both beetle and host are clothed in white pubescence, possible adaptations against moisture loss and overheating in their xeric dune habitats. *Tetraopes mandibularis* Chemsak is affiliated with *Asclepias latifolia* in the Llano Estacado region of northwestern Texas and adjacent Oklahoma (Rice, Turnbow & Hovore, 1985; Farrell, 1991). *Tetraopes varicornis* Castelnau uses *A. notha* in southern Mexico (Farrell, 1991). *Tetraopes sublaevis* and its sole host *A. erosa* are confined to the lower Colorado Desert (Chemsak, 1963; Farrell, 1991).

Two additional species of *Tetraopes* have been reported only from single host *Asclepias* species, and we have no direct observations that contradict these associations though we cannot confirm them first hand. *Tetraopes quinque maculatus* Haldeman was reported to attack *A. amplexicaulis* in the midwestern U.S. (Price & Wilson, 1979) and we have collected several specimens of this species in the vicinity of *A. amplexicaulis* but have not observed adult or larval feeding. *Tetraopes linsleyi* Chemsak reportedly uses *A. linaria*, the only milkweed to occur in its dry Chiricahuan canyon habitats (Hovore, 1983; pers. comm.). We provisionally accept these associations pending confirmation. In contrast, while *Tetraopes elegans* Horn was inferred to use *A. subulata* by Chemsak (1963) on the grounds that this is one of very few milkweeds that occurs throughout its range in Baja California, the distribution of *Asclepias albicans* is also very similar (Woodson, 1954) to that of *T. elegans* and two other *Asclepias* species, very closely-related to *A. albicans*, also occur in Baja. Therefore, we regard the host of *T. elegans* as unknown.

The hosts of *T. annulatus* LeConte are uncertain, but this may be among the very few *Tetraopes* species to use more than one host. While adults of this species have been found on *A. subverticillata* in Arizona, they have also been collected from *A. speciosa* in Utah, and from the closely-related *A. sullivanii* in Missouri (M. Rice, pers. comm.). The remarkable range in body size of this species (Chemsak, 1963; Farrell, 1991) also suggests larval feeding on different hostplants, although this remains unconfirmed.

Like other herbivore groups popular with amateur collectors, *Tetraopes* are sometimes recorded from plant species other than those supporting growth and reproduction. For example, while *Tetraopes tetraphthalmus* adults most commonly feed on and oviposit near *Asclepias syriaca*, we have also seen this beetle feeding on flowers of *A. incarnata* (swamp milkweed), which often occurs in the wet margins of fields occupied by *A. syriaca* and flowers slightly later. However, we have never observed oviposition near *A. incarnata*, and it is very unlikely that the beetle larvae could survive the combination of very wet soil and the very shallow and fine root system offered by this milkweed species (Hartman, 1977). We have also occasionally observed adults of several other species on non-host milkweeds, at the edge of the beetles' range, and after peak adult emergence. Thus, at least some accidental records may be attributable to post-reproductive dispersal of adults, which sometimes undertake long-distance flights (Davis, 1980a,b, 1984; D. McCauley, pers. comm.).

With one exception, adult oviposition and larval habits of *Phaea* species have been heretofore undescribed. The genus appears to consist of two morphological subgroups which are affiliated respectively with Convolvulaceae and Apocynaceae, members of related orders in the subclass Asteridae (Olmstead *et al.*, 1992) which share by convergence the presence of latex canals. *Phaea jucunda* has long been known to bore in the stems of the convolvulaceous vine *Ipomoea pandurata* (Craighead, 1923; Rice *et al.*, 1985), and other morphologically similar species also attack *Ipomoea* species. For example, *P. canescens*, the only other North American species of *Phaea*, attacks the shrubby *Ipomoea leptophylla* (M. Rice, pers. comm.), while the Central American *P. acromela* is affiliated with an as yet unidentified species of arborescent *Ipomoea* (F. Hovore, pers. comm.). The remaining Central American species in this subgroup seem likewise affiliated with woody *Ipomoea* species (J. Chemsak, F. Hovore, E. Giesbert, pers. comm.). In southern Mexico, at least some of the species in the other *Phaea* subgroup oviposit in stems of arborescent Apocynaceae. For example, *Phaea maryannae* and *P. biplagiata* both attack *Stemmadenia palmeri*, while *P. mirabilis* oviposits

in *Thevetia ovata* (Farrell, 1991). Verbal descriptions of the hosts of other *Phaea* species indicate that these are also latex-bearing trees with tubular flowers, strongly suggesting either Apocynaceae or Convolvulaceae, but these await identification.

Adults of species of *Phaea* and *Tetraopes* bear apparently aposematic, bright orange or red markings of varying extent. Aposematism is unusually widespread among herbivores of the Apocynaceae and Asclepiadaceae, and many of these specialized insects, including *Tetraopes*, sequester toxins from their hostplants for defense against predators (Brower & Brower, 1964; Scudder & Duffy, 1972; Rothschild, 1973; Isman, Duffy & Scudder, 1977; Marsh *et al.*, 1977; Nishio, Blum & Takahashi, 1983; Brower *et al.*, 1984a,b; Berenbaum & Miliczky, 1984; Ackery & Vane-Wright, 1984). Variation in adult coloration and flight among *Tetraopes* species may be correlated with host chemistry. The more primitive beetle species, affiliated with apparently less toxic hostplants (see below), have much less surface area brightly-colored (Chemsak, 1963), are smaller in body size on average, and are also more difficult to capture.

#### *Relationships among hostplants*

The following review is summarized in Figure 1. The two main hostplant families of *Tetraopes* and relatives, Asclepiadaceae and Apocynaceae, form a group sometimes termed the Apocynales. Its monophyly is supported by, among other derived characters, the possession of latex canals and cardiac glycosides (Cronquist, 1981). A recent molecular phylogenetic study (Sennblad & Bremer, 1996) has shown the Apocynaceae to be paraphyletic. Of the two apocynaceous hosts of *Phaea*, *Thevetia* and *Stemmadenia*, *Thevetia* is closest to the monophyletic Asclepiadaceae, while the group that includes *Tabernaemontana*, the closest relative of *Stemmadenia* (not included in the study), is more basal (Sennblad & Bremer, 1996). Among the three hostgenera of *Tetraopes*, all in Asclepiadaceae, *Marsdenia* is closer to *Asclepias* than *Matelea* (Woodson, 1941).

*Asclepias* is a North American genus of about 120 species, most recently revised by Woodson (1954). Most of the known *Tetraopes* hosts are in the nominate subgenus, the largest of nine subgenera recognized by Woodson. Woodson grouped the 72 species of this subgenus into eight 'series', among which he postulated the phylogenetic relationships shown in Figure 1.

Striking, independent support for Woodson's arrangement comes from subsequent studies of the distribution of cardenolide types among milkweed species which are host to the monarch butterfly. These have included 20 species representing all but one of Woodson's series (Nelson, Seiber & Brower, 1981). Series 5–8 are unique in producing cardenolides of the labriniformin type, whereas series 1–4 produce cardenolides of only the calotropogenin type, which are widespread across Asclepiadaceae and presumably primitive (Brower *et al.*, 1984a,b). Within the chemically advanced group of series, the levels, locations and identities of characteristic cardenolides can be further arranged into transformation series consistent with Woodson's arrangement (Fig. 1). As detailed in the Discussion, these features, in addition to supporting Woodson's phylogeny, also suggest a sequence of increasingly effective defenses. In summary, while Woodson's phylogeny needs re-examination, it provides a credible first estimate to which the independent estimate for *Tetraopes* can be compared.

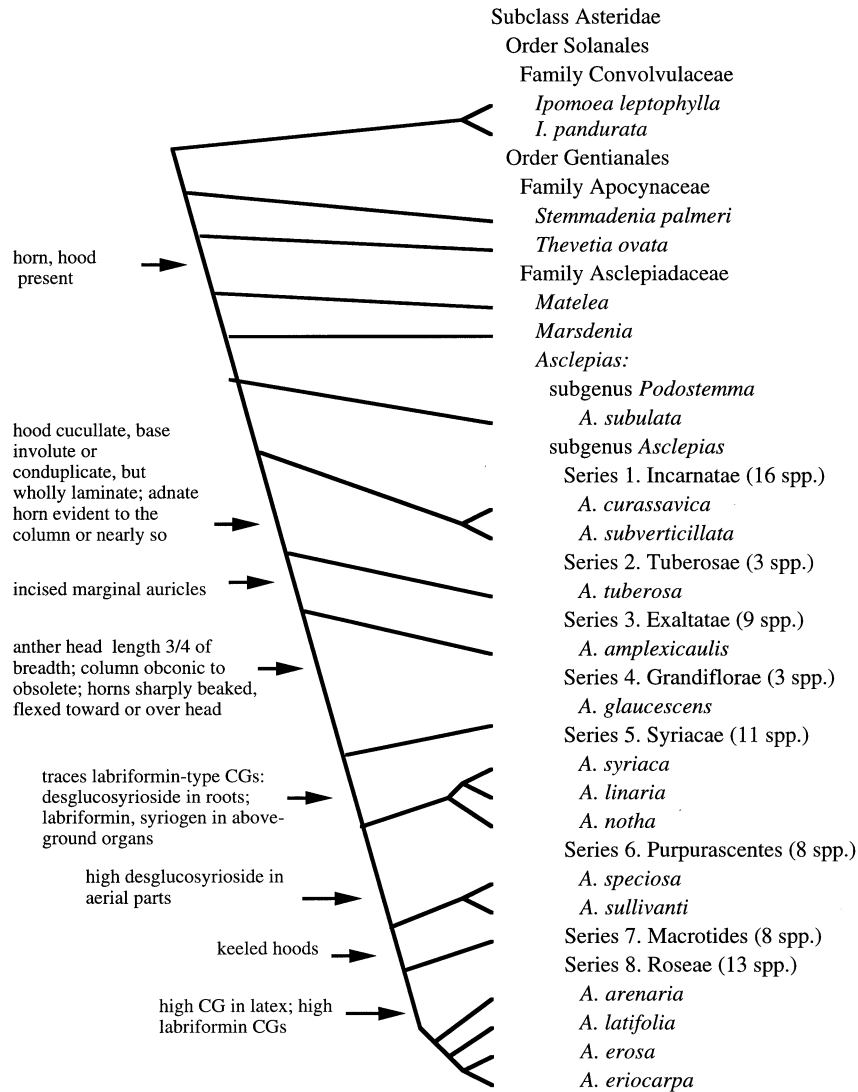


Figure 1. Taxonomic arrangement of hostplants used by *Tetraopes* and *Phaea* following Woodson (1954, 1941) within *Asclepias*; and Sennblad & Bremer (1996) and Leeuwenberg (1994) outside of *Asclepias*.

## MATERIAL AND METHODS

### *Morphological analysis*

Adult morphological characters were scored for all species of *Tetraopes*; seven species of *Phaea*, including all those with known hostplants; and the Philippine genus *Eustathes*, nearest relative of *Tetraopes* + *Phaea*. Morphological features were examined under a Wild dissecting microscope. Dissections were performed with sharpened insect pins and stored in alcohol for further examination. Soft tissues were cleared from genitalic dissections in 10% KOH. The 26 features showing essentially fixed



TABLE 3. Morphological characters in *Tetraopes* and *Phaea* scored for phylogenetic analyses. Numbers following descriptions are ordered character state codings

---

HEAD	
1.	First antennomere longer than scape 0; shorter than scape 1
2.	Mandible sexually monomorphic 0; dimorphic 1
3.	Labial palpi long, narrow 0; subovoid 1; ovoid 2
4.	Antennal annulation absent 0; present 1
5.	Scape lateroapical carinae absent 0; present 1
THORAX	
6.	Pronotal umbone absent 0; present, weakly developed 1; strongly developed 2; strongly developed with lateral ridge 3
7.	Umbonal lateral sulci absent 0; present at frontal 1/2 1; sulci along length of umbone 2; sulci continuous around umbone 3
8.	Lateral macula absent 0; present 1
9.	Umbonal maculae absent 0; present 1
10.	Procoxal cavities widely separated by prosternum 0; cavities contiguous or nearly so 1
11.	Lateral umbone absent 0; present, weakly developed 1; strongly developed 2
ELYTRA	
12.	Elytral disc coarsely punctate 0; smooth 1
13.	Discal chevron absent 0; present 1
14.	Apical chevron absent 0; present 1
15.	Humeral maculae absent 0; present 1
16.	Discal maculae absent 0; present 1
17.	Apical maculae absent 0; present 1
18.	Elytral form mesally constricted, subparallel, slender 0; stout, parallel 1
19.	Lateral macula absent 0; present 1
APPENDAGES	
20.	Claws appendiculate 0; bifid 1
21.	Male metatrochanter spur absent 0; present 1
ABDOMEN	
22.	Female sternal sulcus present 0; absent 1
23.	Aedeagus dorsal piece subtending ventral 0; overlapping ventral 1
24.	Aedeagal apex lanceolate 0; explanate 1
25.	Aedeagus with dorsal piece lateral explanation absent 0; present 1
OVERALL HABITUS	
26.	Exoskeleton with scattered, sparse golden pubescence 0; with dense, white pubescence 1

---

differences among species were coded as discrete characters for phylogenetic analysis (Tables 3,4).

#### *Allozyme electrophoresis*

Fresh material of three species of *Phaea* and 13 species of *Tetraopes*, including all those with confirmed host affiliations, was fresh-frozen in liquid nitrogen in the field, and stored at  $-85^{\circ}\text{C}$ . Whenever possible, electrophoretic analyses for each species included samples from several geographic regions (Table 2).

Starch gel electrophoresis was performed using methods modified from Hillis & Moritz (1990). Frozen individual beetles were homogenized in 7 ml of ice-cold homogenization buffer (200 mM Tris-HCL, pH 8.0, 26 mM sodium metabisulfite, 10 mM  $\text{MgCl}_2$  1.5 mM EDTA, 5% w/v PVP-40, 0.05% 2-mercaptoethanol; modified from Futuyama & McCafferty, 1990), and spun for 5 minutes in a refrigerated microfuge. Half the supernatant was frozen for subsequent runs. The rest was loaded



into cold gels (11% starch from Connaught Laboratories) using 5 mm square wicks of Whatman #2 filter paper. All enzymes were run for 14–15 hours on small (200 ml total volume) or large (450 ml total volume) gels, in one of seven buffer systems determined by an initial survey to be optimal for allele separation and resolution (Appendix). Trays of ice were placed on top of the gels, which were run in a refrigerator at 0–4°C. Gels were sliced two or three times and stained following recipes in Hillis & Moritz (1990). Electromorph differences and identities were confirmed through multiple, side-by-side comparisons.

#### *Phylogenetic analyses*

Morphological characters with more than two states (5 of 26 characters) were coded to reflect transformation series inferred from morphological similarity (Table 3). For the allozyme data, each locus was treated as a character, with alleles as the unordered states (Table 4). Polymorphism was treated as ambiguous.

Morphological and allelic data were first analysed separately for the subset of 16 taxa on which both were obtained, which includes nearly all species with known hosts. As these analyses yielded largely concordant groupings, a third phylogeny estimate was obtained for these species by combining the two data sets. This tree was then compared to that for all 29 species based on both data sets, as a test for the possible effects of missing taxa on the estimate for the reduced set. Finally, a tree was calculated for all taxa with known hosts, using both character sets, and compared to the host phylogeny.

Most parsimonious trees were generated in each analysis using the heuristic search routines in PAUP 3.1.1 (Swofford, 1993), with 100 random addition sequences and successive weighting using mean retention indices. Decay analyses used AutoDecay 1.1 (courtesy of N. Eriksson), with 10 random addition sequences for each reverse constraint tree analysis. Similarity clustering was also performed on the allozyme allele frequencies, using the Manhattan, Nei's and Roger's distance coefficients and the UPGMA routine in NTSYS-pc (Rohlf, 1990).

*Phaea mirabilis* was deleted from the data matrix for calculations based on distances because it showed 100% divergence from the most derived *Tetraopes* species, rendering Nei's distance infinite.

#### *Comparisons of phylogeny estimates*

The problem of measuring correspondence between host and parasite phylogeny estimates has been recently reviewed by Page (1995). Our earlier work (Farrell & Mitter, 1990) applied quantitative methods developed for similar problems in biogeography (Page, 1990) and in evaluation of cladograms generated by different data sets for the same taxa (Shao & Sokal, 1986). Recent conceptual advances permit more realistic evaluation of phylogeny correspondence when host-parasite associations may result from several underlying processes (Page, 1995). We therefore applied cospeciation analysis as implemented in the TreeMap program package of Page (1995). In this approach, one maps the number of cospeciation events that explain the distribution of the observed insect species across hostplant species entirely through parallel phylogenesis, minimizing the total number of host-shifts, sorting

and duplication events. Statistical significance is evaluated by comparing the observed cospeciation count to the distribution of values for randomly generated parasite and/or host phylogenies (Page, 1995). TreeMap permits generation of trees under either Markov or proportional-to-distinguishable models (Page, 1995). We selected the more conservative Markov model of tree generation, which consistently produced higher (i.e. of lower significance) *P* values. We also chose to randomize the beetle phylogeny estimate as this contains fewer taxa for the purposes of this study. When comparing trees of unequal size, randomization of the smaller tree will produce higher *P* values for any given number of cospeciation events, and thus is more conservative.

Using this approach, the one species apparently associated with multiple *Asclepias* hosts, *Tetraopes annulatus*, was scored for association with each host separately, and scored for association with all three hosts. The strikingly allopatric Arizonan and Mexican populations of *Tetraopes discoideus* were treated as separate entities, each associated with a different host, as these populations are also divergent in morphology and allozymes (see Results). We did not include the *Tetraopes* species for which there were no direct observations of host-use.

## RESULTS

### *Allozyme data*

Twenty-three apparent loci were resolved (Appendix), all of which showed interspecific variation. These include all the loci analysed in *Tetraopes tetrophthalmus* by McCauley & Eanes (1987), except that we did not assay leucine amino peptidase or hexokinase. Intra-population polymorphism was uncommon, and there were few instances where more than one allele was shared with other species. Thus, very little phylogenetic information was lost in the discrete coding. In no instance of polymorphism was there significant departure from Hardy–Weinberg genotype proportions.

Nei's distance between the populations of *T. femoratus*, *T. annulatus* and *T. tetrophthalmus* ranged between 0.007 and 0.044, suggesting moderate levels of divergence accompanying geographic isolation. For each of these species, mean distances were used in the phenetic analysis reported below. The small samples of *T. discoideus* from southern Mexico and Arizona differed more strongly. Chemsak (1963) also noted consistent (though not invariant) differences in head color between these populations.

### *Phylogenetic analyses*

For the 16 species scored for both morphology and allozymes, and for which hostplants are known, the morphological data alone yield four most parsimonious trees of which the strict consensus is shown in Figure 2a. The allozyme data alone

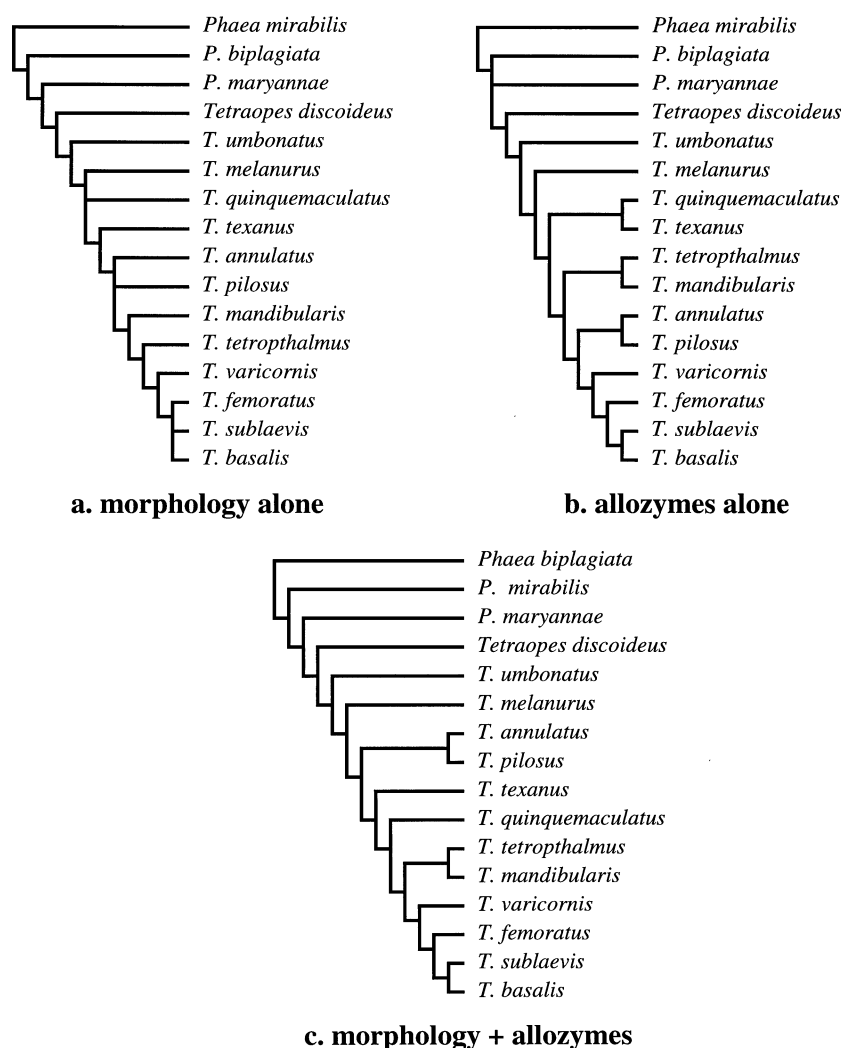


Figure 2. (a) Strict consensus tree of the four most parsimonious trees based on morphology alone, for the subset of *Tetraopes* species for which allozyme data are also available. Morphological characters do not resolve relationships among *T. femoratus*, *T. sublaevis*, and *T. basalis*, and are equivocal about placements of *T. melanurus* and *T. quinquemaculatus*. Length is 42 steps, rescaled consistency index = 0.5. (b) Strict consensus tree of two most parsimonious trees for subset of *Tetraopes* and *Phaea* species based on allozyme data, coded as in Table 4. Length is 90 steps, rescaled consistency index = 0.7. Allozymes resolve all relationships but are equivocal about the placements of *Phaea biplagiata* and *P. maryannae*, which are almost completely divergent from *Tetraopes* species. (c) Single most parsimonious tree for *Tetraopes* and *Phaea* species for which allozyme data are available, based on Table 4 (all data). Length is 140 steps, rescaled consistency index = 0.6.

resulted in two most parsimonious trees, whose strict consensus tree is shown in Figure 2b. UPGMA clustering on allele-frequency distances specifies an almost identical tree (Fig. 3). The allozyme-based estimate is slightly more resolved within

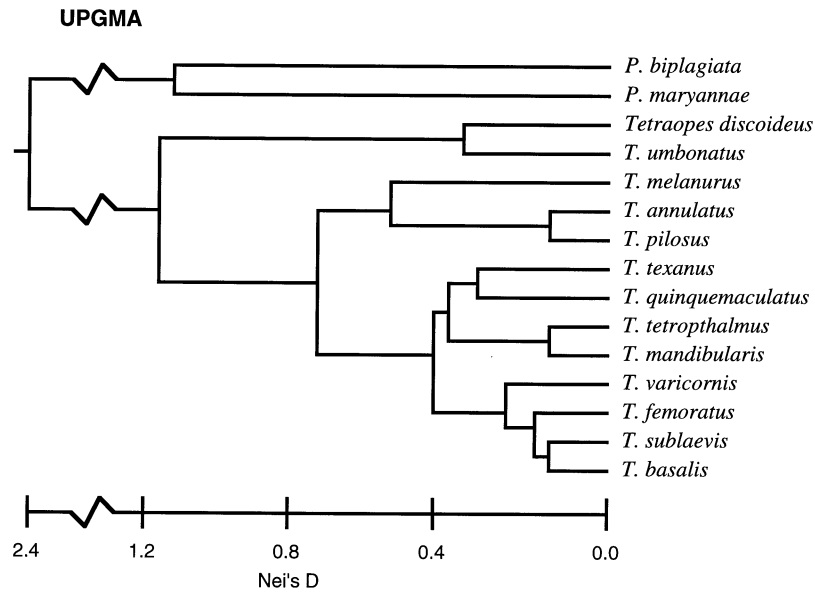


Figure 3. UPGMA phenogram of Nei's distances for *Tetraopes* and *Phaea* species specifies nearly identical relationships to cladistic analysis of same data. UPGMA clustering under Manhattan, Rogers' and Prevosti' distances specifies same tree.

*Tetraopes* than that based on morphology, but less resolved for the outgroup *Phaea* species. The two disagree only by minor re-positioning among nearby taxa, namely *T. quinquemaculatus*, *T. mandibularis*, and the pair *T. annulatus*/*T. pilosus*. The two data sets analysed in combination yield six most parsimonious trees. Following successive weighting by the mean retention indices of these, a single most parsimonious tree results (Fig. 2c) which is completely resolved, with at least one disagreement resolved in favor of each data set. The relationships among these taxa are little changed in the most parsimonious trees resulting when the 13 species scored only for morphology are added to the combined data set (Fig. 4). This tree confirms the monophyly of *Tetraopes* and suggests that *Phaea* is paraphyletic, with the Apocynaceae-feeding group most closely allied to *Tetraopes*.

#### *Concordance of insect and plant cladograms*

The phylogeny for the beetle species with recorded hosts, extracted from the phylogeny for all species based on the combined data sets (Fig. 4), is compared to the literature-synthesized estimate of host relationships (Fig. 1) in Figure 6. There are several points of disagreement, of which the most striking involve *T. pilosus* and *T. mandibularis*, both of which appear to represent colonization of the advanced host series Roseae from ancestors affiliated with more primitive milkweeds. Overall, however, the phylogenies appear to match fairly well, an impression supported by heuristic statistical analyses. Under cospeciation analysis as implemented in the TreeMap package of Page (1995), 13 cospeciation events explain the distribution of beetle across host species when *Tetraopes annulatus* is scored for association with either

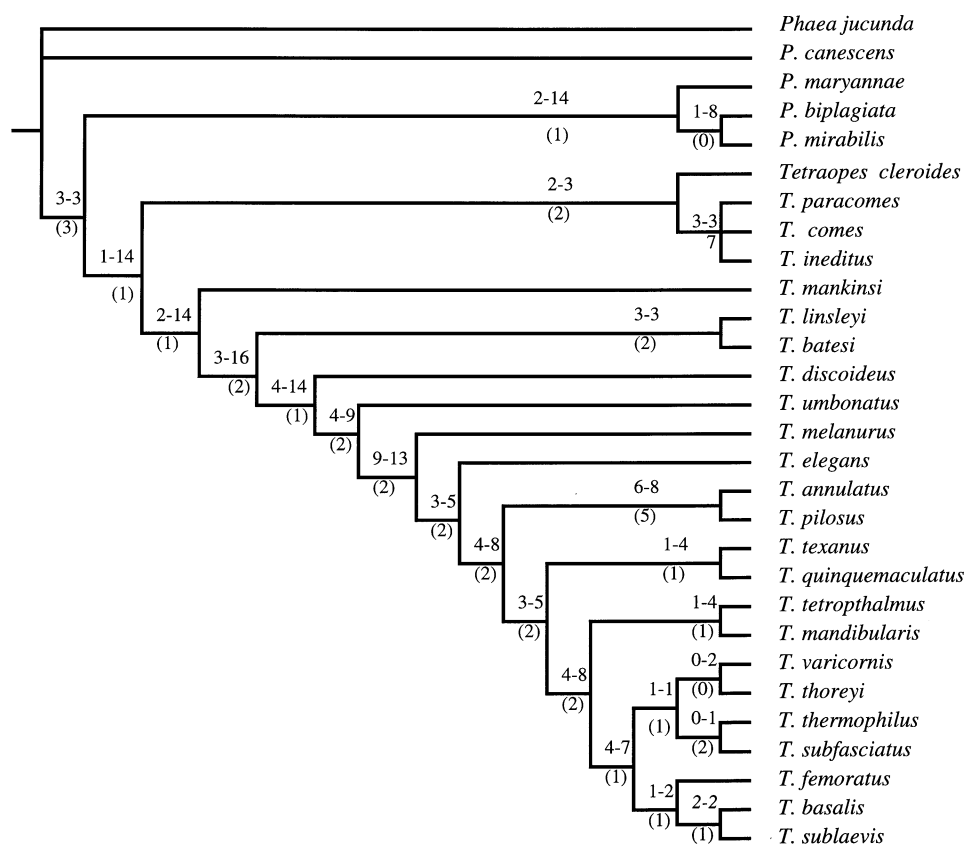


Figure 4. One of the six most parsimonious trees for all species of *Tetraopes* plus a subset of *Phaea* species, based on all available data. Trees were obtained under heuristic search routine and 100 random addition sequences in PAUP (vers. 3.1.1:courtesy of D. Swofford). Length is 173 steps, consistency index = 0.62, retention index is 0.79. Character changes were optimized on branches under DELTRAN routine, which favors parallelisms over reversals. The numbers adjacent to internal nodes are the numbers of unambiguous changes followed by the maximum number of changes for each grouping, with the decay index in parentheses.

*Asclepias sullivantii* or *A. speciosa* ( $P=0.01$ ). Ten cospeciation events result in the analysis when *Tetraopes annulatus* is scored for association with *A. subverticillata* or all three plant species ( $P=0.07$ ). When *Tetraopes annulatus* is omitted from the analysis, on the grounds that its affiliations are ambiguous, the estimate is 12 cospeciation events ( $P=0.038$ ). If *Phaea* is also removed, confining the analysis to herbivores of Asclepiadaceae, the estimate is 10 cospeciation events. Ten or more cospeciation events are significant when the host or both host and beetle phylogeny estimates are randomized ( $P<0.05$ ).

#### DISCUSSION

The agreement between morphological and allozyme analyses suggests that the *Tetraopes* phylogeny estimate is reasonably robust, but addition of other molecular

character sets, now in progress, is needed to settle several weakly-resolved regions of the tree. The need for modern re-examination of *Asclepias* phylogeny, also underway, is even more evident, and dictates caution about any conclusion to be drawn here.

Nonetheless, the correspondence between the beetle and plant phylogenies appears stronger than expected by chance, and seems more likely to increase than decrease as those independent estimates are improved (i.e. unless error in the current estimates produced this pattern, correspondence should become more clear as error decreases). The match is inexact, suggesting that pairs of associated species have typically arisen most immediately by beetle colonizations from a related host rather than parallel speciation, but it is consistent with broadly synchronous diversification between lineages, providing the opportunity for long-term coevolution.

#### *Ages of associated beetles and hosts*

The earliest fossils of Apocynaceae are Paleocene (Muller, 1984), with extant genera appearing in the Eocene. The divergence between Apocynaceae + Asclepiadaceae and Convolvulaceae, which are in different orders, was probably considerably earlier. The earliest fossils of Asclepiadaceae appear in the Oligocene and Miocene (Muller, 1984); as these already represent extant genera, including *Asclepias*, the family is likely to be older. Fossil datings of subgroups within *Asclepias* are not available.

*Tetraopini* are represented by a single, Oligocene fossil, which cannot be further placed (Statz, 1938). Biogeography suggests that the *Phaea* + *Tetraopes* clade itself is this old or older: the disjunction between this New World lineage and its Southeast Asian sister group *Eustathes* mirrors the distributions of many pantropical groups for which the fossil record suggests Late Eocene to Early Oligocene origins (Tiffney, 1985; 35–>45 Mya), before northerly dispersal routes were cut off by Oligocene cooling.

Mapping of species distributions on the phylogeny estimate (Fig. 5) suggests a tropical lowland origin for *Tetraopes*, followed by more recent occupation of temperate upland savannah and most recently, Sonoran desert and midwestern Sandhills. A similar biogeographic history is suggested for *Asclepias* by Woodson's arrangement. Most species in the relatively primitive series 1–4 occupy mesic tropical habitats and are distributed from Mexico south, while most species in the derived series 5–8 occupy grassy temperate savannah habitats. Species within the highly-derived Roseae (series 8) have invaded the youngest, most severe habitats, the Sonoran Desert and central Sandhills of the United States.

These sequences of habitat occupation parallel the order of appearance of the various habitats in the plant fossil record (Axelrod, 1979; Wolfe, 1978, 1985; Tidwell & Nambudiri, 1989), as expected if endemic beetle and milkweed lineages and their habitats differentiated synchronously. However, they also parallel the order of severity of these habitats with respect to moisture. It is conceivable that both beetle and milkweed colonization would have been constrained to follow a similar sequence, even if they occurred entirely after differentiation of the habitats, by the need for successive pre-adaptations to successively harsher environments. This alternative seems less parsimonious to us, but we cannot entirely rule it out. If we provisionally accept the time scale implied by our habitat datings, the origin of association with



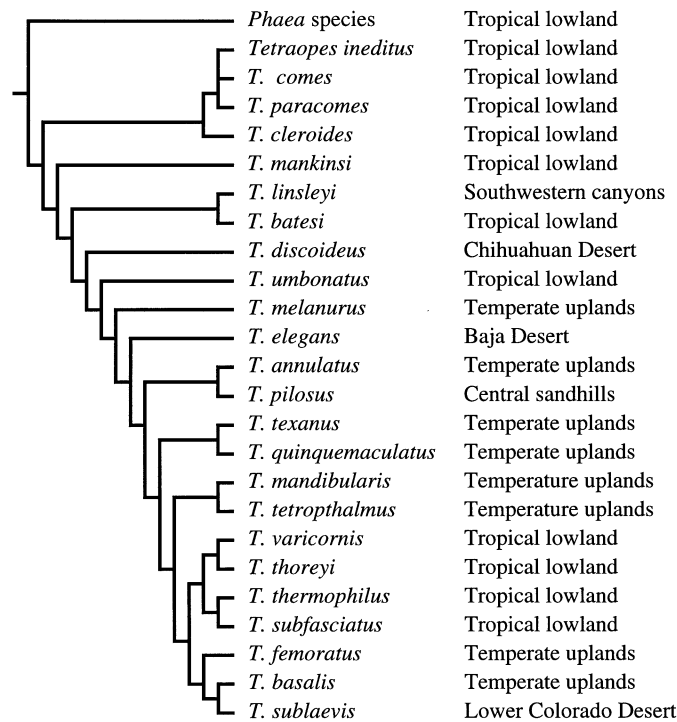


Figure 5. Biogeographic distributions optimized on tree from Figure 4 imply tropical origin followed by colonization of more temperate latitudes, and at least one secondary invasion of the tropics (by the ancestor of *T. thermophilus*, *T. subfasciatus*, *T. thoreyi*, and *T. varicornis*).

Asclepiadaceae, corresponding to the split between *Tetraopes* and *Phaea*, is dated to 40–47 Mya; association with subgenus *Asclepias* at more than 20 Mya (pre-dating divergence of *T. discoideus*); and exclusive association with the advanced series (5–8) of *Asclepias* (*T. mandibularis* through *T. basalis* in Figure 6) at ~7 Mya. These projections, like the sparse fossil evidence, are at least consistent with the hypothesis that *Tetraopes* and its subgroups have diversified in approximate synchrony with their asclepiadaceous hosts.

In contrast, the divergence between the *Phaea* groups feeding on Convolvulaceae versus Apocynaceae is probably not as old as that between their more distantly related host groups. Transfers between these families are relatively common, and may reflect both shared placement in the subclass Asteridae and the convergent possession of latex canals, which together account for a majority of the insect faunal connections between Apocynaceae/Asclepiadaceae and other plant families (Farrell & Mitter, 1993).

#### *Coevolution?*

The phylogenetic and temporal evidence adduced above suggests that *Tetraopes* and *Asclepias*, particularly subgenus *Asclepias*, have been associated during much of their respective histories, providing abundant opportunity for evolution in response

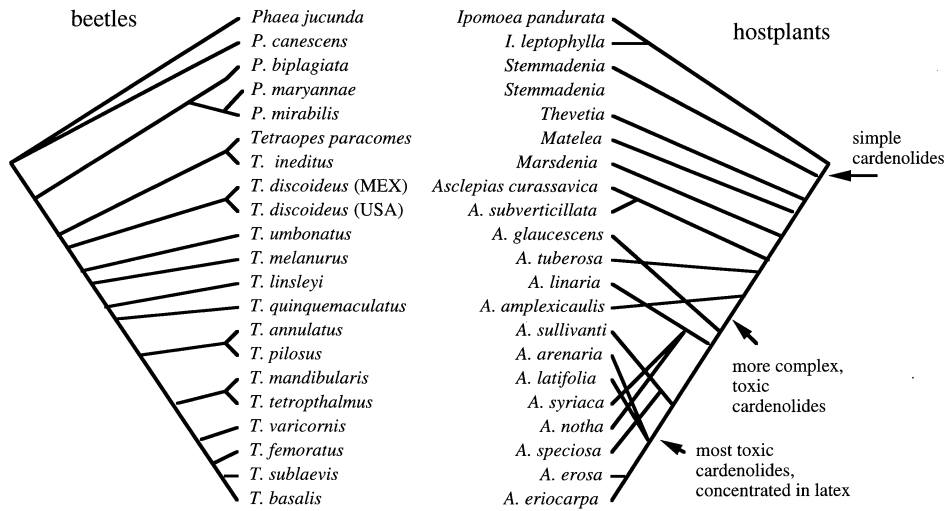


Figure 6. Comparison of most parsimonious tree, based on all data, for *Tetraopes* and *Phaea* species with known hosts, to hostplant phylogeny of Figure 1. There are 10–13 cospeciation events, depending on which reported host of *T. annulatus* is scored. Correspondence is significant under 2/3 scorings ( $P < 0.01$ ), and if *T. annulatus* is omitted on the grounds that its host affiliations are ambiguous ( $P = 0.038$ ; see text for details). \**T. annulatus* is here depicted opposite its host *A. sullivantii*, though adults of this species have also been collected from *A. subverticillata* and *A. speciosa* (see Introduction).

to their interaction. Direct evidence for coevolution has not been sought, but several suggestive observations point to directions for future study.

Reciprocal adaptation between particular pairs of plant and phytophagous insects is considered rare (Futuyma & Keese, 1992; Farrell & Mitter, 1993), and has been reported in just a few cases involving long-standing, highly specific interactions such as exclusive plant/pollinator associations (Thompson, 1994). However, such pairwise coevolution has rarely been directly looked for in antagonistic interactions. For *Tetraopes* and *Asclepias*, pairwise coevolution should most profitably be sought in paired endemics of distinctive, extreme habitats, such the affiliation of *Tetraopes pilosus* and *Asclepias arenaria* in the Central Sandhills. Such species seem especially likely to have differentiated together, and the harshness of their environment may both restrict the number of other herbivores with which the plant must contend, and place a premium on effective defense (Coley, Bryant & Chapin, 1987). *Tetraopes* is clearly capable of impairing milkweed fitness (see earlier references), and its combination of larval root feeding and adult feeding on reproductive parts may impose especially severe selection on hostplants (Brown, 1990).

Diffuse coevolution as embodied in Ehrlich & Raven's model (1964)—evolution of plant lineages in simultaneous response to suites of herbivore species and vice versa—has been accorded a wider influence on the structure and diversity of insect/plant communities (Futuyma & Keese, 1992). However, there are as yet few plausible instances, let alone convincing demonstrations, of such coevolution effects. Berenbaum (1983) spelled out the predictions of Ehrlich & Raven's (1964) model, and built a persuasive case for stepwise elaboration of coumarin compounds, in Apiaceae and other plant families, in response to counter-adapting herbivores. However, the phylogenies needed to secure this inference are not yet available

(Miller, 1987). Coevolutionary 'escape' may also explain the supplementation or replacement of glucosinolates by other, very different secondary compounds in some lineages of crucifers (Feeny, 1977). Perhaps the strongest case for Ehrlich & Raven's scenario is provided by secretory canals containing latex or resin. These structures are effective defenses against a spectrum of enemies, and no other hypothesized function for them seems plausible (Dussourd & Eisner, 1987). The multiple lineages in which they have independently arisen show consistently elevated diversification rates (Farrell, Dussourd & Mitter, 1991). They have in turn evoked characteristic counter-adaptations by some insect herbivores, some of which circumvent the canals by severing them before feeding (Dussourd, 1993).

The subgenus *Asclepias* presents a *prima facie* case for coevolution *sensu* Ehrlich and Raven with enemies including *Tetraopes*, that parallels on a smaller scale the examples just cited. There is, first, a phylogenetic progression in the types and tissue distribution of cardenolides, alluded to earlier (Fig. 1), which can be plausibly read as a stepwise escalation of defense (Nelson *et al.*, 1981). Cardenolides in the more primitive of Woodson's series (1–4), like those in other subgenera and genera of asclepiads, are of the simpler, presumably primitive calotropogenin type. Series 5–8 are unique in producing cardenolides of the structurally complex labriformin family. These compounds are among the most emetic and toxic cardenolides known (Detweiler, 1967; Brower *et al.*, 1982, 1984a,b). In series 5 (Syriacae, host to *T. tetropthalmus* and *T. linsleyi*), the labriformin-type cardenolides are present in only trace amounts; they increase in successive series to a maximum in series 8 (Roseae, host to *T. sublaevis*, *T. pilosus*, *T. basalis* and *T. mandibularis*). In Roseae, moreover, cardenolides are confined principally to the latex, where their deterrent effects on herbivores should be maximal (Nelson *et al.*, 1981). Species in this series have the largest laticifers in the subgenus (Nelson *et al.*, 1981), and the highest concentration of cardenolides known in any milkweed. Perhaps the ability of these plants to invade harshly xeric habitats unoccupied by other milkweeds derives in part from enhanced defences.

While this phylogenetic pattern suggests escalation of defence, demonstrating that such plant traits evolved as defences at all, let alone as a response to any particular enemy, has proven notoriously complex (review in Futuyma & Keese, 1992). There is some evidence that cardenolides are toxic or repellent to insects and vertebrates that do not feed on plants containing them (Detweiler, 1967; Cohen, 1983), but there has been little systematic attempt to compare the defensive effectiveness of the varying cardenolide profiles and deployments within subgenus *Asclepias*. However, preliminary field and lab observations suggest that milkweeds in series Roseae are free from the assemblages of oligophagous ctenuchine arctiid moths and chrysomelid beetles that attack chemically and phylogenetically more primitive congeners. Indeed, *Tetraopes* and the monarch butterfly are the only known folivores of Roseae. Thus, enhanced defences may have allowed these plants to escape former enemies, including most insects. Support for this inference, for example, would come from demonstration of negative effects of advanced cardenolides on the fitness of primitive beetles.

Extending this scenario to the limit, one could further interpret Woodson's phylogeny as supporting Ehrlich and Raven's conjecture that origin of novel defenses promotes diversification. That is, the three nested *Asclepias* subgroups characterized by the successive defence innovations postulated above are each more species-rich than their apparent sister groups (Fig. 1). In turn, the *Tetraopes* clade associated with the chemically advanced *Asclepias* series (circumscribed by *T. linsleyi* and *T. basalis* in

Fig. 5) is likewise more diverse than its sister group, suggestive of beetle radiation following colonization of a newly-diverse host clade.

Tests of this scenario for *Asclepias* faces the difficulty that each of its component adaptations has evolved only once. This makes unavailable the criterion of repeatability across independent lineages, a powerful form of evidence on both the adaptive value of traits (Williams, 1992), and their consequences for diversification (Heard & Hauser, 1995). On the other hand, the most derived, and apparently most toxic, series Roseae has been colonized by beetles three times (*T. pilosus*, *T. mandibularis* and the common ancestor of *T. sublaevis* and *T. basalis*), thus providing opportunity for comparative study of the necessary adaptations and possible advantages involved in use of these plants. Moreover, the phylogeography of the *Tetraopes/Asclepias* assemblage suggests that extant species and their adaptations evolved largely in the habitats, often quite restricted, that they currently occupy. The ecological circumstances under which putative reciprocal adaptations have arisen in these relatively young, still-localized lineages may be better preserved than those surrounding the origin of older, now widespread traits such as the possession of cardenolides per se. This should permit correspondingly more compelling experimental tests of hypotheses about both the adaptive origins of traits, and the mechanisms whereby improved adaptation translates into increased diversification (Sanderson & Donoghue, 1994). Experimental studies of examples such as Roseae are needed for rigorous evaluation of Ehrlich and Raven's model, now that broad statistical approaches have provided initial, presumptive evidence for diffuse coevolution between insects and plants.

#### *Concluding observations*

Hostplant use in *Tetraopes* is unusually conservative: our evidence suggests that these beetles' exclusive association with Asclepiadaceae dates to the mid Tertiary, perhaps to as long as 40 Mya. Moreover, unlike that of most insect groups which have been similarly examined, the phylogeny of the *Tetraopes/Phaea* lineage shows significant concordance with that of its hosts. This concordance is plausibly ascribed to approximately synchronous diversification, at least between *Tetraopes* and *Asclepias*. These findings parallel results of an earlier analysis of *Phyllobrotica* leaf beetles (Farrell & Mitter, 1990). Thus, they support the conjecture that parallel phylogenesis with hostplants, with its attendant opportunity for long-term coevolution, is promoted by shared features of these two assemblages which probably reinforce specificity and conservatism of their habits. These traits include larval endophagy, adult feeding and mating on the larval host, and 'toxic' host chemistry on which the apparently aposematic adult beetles may be dependent for defence.

The hostplants of *Tetraopes* and *Phyllobrotica*, Asclepiadaceae and Lamiaceae, like the coumarin-rich Apiaceae (Berenbaum, 1983), belong to the derived angiosperm subclass Asteridae *s.l.* (Olmstead *et al.*, 1992), which is characterized by an exceptional prevalence and diversity of toxic and repellent compounds (Cronquist, 1981). All three families are derived relatively recently (mid-Tertiary) from woody tropical ancestors, and consist partly (milkweeds) to mostly of temperate herbs that have presumably diversified with the spread of open, seasonal habitats since the Eocene (Judd, Sanders & Donoghue, 1994). For reasons advanced earlier, such plant

lineages should provide test cases for the prevalence and detectability of escape and radiation coevolution, rigorous study of which has barely begun.

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## APPENDIX

Enzyme loci scored for phylogenetic analyses. The suffix 2 indicates a cathodal locus. 2. TBE: Tris-borate-EDTA; TM: Tris-maleate; LIOH: discontinuous Lithium hydroxide; TC7, 8: Tris citrate; TVB; Salb: Salamander B (Hillis & Mortiz, 1990; Richardson *et al.*, 1986; Werth, 1985). The matrix of allele frequencies will be supplied on request by the senior author.

Enzyme	E. C. #	Locus <sup>1</sup>	Buffer <sup>2</sup>
1. Aconitase	4.2.1.3	<i>Aco-1</i>	1/2 LIOH
2. Aconitase	4.2.1.3	<i>Aco-2</i>	TVB
3. Aldolase	4.1.2.13	<i>Ald</i>	SalB
4. Catalase	1.11.1.6	<i>Cat</i>	SalB
5. Fructose-1,6 diphosphate	3.1.3.11	<i>Fdp</i>	TBE
6. Glyceraldehyde-3-phosphate dehydrogenase	1.2.1.12	<i>G3p</i>	TC7
7. Glucose-6-phosphate dehydrogenase	1.1.1.49	<i>G6p</i>	TC8dil
8. Aspartate aminotransferase	2.6.1.1	<i>Got1</i>	TC8dil
9. Aspartate aminotransferase	2.6.1.1	<i>Got2</i>	TBE
10. Hydroxybutyrate dehydrogenase	1.1.1.30	<i>Hbd</i>	TC8dil
11. Alpha-glycero-phosphate dehydrogenase	1.1.1.8	<i>Agpd</i>	TC8dil
12. Isocitrate dehydrogenase	1.1.1.42	<i>Idh</i>	TC7
13. Malate dehydrogenase	1.1.1.37	<i>Mdh-1</i>	TC7
14. Malate dehydrogenase	1.1.1.37	<i>Mdh-2</i>	TC7
15. Malic enzyme	1.1.1.40	<i>Me</i>	TC7
16. Mannose-6-phosphate isomerase	5.3.1.8	<i>Mpi</i>	TC8dil
17. Phosphoglucomutase	5.4.2.2	<i>Pgm</i>	TM
18. Glucose phosphate isomerase	5.3.1.9	<i>Phi</i>	1/2 LIOH
19. 6-phosphogluconate dehydrogenase	1.1.1.44	<i>Gp6</i>	TC8dil
20. Sorbitol dehydrogenase	1.1.1.14	<i>Sdh</i>	TBE
21. Superoxide dismutase	1.15.1.1.	<i>Tox</i>	TBE
22. Xanthine dehydrogenase	1.1.1.204	<i>Xdh</i>	LIOH
23. Adenylate kinase	2.7.4.3	<i>Ak</i>	LIOH