Evolution of Parasitism in the Lycaenidae (Lepidoptera)

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ABSTRACT

Of the four most diverse insect orders, the Lepidoptera contain remarkably few predatory and/or parasitic taxa, and while species with carnivorous life histories have evolved independently numerous times in moths and butterflies, this has rarely led to diversification. As a rule, aphytophagous taxa seem prone to extinction. In this dissertation, I explore the ecological and evolutionary consequences of entomophagy in the butterfly family Lycaenidae using several approaches: natural history observation, phylogenetics, population genetics and stable isotope chemistry.

A striking exception to the lack of radiation and persistence in aphytophagous lineages is the lycaenid subfamily Miletinae, which with 13 genera and 190 species is among the largest and most diverse groups of aphytophagous Lepidoptera. Most miletines eat Hemiptera, although some consume ant brood or are fed by trophallaxis from their host ant. I inferred the higher-level phylogeny of this group using data from one mitochondrial and six nuclear genes sampled from representatives of all genera and nearly half the described species. Biogeographic analyses indicate that Miletinae likely diverged from an African ancestor near the start of the Eocene, and four lineages dispersed between Africa and Asia. Phylogenetic constraint in prey selection is apparent at two levels: related miletine species are more likely to feed on related Hemiptera and

are also more likely to associate with closely related ants species, either directly by eating the ants, or indirectly by eating hemipteran prey attended by those ants.

I then examined the influence of diet on the population structure of lycaenid butterflies, and more specifically, I investigated whether particular feeding habits are correlated with traits that might make species vulnerable to extinction. To do this, I compared the phylogeography and population genetics of two endemic lycaenid species of roughly similar age from southern Africa: *Chrysoritis chrysaor*, whose caterpillars are strictly herbivorous, and *Thestor protumnus*, whose cuckoo-like caterpillars survive by soliciting regurgitations from their host ants. I sampled both species from populations throughout their entire known ranges, and found that in contrast to *C. chrysaor*, *T. protumnus* has exceedingly small effective population sizes and individuals disperse poorly. With its aphytophagous life history, *T. protumnus* exhibits a high degree of host dependence and specialization. Although these results are correlative and based on only a single comparison, it seems likely that small population sizes and extreme ecological specialization make populations of *T. protumnus* more susceptible to disturbance and prone to extinction.

Having focused in detail on the population biology of just one species, I then analysed the evolution of *Thestor* as a whole. This genus is exceptional because all of its 27 described species are thought to be entomophagous, and all are thought to be predators or parasites of a single species of ant, *Anoplolepis custodiens*. Using representatives sampled from all known species and populations of *Thestor* as well as 15 outgroup species, I inferred the phylogeny of the genus in two ways: first by using characters from mitochondrial and nuclear genes, and second by analyzing genome-wide SNPs generated

for each species using double digest RADseq. I also sequenced the ants associated with each of these taxa using ddRADseq. This investigtion showed that all 24 of the species in the Western Cape utilize *Anoplolepis custodiens*, while *T. protumnus*and *T. dryburghi* (the two species that are found in the north-western part of South Africa) use a closely related, but different species of *Anoplolepis,* and *T. basutus* (the species found in the eastern part of South Africa) utilizes yet a third species. Thus factors driving diversity in the genus *Thestor* may have initially involved ant associations and/or geographic isolation, but other forces are likely to be responsible for generating and maintaining the more recent diversity in the group. Flight time may have separated the "black" and "yellow" groups of *Thestor*: the black group fly predominantly in the summer months, while the yellow group fly predominantly in the spring. And while species spread across the genus fly in the spring and summer months, only members of the yellow group fly during the winter and fall months. Despite these broad scale differences, species in the genus *Thestor* show little evidence of niche partitioning, especially those in the Western Cape, and represent an extreme example of the coexistence of 24 species apparently utilizing a single food resource.

While working on the previous three projects, I was surprised by the number of species of South African Lycaenidae with incomplete life histories despite decades of work by avid lepidopterists in the region. For example, in the genus *Thestor*, although all 27 species are assumed to be aphytophagous, partial life histories have been described for only four species. In part the paucity of data is due to the difficult terrain occupied by these butterflies, and the fact that those whose caterpillars associate with ants often spend significant portions of their lives hidden in ant nests in crevices of rock that are

intractable for excavation and observation. To deepen our understanding of South African lycaenid life histories, I used nitrogen and carbon stable isotopic methods to survey a large number of species and their potential food sources. With these methods, I confirmed some known or suspected life histories and showed that in any one area, a species can have a highly variable diet. I also discovered that some of the nitrogen stable isotope values are much higher than expected for land animals, implying longer than average food chains and/or extreme environmental conditions.

Together, these studies shed light on how carnivorous life histories affect the evolution of lycaenid butterflies, and help to explain why entomophagous lineages appear to be an evolutionary "dead end" in contrast to their herbivorous counterparts.

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This thesis is dedicated, with love and gratitude, to my parents,

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for instilling in me a love of nature from a very early age.

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And thank you to Vinny, for changing my life in ways I could never have imagined.

IDYLLA MALEŃKA TAKA

Idylla maleńka taka:
Wróbel połyka robaka,
Wróbla kot dusi niecnota,
Pies chętnie rozdziera kota,
Psa wilk z lubością pożera,
Wilka zadławia pantera.
Panterę lew rwie na ćwierci,
Lwa - człowiek; a sam,
Po śmierci staje się łupem robaka.
Idylla maleńka taka.

-Mikołaj Biernacki-Rodoć

INTRODUCTION

Interactions between insects and plants have generated much of the organic diversity we see today (Ehrlich and Raven 1964; Mitter *et al.* 1981), and this is especially true in the Lepidoptera, where more than 99% of all taxa feed on plants (Pierce 1995; Powell *et al.* 1998). Although much of the research on diversification in the Lepidoptera has focused on the evolutionary consequences of overcoming the 'hurdle of phytophagy', relatively little work has addressed the question of reversals: why are there so few predatory and parasitic Lepidoptera? While such reversals have occurred multiple times, especially amongst taxa that associate with ants (Pierce 1995), they have rarely led to radiations. This is in contrast to other holometabolous orders, such as Diptera, Hymenoptera and Coleoptera that each have large aphytophagous clades.

This thesis explores the ecological and evolutionary context and consequences of parasitic and predatory insects whose ancestors were phytophagous, comparing and contrasting aphytophagous and phytophagous members of the family Lycaenidae (Lepidoptera), the blues, coppers and hairstreaks.

In Chapter 1, we look at the biogeography and life history evolution of the Miletinae. The phylogeny of the subfamily Miletinae was inferred using molecular characters in order to explore the biogeography and life history evolution of the largest radiation of aphytophagous butterflies. Of the four most diverse insect orders, Lepidoptera contains remarkably few predatory and parasitic species. The wholly aphytophagous subfamily Miletinae is a conspicuous exception, consisting of nearly 190 species. Most miletines eat Hemiptera, although some also consume ant brood or

are fed by ant trophallaxis. I inferred a phylogeny using 4,915 bp from seven markers sampled from representatives of all 13 recognized genera and nearly half the described species. Phylogenetic constraint in prey selection is apparent at two levels: closely related miletine species are more likely to feed on closely related Hemiptera, and to associate with closely related ants. This, in conjunction with field observations, suggests that female miletines may use ants as cues to locate their hemipteran prey.

Chapter 2 explores how extinction risk in predaceous butterflies may be driven by population size by looking at a case study comparing two species in South Africa. A comparison of the biogeographic distributions and population genetics was used to study the evolutionary consequences of life history variation in two lycaenid species that are endemic to southern Africa: *Chrysoritis chrysaor*, whose members are strictly herbivorous, and *Thestor protumnus*, whose members trick ants to regurgitate food for their consumption. I found that in contrast to *C. chrysaor*, *T. protumnus* have small effective population sizes and disperse poorly. With its aphytophagous life history, *T. protumnus* appears to exhibit a higher degree of host dependence and specialization, potentially acting as a biological barrier to dispersal. Such specialization may also make populations of *T. protumnus* more susceptible to disturbance and prone to extinction.

Chapter 3 takes a closer look at a single aphytophagous genus, *Thestor* (Lepidoptera, Miletinae). Characters generated from throughout the genome are used to infer phylogenies of the genus *Thestor* as well as the ants that it parasitizes. *Thestor* (Lepidoptera: Lycaenidae) comprises 27 recognized species, all of which are endemic to southern Africa. All species are believed to be parasitic, feeding on homopterans

and/or the brood, regurgitations, and/or workers of the ant, *Anoplolepis custodiens*. A 6-gene phylogeny, COI barcoding dataset and double digest RAD tags of the butterflies, as well as double digest RAD tags of the associated ants are used to investigate how *Thestor* has achieved such diversity despite apparent overlap in ecological niche (i.e. association *with A. custodiens*). It appears that all 24 of the species in the Western Cape utilize the same ant, while the two species that are found in the north-western part of South Africa use a closely related, but different ant, and the species found in the eastern part of South Africa utilizes yet a third ant. This suggests that factors driving the diversity in the genus *Thestor* may have been ants and/or geography when the genus originated, but that other forces are responsible for generating the more recent diversity in the group. One possibility may have been temporal partitioning: the "black" group of *Thestor* species (*T. murrayi, T. kaplani, T. compassbergae, T. camdeboo, T. pringlei, T. penningtoni, T. holmesi, T. stepheni, T. claassensi, T. overbergensis, T. rileyi, T. yildizae, T. barbatus, T. petra* and *T. brachycerous*) fly predominantly in the summer months, while the "yellow group" (*T. braunsi, T. malagas, T. dicksoni, T. vansoni, T. pictus, T. rooibergensis, T. swanepoeli, T. rossouwi, T. strutti* and *T. montanus*) fly predominantly in the spring. And while species spread across the phylogeny fly in the spring and summer months, only members of the yellow group fly during the winter and fall months. Thus flight time may have driven the separation of the yellow and black groups of *Thestor.*

Chapter 4 contains a survey of the isotopic variability in butterfly species in the Cape Region and addresses the life history aspects that are difficult to learn from just field observations. The biology of the majority of lycaenid species in southern Africa

is unknown, largely because of the enormous difficulty of following them in ant nests, many of which are under rocks and in crevices that are impossible to excavate. This study summarizes stable isotope measurements to determine trophic status of over 600 specimens of plants, ants, Hemiptera and butterflies collected from 33 sites.

In the appendices I discuss taxonomy of the subfamily Miletinae and explore life histories of South African butterflies through field observations.

CHAPTER 1

When caterpillars attack: Biogeography and life history evolution of the Miletinae (Lepidoptera: Lycaenidae)[1]

Co-authors: David J. Lohman, Kathrin Sommer, Glenn Adelson, Douglas B. Rand, John Mathew, Gerard Talavera, and Naomi E. Pierce

Abstract

Of the four most diverse insect orders, Lepidoptera contains remarkably few predatory and parasitic species. Although species with these habits have evolved multiple times in moths and butterflies, they have rarely been associated with diversification. The wholly aphytophagous subfamily Miletinae (Lycaenidae) is an exception, consisting of nearly 190 species distributed primarily throughout the Old World tropics and subtropics. Most miletines eat Hemiptera, although some consume ant brood or are fed by ant trophallaxis. A well-resolved phylogeny inferred using 4915 bp from seven markers sampled from representatives of all genera and nearly one-third the described species was used to examine the biogeography and evolution of biotic associations in this group. Biogeographic analyses indicate that Miletinae likely diverged from an African ancestor near the start of the Eocene, and four lineages dispersed between Africa and Asia. Phylogenetic constraint in prey selection

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is apparent at two levels: related miletine species are more likely to feed on related Hemiptera, and related miletines are more likely to associate with related ants, either directly by eating the ants, or indirectly by eating hemipteran prey that are attended by those ants. These results suggest that adaptations for host ant location by ovipositing female miletines may have been retained from phytophagous ancestors that associated with ants mutualistically.

Introduction

Evolutionary shifts to herbivory are associated with increased diversification in insects (Farrell *et al.* 1992). More than one-quarter of the earth's described species are phytophagous insects that feed obligately on living plant tissue during at least part of their life cycle (Strong *et al.* 1984; Grimaldi and Engel 2005). Although less than one-third of insect orders include herbivores (Orthoptera, Phasmatodea, Blattodea (including termites), Hemiptera, Thysanoptera, and the four “mega diverse” orders: Hymenoptera, Diptera, Coleoptera, and Lepidoptera), these orders are disproportionately species rich (Strong *et al.* 1984; Mitter *et al.* 1988; Winkler and Mitter 2008; Futuyma and Agrawal 2009). Conversely, insect lineages that have shifted from herbivory to parasitism tend to be less diverse than their plant feeding relatives, perhaps because ascending the trophic pyramid restricts population sizes and densities, thereby decreasing opportunities for speciation while increasing extinction likelihood (Wiegmann *et al.* 1993).

Of the four largest holometabolous orders, only Lepidoptera are almost exclusively phytophagous. Although a significant proportion of Hymenoptera,

Diptera, and Coleoptera derive their nutrition from animal sources at some point during their life cycle, less than 2% of Lepidoptera, or as few as 200 to 300 species, have been recorded as "aphytophagous" and feed obligately on something other than living plants for at least some portion of their life cycle (Pierce 1995). Moreover, these few hundred species are taxonomically widespread throughout Lepidoptera, compared with the other megadiverse orders in which parasitic or carnivorous behavior is restricted to a few lineages. The taxonomic distribution of aphytophagy suggests that the habit has evolved multiple times independently in the Lepidoptera, particularly in the butterfly family Lycaenidae (Cottrell 1984; Pierce 1995). Some aphytophagous Lepidoptera are predators that eat other animals, primarily insects, and others are parasites, that potentially lower their host's fitness without killing them (e.g., via trophallaxis with ants). Other aphytophagous taxa feed on detritus, lichen, and keratin. Despite multiple shifts away from herbivory within the Lepidoptera, these shifts appear to be evolutionarily transient, or "tippy" in their distribution—aphytophagous lepidopteran lineages rarely persist and radiate (Pierce 1995). Most shifts to predation/parasitism in the family Lycaenidae have occurred within otherwise phytophagous clades, whose species associate mutualistically with ants, and have given rise to only one or two parasitic species.

The caterpillars of approximately three-quarters of the species in the family Lycaenidae associate with ants (Pierce *et al.* 2002). A significant number of species in the sister family of the Lycaenidae, the Riodinidae, are also known to associate with ants (e.g., DeVries 1991b; DeVries and Penz 2000; Kaminski *et al.* 2013), whereas species in other lepidopteran families, with rare exceptions, do not. These associations

can range from facultative to obligate interactions, and from mutualism to parasitism. Ant-associated Hemiptera are often involved. The first innovation in the evolution of myrmecophily in the Lycaenidae is likely to have involved tolerance of the caterpillar by ants (termed "myrmecoxeny"). In most circumstances, foraging ants encountering a caterpillar will regard it as potential prey. However, once lycaenid caterpillars evolved a means to appease aggressive ants, myrmecophilous lycaenids would have had the great advantage of occupying "enemy free space" (Atsatt 1981), and more complicated interactions would have been possible, both with ant-associated Hemiptera and with the ants themselves. Presumably at the same time, or shortly thereafter, lycaenid caterpillars that could appease ants also began to reward them with nutritious secretions in exchange for defense against parasites and predators. These interactions were facilitated by a number of adaptations, including specialized exocrine glands, an unusually thick cuticle, a retractable head, and various stridulatory organs used to communicate with ants and conspecifics (Hinton 1951; Malicky 1970; DeVries 1991a; Travassos and Pierce 2000). The great majority of lycaenid–ant interactions involve ants associating apparently mutualistically with caterpillars feeding on plants, but a smaller proportion—less than 5% of the species with described life histories—associate parasitically with ants and are aphytophagous, feeding either on Hemiptera attended by ants or on the ants themselves (Pierce *et al.* 2002).

In general, predatory and parasitic Lepidoptera consume organisms that cohabit the plants on which they live: ants, Hemiptera, and insect eggs. It is perhaps because of their proximity to ants and ant-attended Hemiptera that shifts to parasitism

and predation have occurred so frequently in the Lycaenidae relative to other lepidopteran taxa (Pierce 1995; Pierce *et al.* 2002). The lycaenid subfamily Miletinae, which comprises approximately 190 species in 13 currently recognized genera, is the largest radiation of aphytophagous butterflies. The larvae of all Miletinae whose life histories have been described are predatory, parasitic, or otherwise aphytophagous, and it is expected that all species in this subfamily share this trait (Cottrell 1984; Savela 2014).

Several hypotheses have been proposed to explain the evolutionary steps leading to this unusually successful radiation and to the diversity of diets within it (Table 1.1). Balduf (1938) speculated that the Miletinae arose from the lichen-feeding subfamily Lipteninae. Cottrell (1984) argued that lycaenid larvae and Hemiptera both prefer to eat and occasionally live on nitrogen-rich plant parts, and that a shift to carnivory may have followed. Maschwitz *et al.* (1988) hypothesized that feeding on ant-attended aphids was the ancestral pattern in the subfamily that gave rise to other derived strategies. A phylogenetic estimate of the Miletinae and determination of their sister taxon facilitates an evaluation of these hypotheses.

Table 1.1 Distribution, dietary, and ant associate information for all miletine species with known life histories, including distribution information for species that are included in this study but have no life-history information.

			Associated ants				Geographic distribution								Larval food											
Taxon	Sampled	Immatures unknown	Dolichoderinae	Formicinae	Myrmicinae	Species	Eastern North America	Africa	India/the Himalayas	China/Indo-China	Sundaland	Philippines	Sulawesi/lesser Sundas	Maluku and New Guinea	Australia	Aphididae	Pemphigidae	Hormaphidiidae	Greenideidae	Coccidae/Pseudococcidae	Membracidae	Cicadellidae	Psyllidae	Ant brood/trophallaxis	Insect prey in ant nests	Hemipteran honeydew	Extrafloral nectar	References
Miletinae: Lachnocnemini																												
Lachnocnema bibulus	X			2	2	*Camponotus acvapimensis*, *C. maculatus*, *Crematogaster* sp., *Pheidole* sp.		X													X	X	X	X		X		1–6
L. brimo	X			1		*Camponotus* sp.		X													X		X					6, 7
L. divergens	X	X						X																				8
L. durbani	X							X												X	X							5, 7
L. magna								X													X			X				7
Thestor basutus				1		*Anoplolepis custodiens*		X												X			X	X				5, 9
T. brachycerus				1		*A. custodiens*		X																				5
T. braunsi	X			1		*A. custodiens*		X																				9
T. dicksoni				1		*A. custodiens*		X																X				5, 7
T. holmesi				1		*A. custodiens*		X																X				7, 9
T. kaplani	X			1		*A. custodiens*		X												[X]			[X]	[X]				9
T. montanus	X			1		*A. custodiens*		X																				9
T. overbergensis	X			1		*A. custodiens*		X																				9
T. penningtoni	X			1		*A. custodiens*		X																[X]				9
T. protumnus				1		*A. custodiens*		X												X				X				5, 10
T. rileyi				1		*A. custodiens*		X																X				7
T. rooibergensis	X			1		*A. custodiens*		X																				9
T. stepheni	X			1		*A. custodiens*		X																				9
T. swanepoeli	X			1		*A. custodiens*		X																				9
T. yildizae	X			1		*A. custodiens*		X																X				11
Miletinae: Liphyrini																												
Aslauga aura	X	X		[1]	[2]			X												[X]	[X]							
A. lamborni				1	2	*Oecophylla longinoda*, *Crematogaster buchneri*, *Pheidole rotundata*		X												X	X							1, 4

Table 1.1 (Continued) Distribution, dietary, and ant associate information for all miletine species with known life histories, including distribution information for species that are included in this study but have no life-history information.

			Associated ants				Geographic distribution									Larval food												
Taxon	Sampled	Immatures unknown	Dolichoderinae	Formicinae	Myrmicinae	Species	Eastern North America	Africa	India/the Himalayas	China/Indo-China	Sundaland	Philippines	Sulawesi/lesser Sundas	Maluku and New Guinea	Australia	Aphididae	Pemphigidae	Hormaphidae	Greenideidae	Coccidae/Pseudococcidae	Membracidae	Cicadellidae	Psyllidae	Ant brood/trophallaxis	Insect prey in ant nests	Hemipteran honeydew	Extrafloral nectar	References
A. latifurca								X												X	X			X				7, 12, 13
A. orientalis	X				[1]			X												X								12
A. purpurescens								X												X	X							4, 12, 13
A. vininga					1	*C. buchneri*		X												X								1, 7
Euliphyra hewitsoni	X			1		*O. longinoda*		X																X				8
E. leucyania				1		*O. longinoda*		X																X	X			8, 14
E. mirifica				1		*O. longinoda*		X																X	X			14, 15
Liphyra brassolis	X			1		*Oecophylla smaragdina*			X	X	X	X			X									X				16–18
L. grandis				1		*O. smaragdina*								X										X				19
Miletinae: Miletini																												
Allotinus apries	X				1	*Myrmicaria arachnoides*					X	X								X				X				20
A. borneensis	X	X									X																	21
A. corbeti	X	X									X	X																21
A. davidis	X			1	1	*Anoplolepis gracilipes*, *Crematogaster difformis*					X					X													21, 22
A. drumila	X	X							X	X																		21
A. fallax	X			1		*A. gracilipes*					X	X										X							21, 23
A. horsfieldi	X										X					X												7, 21, 24
A. leogoron	X	X									X																	21
A. major	X			1		*A. gracilipes*							X								X							21, 25
A. nicholsi	X	X									X																	21
A. nivalis	X	X									X	X																21
A. portunus	X	X									X																	21
A. punctatus	X	X										X																21
A. samarensis	X	X										X	X															21

Table 1.1 (Continued) Distribution, dietary, and ant associate information for all miletine species with known life histories, including distribution information for species that are included in this study but have no life-history information.

			Associated ants				Geographic distribution								Larval food												
Taxon	Sampled	Immatures unknown	Dolichoderinae	Formicinae	Myrmicinae	Species	Eastern North America	Africa	India/the Himalayas	China/Indo-China	Sundaland	Philippines	Sulawesi/lesser Sundas	Maluku and New Guinea	Australia	Aphididae	Pemphigidae	Hormaphididae	Greenideidae	Coccidae/Pseudococcidae	Membracidae	Cicadellidae	Psyllidae	Ant brood/trophallaxis	Insect prey in ant nests	Hemipteran honeydew	Extrafloral nectar	References
A. sarrastes	X	X									X	X															21
A. strigatus	X	X									X																21
A. substrigosus	X		1		1	*Technomyrmex* sp., *Crematogaster* sp.				X	X	X						X		X	X							20–22
A. subviolaceus	X			1		*A. gracilipes*				X	X	X									X							20, 21
A. unicolor	X			1		*A. gracilipes*				X	X	X	X			X		X		X	X		X			X		7, 21–23, 26
Logania distanti	X	X							X	X	X	X												[X]				21
L. hampsoni			2			*Anonychomyrma nitidiceps*, *Technomyrmex albipes*								X											X				21, 27
L. malayica	X				2	*Rhoptromyrmex wroughtonii*					X	X				X		X		X	X		X	X		X	X	20, 21, 28
						Leptothorax sp.																						
L. marmorata	X		1			*Dolichoderus* sp.					X	X									X					X	X	21, 28
L. regina	X	X	[2]								X																	21
Lontalius eltus	X	X									X																	21
Megalopalpus zymna	X			1	1	*Pheidole aurivillii*, *C. acvapimensis*		X													X	X						1, 7, 29, 30
Miletus ancon	X									X	X									X								7, 21
M. biggsii	X		1			*Dolichoderus* sp.				X	X					X		X		X								20, 22, 23, 31
M. boisduvali			1			*Dolichoderus bituberculatus*					X		X	X				X		X								31, 32
M. cellarius	X	X									X																	31
M. chinensis	X		2	1		*Dolichoderus* sp., *D. bituberculatus*, *Polyrhachis dives*				X	X					X													31, 33, 34
M. drucei	X	X									X	X																31
M. gaesa	X	X									X																	31
M. gallus	X	X									X																	31
M. gopara	X	X									X																	31
M. heracleion	X	X									X																	31
M. leos	X	X											X	X														31

Table 1.1 (Continued) Distribution, dietary, and ant associate information for all miletine species with known life histories, including distribution information for species that are included in this study but have no life-history information.

			Associated ants				Geographic distribution								Larval food												
Taxon	Sampled	Immatures unknown	Dolichoderinae	Formicinae	Myrmicinae	Species	Eastern North America	Africa	India/the Himalayas	China/Indo-China	Sundaland	Philippines	Sulawesi/lesser Sundas	Maluku and New Guinea	Australia	Aphididae	Pemphigidae	Hormaphididae	Greenideidae	Coccidae/Pseudococcidae	Membracidae	Cicadellidae	Psyllidae	Ant brood/trophallaxis	Insect prey in ant nests	Hemipteran honeydew	Extrafloral nectar	References
M. mallus	X		1			*Dolichoderus* sp.				X						X			X									23, 31
M. nymphis	X									X	X									X								6, 31
M. symethus	X		1			*Dolichoderus* sp.					X	X								X								31, 35
Miletinae: Spalgini																												
Feniseca tarquinius	X			3	1	*Camponotus* spp., *Formica* spp., *Lasius pallitarsus*, *Myrmica* spp.	X										X											36, 37
Spalgis epius	X			2	1	*A. gracilipes*, *O. smaragdina*, *Crematogaster* sp.			X	X	X	X	X	X						X								29, 38, 39
Spalgis lemolea	X			2	1	*Anoplolepis* sp., *O. longinoda*, *Crematogaster* sp.		X												X								1, 29
Spalgis substrigata																				X								40
Miletinae: Tarakini																												
Taraka hamada	X			1		*Formica japonica*			X	X	X						X	X								X		29, 34, 39, 41
Taraka mahanetra	X	X								X	X																	42, 43

Sampled = species that were included in the present phylogenetic study; Dolichoderine, Formicinae, Myrmicinae = number of species from each of these subfamilies that have been recorded in association with a particular miletine species. Occasionally, life-history traits were inferred from closely related species because information about a sampled species was unavailable. Symbols indicating these inferred life-history traits are surrounded by square brackets []. Where necessary, ant names have been updated to reflect current taxonomy.

References: 1 = Lamborn et al. (1914); 2 = Cripps and Jackson (1940); 3 = Farquharson (1922); 4 = van Someren (1974); 5 = Clark and Dickson (1971); 6 = Fiedler (1991); 7 = Robinson et al. (2009); 8 = Larsen (200)5; 9 = Heath and Claassens (2003); 10 = Migdoll (1988); 11 = Heath and Claassens (2000); 12 = Cottrell (1981); 13 = Jackson (1937); 14 = Dejean and Beugnon (1996); 15 = Hinton (1951); 16 = Cottrell (1987); 17 = Johnson and Valentine (1986); 18 = Dodd (1902); 19 = Parsons (1991); 20 = Maschwitz et al. (1988); 21 = Eliot (1986); 22 = Maschwitz et al. (1985); 23 = Lohman and Samarita (2009); 24 = Bingham (1907); 25 = Kitching (1987); 26 = Fiedler and Maschwitz (1989); 27 = Parsons (2000); 28 = Fiedler (1993); 29 = Cottrell (1984); 30 = Ackery (1990); 31 = Eliot (1961); 32 = Roepke (1919); 33 = Kershaw (1905); 34 = Bascombe et al. (1999); 35 = Eliot (1980); 36 = Mathew et al. (2008); 37 = Lohman et al. (2006); 38 = Venkatesha et al. (2004); 39 = Hsu (2006); 40 = Smith (1914); 41 = Banno (1990); 42 = Corbet et al. (1994); 43 = Ek-Amnuay (2006).

The biogeographic history of the Miletinae appears to be complex. Of the 13 genera, four genera are wholly Asian (*Allotinus*, *Lontalius*, *Miletus*, and *Taraka*), two are primarily distributed in Southeast Asia, but have species that inhabit the Australian region including New Guinea (*Liphyra* and *Logania*), five are entirely Afrotropical (*Aslauga*, *Euliphyra*, *Lachnocnema*, *Megalopalpus*, and *Thestor*), *Spalgis* species are found in all three of these regions (Oriental, Australian, and Afrotropical), and the monotypic genus *Feniseca* is strictly Nearctic (Eliot 1973, 1986). The distribution of various genera and higher taxa within Miletinae implies that lineages have dispersed between Africa and Asia repeatedly; however, the number and directionality of dispersal events are unclear.

Phylogenetic patterns of association between the three interacting taxa—butterflies, ants and Hemiptera—may likewise be complex. Related phytophagous butterfly caterpillars tend to feed on related plants (Ehrlich and Raven 1964; Janz *et al.* 2006) in part because adaptations to the chemical defenses of particular plant lineages restrict the dietary choices of herbivorous insects (e.g., Berenbaum 1995; Futuyma and Agrawal 2009). Predacious insects (not including more specialized parasites and parasitoids) tend not to be dietary specialists, and individual miletine species have been recorded feeding on a variety of different hemipteran taxa. For example, three miletine species in three different genera have been reported eating members of all four superfamilies of Hemiptera (Table 1.1). However, some species are notably selective in their prey choice, including *Feniseca tarquinius*, which specializes on Woolly Alder Aphids, *Paraprociphilus tessellatus* (Mathew *et al.* 2008).

The species of Hemiptera eaten by miletine caterpillars have several important similarities. They are usually soft bodied, restricted to their host plants by limited mobility, and are typically attended by ants. Thus, once miletine caterpillars began to consume Hemiptera, they presumably could easily switch to eating any kind of Hemiptera. It is also possible that because attendant ants defend Hemiptera against predators, selection has favored caterpillars that are able to fool ants semiochemically to elude detection by their prey, as has been demonstrated in the species *F. tarquinius* (Youngsteadt and DeVries 2005; Lohman *et al.* 2006).

Some taxonomic associations between miletine butterfly larvae and ants are apparent. *Miletus* caterpillars, for example, have always been found in association Hemiptera attended by *Dolichoderus* ants, and *Liphyra* and *Euliphyra* feed exclusively on the immatures of *Oecophylla* ants. However, it is unclear whether these taxonomic patterns translate into a relatively small number of transitions to novel ant associations with the family, or whether ant associations are more evolutionarily labile. Unlike mutualistic interactions between lycaenid larvae and ants, miletine larvae do not interact directly with the ants attending their hemipteran prey. Although mutualistically myrmecophilous lycaenids entrain the defensive assistance of ants with nutritious rewards offered from specialized glands, the caterpillars of miletine species universally lack a dorsal nectary organ for provisioning nutritious secretions, and only a few retain tentacle organs (Cottrell 1984). Nevertheless, they all retain the single celled "pore cupola organs" thought to be critical for ant appeasement (Cottrell 1984), suggesting that adaptation for some kind of association with ants may still be present in this group.

The species of ants associated with miletine caterpillars are in the largest and most common subfamilies. They share characteristics common to many "agricultural" ants that associate closely with other Lycaenidae (Pierce and Elgar 1985; Eastwood and Fraser 1999; Fiedler 2001). They tend to be dietary generalists; spend much of their time above ground, frequently in tree canopies and sometimes nesting in trees; and possess large, polydomous colonies with impressive mass recruitment systems of defense (Hölldobler and Wilson 1990). The workers are typically opportunistic foragers, and representatives of each subfamily have been recorded attending many different species of Hemiptera. Because aphytophagous lycaenids rely predominantly on Hemiptera or on Hemiptera-associated ants for their sustenance, it seems likely that associating with dominant, ecologically "apparent" ants with large colonies may be important for maintaining parasitic relationships over long periods of time.

We therefore hypothesize that there will be strong phylogenetic associations between Miletinae and their hemipteran hosts. Despite the fact that the larvae of Miletinae do not possess a dorsal nectary organ to reward attendant ants, we also speculate that a relationship with ants may nevertheless persist as the "ghost of ant association past." Miletinae are likely to have evolved from a lycaenid lineage that associated with ants mutualistically, and behavioral or other adaptations for maintaining these interspecific interactions may have been retained because of at least two main selective advantages that they conferred: to enable miletines to avoid detection and attack by ants, which normally defend their hemipteran mutualists, and to facilitate ovipositing females in the location of suitable host prey, because ant attendance typically makes associated Hemiptera easier to find.

In this study, we reconstruct the phylogeny of the Miletinae (Lepidoptera: Lycaenidae) using 90 specimens comprising 68 exemplar ingroup taxa and 22 taxa representing a taxonomically broad sample of all possible outgroup lineages. We use this phylogeny to examine the evolution of aphytophagy, shifts in diet breadth and preferences, and ant associations with hemipteran prey. In addition, we examine the biogeographic history of the group and discuss the causes and effects of dramatic dietary shifts between different trophic levels.

Methods

Specimen Collection and Taxon Sampling

Wings were removed from wild-caught specimens and stored in paper envelopes as vouchers; bodies were immediately transferred into 100% ethanol and ultimately stored at −80°C. All specimens and their genomic DNA are deposited in the DNA and Tissues Collection of the Museum of Comparative Zoology at Harvard University in Cambridge, Massachusetts. The specimens sequenced for this study include 63 species from all 13 currently recognized genera (Table S1). The two large genera, *Allotinus* and *Miletus*, were sampled most extensively and enabled us to evaluate the monophyly of Eliot's subgeneric designations (1986; Corbet *et al.* 1994). Our ingroup sample includes representatives of approximately two-thirds of the species for which life histories have been documented (Table 1.1), and one-third of all valid Miletinae species that have been described (Bridges 1988). Representatives of all putative miletine sister groups were included as outgroups (Lipteninae, Poritiinae,

Aphnaeinae) and the tree was rooted with two specimens from the subfamily Curetinae.

DNA Extraction, Sequencing, and Alignment

Genomic DNA was extracted from three legs or a small piece of abdominal tissue using a DNeasy Tissue Kit (Qiagen, Inc., qiagen.com). Seven markers comprising 4915 bp were amplified using complementary primer pairs (Table S2): mitochondrial *cytochrome c oxidase* I (1197 bp, COI); nuclear rDNA 28S (580 unambiguously aligned bp out of about 820 bp sequenced); and the five nuclear, protein-coding markers: *elongation factor* 1α (1065 bp, EF1α), *wingless* (402 bp, wg), *histone 3* (327 bp, H3), *carbamoylphosphate synthase* (747 bp, CAD), and *glyceraldehyde-3-phosphate dehydrogenase* (597 bp, G3PD). All PCRs comprised 16.65 µl ultra pure water, 1 µl 25 mM $MgCl_2$, 2.5 µl 10X PCR buffer, 1 µl 10 mg/mL bovine serum albumin, 0.25 µl 100 mM dNTPs, 0. 2µl 5 U/µl Taq polymerase (Qiagen, Inc., qiagen.com), and 1.2 µl of each primer (10 mM) for a total volume of 25 µl. The reactions were run with a touchdown cycling profile. Typical reaction conditions were: 2 min at 94°C followed by 20 cycles of 50 sec at 94°C, 40 sec at 48°C (decreasing by 0.5°C per cycle), and 80 sec at 70°C followed by 20 similar cycles with the annealing temperature constant at 50°C and ending with a final annealing step of 73°C for 5 min. The only exception was histone 3, in which the third phase of each cycle (the extension phase) was decreased to 60 sec. PCR products were purified by incubating samples at 37°C for 35 min with *Escherichia coli* enzyme exonuclease I and Antarctic phosphatase (EXO-AP), and subsequently raising the

temperature to 80°C for 20 min to deactivate the enzymes. Cycle sequencing was done using BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kits, and sequencing was performed on Applied Biosystems 3100 or 3470 automated sequencers. The resulting electropherograms were assembled and edited in Sequencher 4.2 (Gene Codes Corp., genecodes.com). All markers were aligned using MAFFT 5 (Katoh *et al.* 2005) and concatenated with MacClade 4.06 (Maddison and Maddison 2003). Several portions of 28S could not be aligned unambiguously, and about 240 bp were excised from the alignment in MacClade and not used in the analyses, resulting in a total of about 580 bp of 28S sequence. Although 28S rDNA is present in multiple copies in most genomes, these copies generally evolve synchronously via concerted evolution (Hillis and Dixon 1991). This was not the case in the genus *Thestor*; different copies of 28S were amplified when using different primer sets for several individuals. The marker 28S could only be amplified in four of ten *Thestor* species using the S3660-A335 primer pair, and only these sequences are included in our dataset. GenBank numbers for all sequences are provided in Table A1.1 and the DNA sequence alignment is provided as online Supporting Information.

Phylogenetic Analysis

Maximum likelihood, Bayesian, and maximum parsimony methods were used to infer the phylogeny of Miletinae. Maximum likelihood (ML) trees were inferred for individual genes and the full dataset using GARLI 0.951 (Zwickl 2006). The GTR+I+G model of sequence evolution was selected by Modeltest 3.7 (Posada and Crandall 1998) for each gene and the concatenated dataset using the Akaike

information criterion (AIC). All model parameters were estimated from the data. Confidence in the most likely tree based on all genes was assessed with 1000 bootstrap replicates performed in GARLI. Each replicate automatically terminated after the search algorithm progressed 10,000 generations without improving the tree topology by a log likelihood of 0.01 or better. A majority-rule consensus tree was calculated with PAUP* 4.0b10 (Swofford 2002).

Bayesian phylogenetic analyses were performed with MrBayes 3.1.1 (Ronquist and Huelsenbeck 2003). The data were partitioned by gene, using the GTR+I+G model for each gene. The substitution rates, character state frequencies, gamma shape parameters, and proportions of invariant sites were unlinked among each of the seven partitions. An analysis of 10 million generations consisted of two independent runs of four chains each with the heating temperature (temp) constrained to 0.2. Trees were sampled every 100 generations, resulting in 100,001 trees. The first 500 trees (0.5%) were discarded before a majority rule consensus tree and posterior probability branch support values were calculated from the remaining trees. Changes in the posterior probabilities of up to 20 splits were plotted over the generations of the analysis with the computer program "Are We There Yet?" (Nylander *et al.* 2008) to assess whether the chains had converged by the end of the analysis. The phylogenetic tree presented in Figure 1.1 is archived on treebase.org (submission 17026).

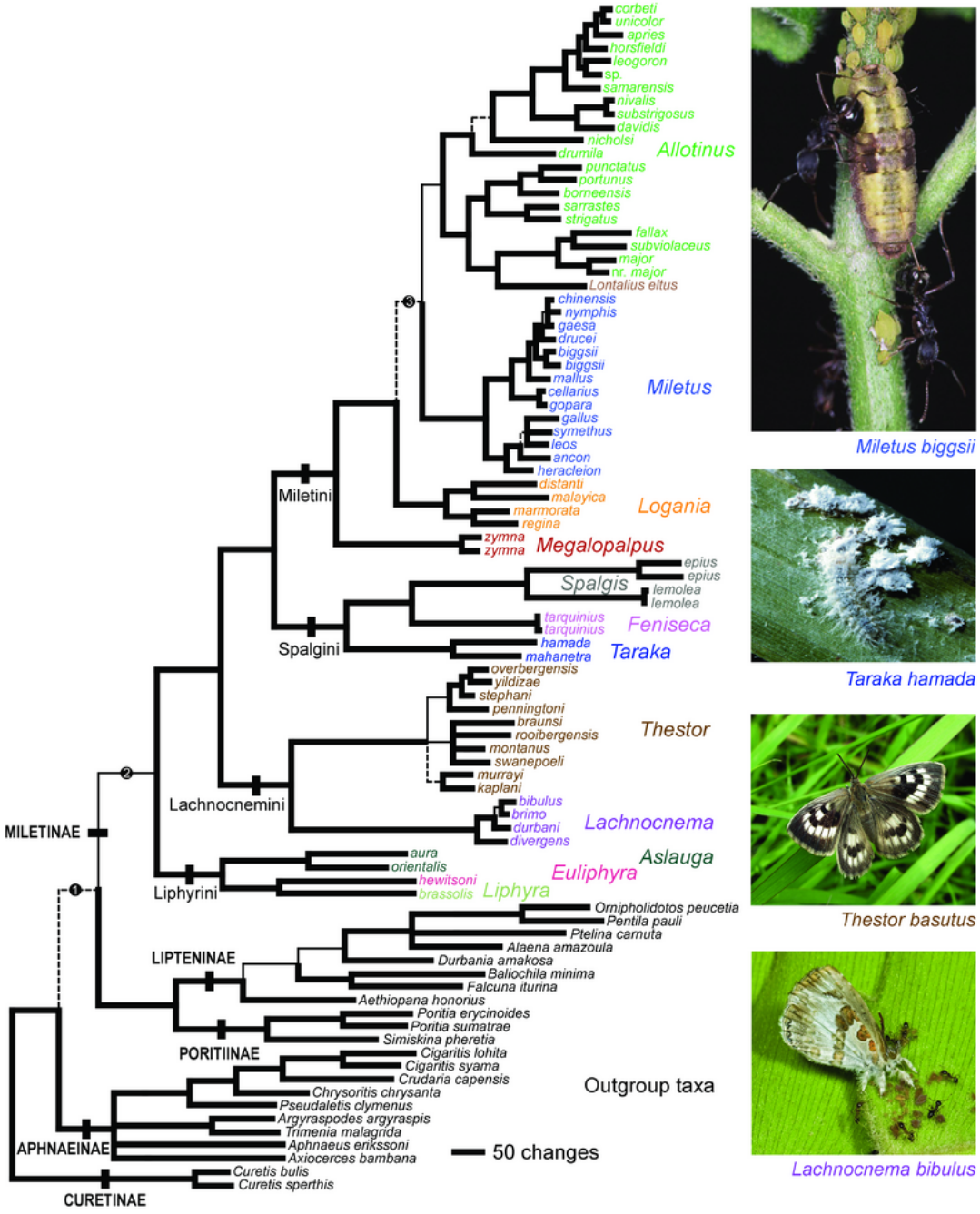

Figure 1.1 Maximum likelihood phylogenetic estimate of 63 Miletinae and 22 outgroup species based on seven markers totaling 4915 bp. Thick branches indicate maximum likelihood bootstrap support (ML) ≥ 90, Bayesian posterior probability (B) ≥ 0.99, and parsimony bootstrap (MP) ≥ 80. Thin branches indicate ML ≥ 70, B ≥ 0.90, and MP ≥ 55; dashed branches denote ML ≥ 50, B < 0.50, and MP < 50. Species names at each terminal node are color-coded by genus. Support for numbered nodes: ML = 62, B = 0.98, and MP < 50; ML = 78, B = 1, and MP = 64; ML = 52, B < 50, and MP < 50. Taxonomy follows Eliot (1973).

PAUP* was used to find the most parsimonious tree using the concatenated dataset of all seven markers. One hundred random addition searches were conducted using heuristic search methods with the TBR branch swapping, collapsing zero-length branches, and weighting all characters equally. Branch support was assessed using 1000 bootstrap replicates. To visualize genetic distances among and within genera, uncorrected pairwise (p-) distances were calculated between all ingroup samples using PAUP*, and frequency distributions of p-distances between congeneric species were plotted along with the distribution of p-distances between species from different genera. These histograms were then redrawn after all *Allotinus* intrageneric distances were removed.

Biogeographic Inference and Divergence Time Estimation

Ancestral areas and ingroup dispersal events were inferred using the programs DIVA version 1.2 (Ronquist 1997) and LAGRANGE (Ree and Smith 2008) in conjunction with a time-calibrated version of the most likely tree inferred with the software BEAST 1.7.5 (Sanderson 2003). All possible area combinations were permitted, and the biogeographic model used to infer historical patterns was constant through time. Genera were coded as belonging to one of three biogeographic regions: Afrotropical, Oriental, or Nearctic. The only exception was *Spalgis*, which is the only genus found in more than one of these regions: *Spalgis epius* was coded as Oriental and *S. lemolea* as African. Note that *Liphyra*, *Logania*, and *S. epius* extend from the Oriental into the Australian region (Parsons 2000), but, for simplicity, we classified these as Oriental, as their distributions are centered there.

Unfortunately, there are no fossilized Miletinae to aid in tree calibration. Thus, we used normally distributed tmrca (time to the most recent common ancestor) priors including maximum and minimum ages within the 95% HPD (highest posterior density) distribution on five nodes as calibrated by Heikkilä *et al.* (2012; Appendix Table 1.3), and a Bayesian phylogeny was inferred with BEAST. Heikkilä *et al.* (2012) used BEAST to calibrate divergence times using four fossils within the Nymphalidae and Pieridae to calibrate their Bayesian tree to estimate dates for the origin and diversification of the seven butterfly families. The uncorrelated relaxed clock (Drummond *et al.* 2006) and a constant population size under a coalescent model were set as priors. Two independent chains were run for 50 million generations each, sampling values every 5000 steps. A conservative burn-in of 500,000 generations was applied after checking Markov chain Monte Carlo (MCMC) convergence in Tracer version 1.5 (Rambaut and Drummond 2007).

Character Mapping and Ancestral Character State Reconstruction

Larval food source(s), taxa of associated Hemiptera, and taxa of associated ants (where known; Table 1.1) were mapped onto the best ML tree. Ant associations were coded by ant subfamily. If a given lycaenid species was known to associate with ant species from two or more subfamilies, then it was scored as being associated with multiple subfamilies. If a particular lycaenid species was known to associate with ants from multiple genera within a single ant subfamily, then it was scored in the same way as a lycaenid that associated with only one species of ant within that subfamily. Field observations of miletine caterpillars are few, and we were not always able to sample

species with available life history information. In a few cases, we inferred life history information from close relatives; these life history inferences are marked in Table 1.1. Hemiptera associations were coded by both hemipteran family and superfamily.

Phylogenetic distributions of life history characters (feeding habit, ant association, and taxon of hemipteran associate) were examined using MacClade 4.06, and ancestral states were reconstructed using the ACCTRAN algorithm. The directionality of character shifts between different feeding habits and between associating with ants from different subfamilies was confirmed with a reversible jump MCMC analysis implemented in the BayesMultiState module of BayesTraits 1.1 (Pagel *et al.* 2004). A regular MCMC analysis was done first, and the ratedev parameter was varied until the acceptance rate was around 30% to estimate priors to be used in the reverse jump analysis. The ratedev parameter used in the final reverse jump analysis was 55 with the prior set to exp(0, 50). The number of rates allowed was 6. The analysis was run for 5,050,000 generations, the first 50,000 of which were discarded as burnin. The BayesTraits results were then compiled in Microsoft Excel and graphed in JMP 7.0 (SAS 2007).

The permutation tail probability test (PTP) implemented in PAUP* was used to determine whether characters had a random distribution on the phylogeny or whether they tended to cluster. More specifically, this method was used to determine whether the diets of Miletinae are phylogenetically conserved by addressing the question: Do related miletine species feed on prey from the same hemipteran superfamily? A clustered character distribution would suggest that transitions between character states (e.g., feeding on Coccidoidea vs. Aphioidea) requires some degree of evolutionary

adaptation and is not labile. Each analysis was replicated 1000 times using all ingroup taxa. For easier interpretation, the inferred character states were then mapped onto a penalized likelihood rate-smoothed version of the most likely tree.

Results

Phylogenetic Relationships of the Miletinae

The best ML tree (−ln = 71009.4), the Bayesian consensus tree, and the most parsimonious tree were all highly congruent. Low branch support and slight differences in topology are indicated with thin or dashed lines in Figure 1.1. None of the gene trees recovered the topology of the full dataset or had strong support at deep nodes (Appendix Figure A1.2), underscoring the importance of our multigene dataset. Most nodes were strongly supported; more than half of all nodes had Bayesian posterior probabilities of 1, and all but three nodes had posterior probabilities >0.90. Notably, however, the sister-group relationship between the Miletinae and Lipteninae + Poritiinae was poorly supported, as was the sister-group relationship of *Allotinus* and *Miletus*. The inclusion of monotypic *Lontalius eltus* within the genus *Allotinus* is strongly supported, indicating that this species should hereafter be known as *Allotinus eltus* (Eliot). All other genera are monophyletic. *Allotinus* comprises two strongly supported clades that are united by weak parsimony bootstrap (62) and ML (78) support. The Miletinae as a whole are monophyletic with strong Bayesian posterior probability (1) and weaker ML (78) and parsimony (64) support. Taxonomic and systematic implications of this work are discussed in Appendix 1.

Comparison of inter- and intrageneric pairwise distances revealed that genetic distances between species are similar in magnitude, and overlap with distances between other species in different genera within the Miletinae. For example, there was, in some cases, a greater genetic distance between two species of *Allotinus* than between species in two different miletine genera. This was also true of species of *Spalgis*, which is the only genus with species in both Afrotropical and Oriental regions.

Biogeographic Inference and Divergence Time Estimation

Ancestral area reconstruction analyses were performed with LAGRANGE and DIVA. DIVA analyses frequently suggested several possible biogeographic scenarios. However, in all instances, at least one of the optimal solutions from the DIVA analysis was consistent with the most optimal solution in LAGRANGE. Both methods agreed that the extant distribution of Miletinae taxa required five dispersal events (four between the Afrotropical and Oriental region and one from the Oriental into the Nearctic). According to LAGRANGE, the Miletinae originated in Africa with a relative probability greater than 0.98 and then several lineages dispersed to the Orient, where they radiated (Figure 1.2). DIVA analysis suggested that an Afrotropical or an Afrotropical + Oriental origin were equally likely.

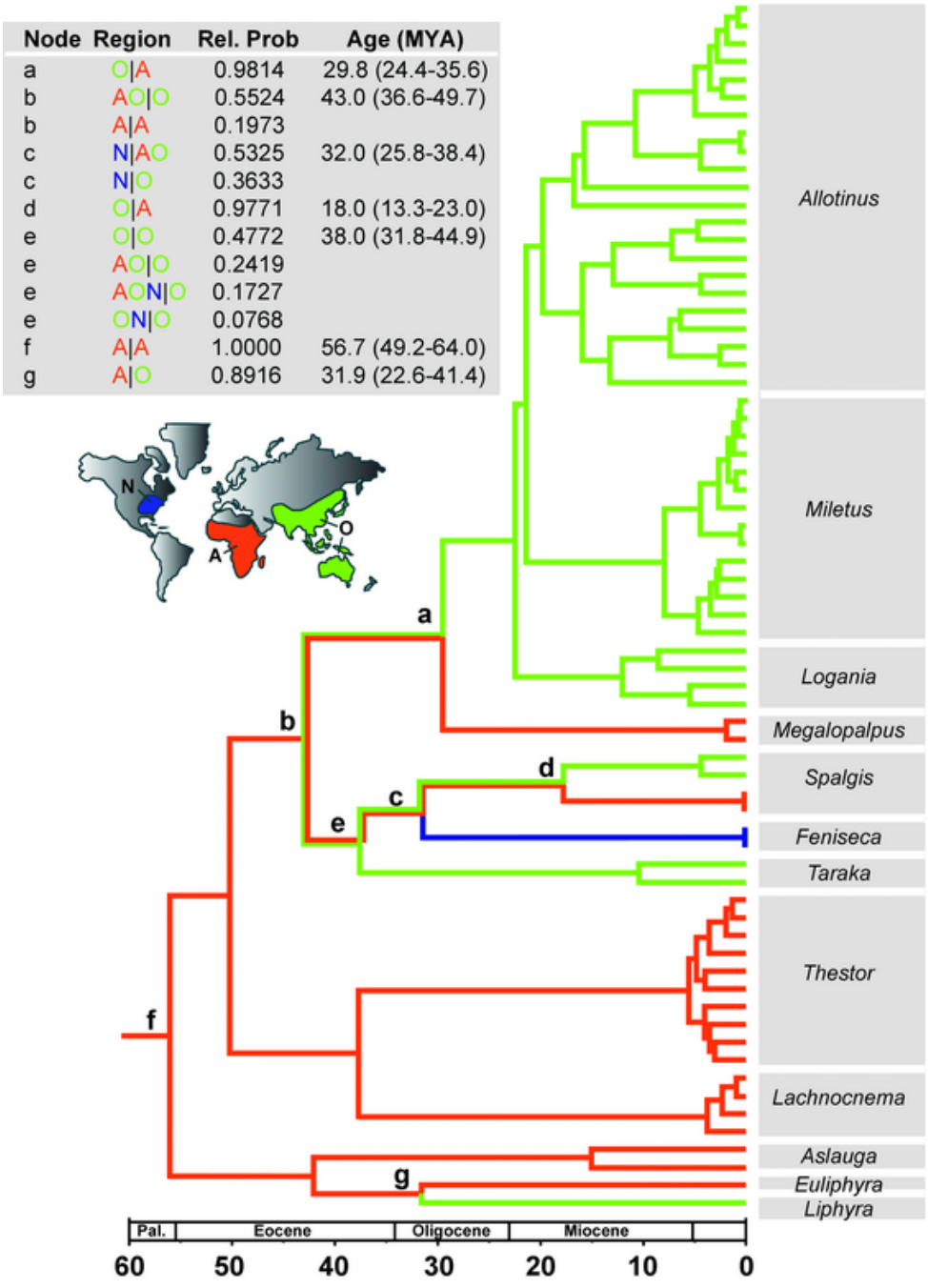

Figure 1.2 Geographic distribution of the Miletinae inferred with LAGRANGE (Ree *et al.* 2005; Ree and Smith 2008). Nodes labeled a–g refer to probabilities of daughter lineages inheriting particular ranges as described in the inset table. These are presented as probabilities that [upper branch inherited given range] | [lower branch inherited given range]. At node d, for example, there is a 98% probability that the ancestor of *Spalgis epius* (upper branch) inherited an Oriental distribution and the ancestor of *S. lemolea* (lower branch) inherited an African distribution from their common ancestor. Colored branches indicate the inferred ancestral areas inherited by each lineage.

Our analyses place the origin of the Miletinae near the start of the Eocene, 57 (95% CI, 49–64) million years ago. The *Liphyra* lineage dispersed out of Africa 32 (23–41) million years ago, *Taraka* and its relatives dispersed out of Africa about 38 (32–45) million years ago, and the monophyletic Oriental Miletini (*Allotinus*, *Miletus*, and *Logania*) clade migrated out of Africa 30 (24–36) million years ago. The *Spalgis lemolea* lineage dispersed back into Africa from Asia approximately 18 (13–23) million years ago, and the *Feniseca* lineage dispersed into North America from Asia 32 (26–39) million years ago (Figure 1.2).

Character Mapping and Ancestral Character State Reconstruction

The immature stages of many miletine species are unknown, and ancestral state reconstruction was therefore used to infer probable life history characteristics (food type and taxon of ant associate) of species for which information is lacking (Figure 1.3). Miletine larvae have been recorded feeding on at least seven different types of food: Hemiptera, ant brood, ant trophallaxis, detritus, insect prey in ant nests, hemipteran honeydew, and extrafloral nectar (Table 1.1). Most feed on Hemiptera, although many supplement this with additional food types. When we mapped all food types onto the best ML tree, the distribution of these seven feeding habits was not significantly clustered ($P = 1.0$); grouping detritus with insect prey and ant trophallaxis with ant brood resulted in five feeding categories that were significant ($P = 0.017$). However, when we grouped feeding behaviors into three categories: "Hemiptera only," "Hemiptera + Other," and "Other only" (where "Other" refers to ant brood, ant trophallaxis, hemipteran honeydew, extrafloral nectar and/or detritus),

then the phylogenetic association was highly significant ($P = 0.005$) and several trends in feeding behavior became evident.

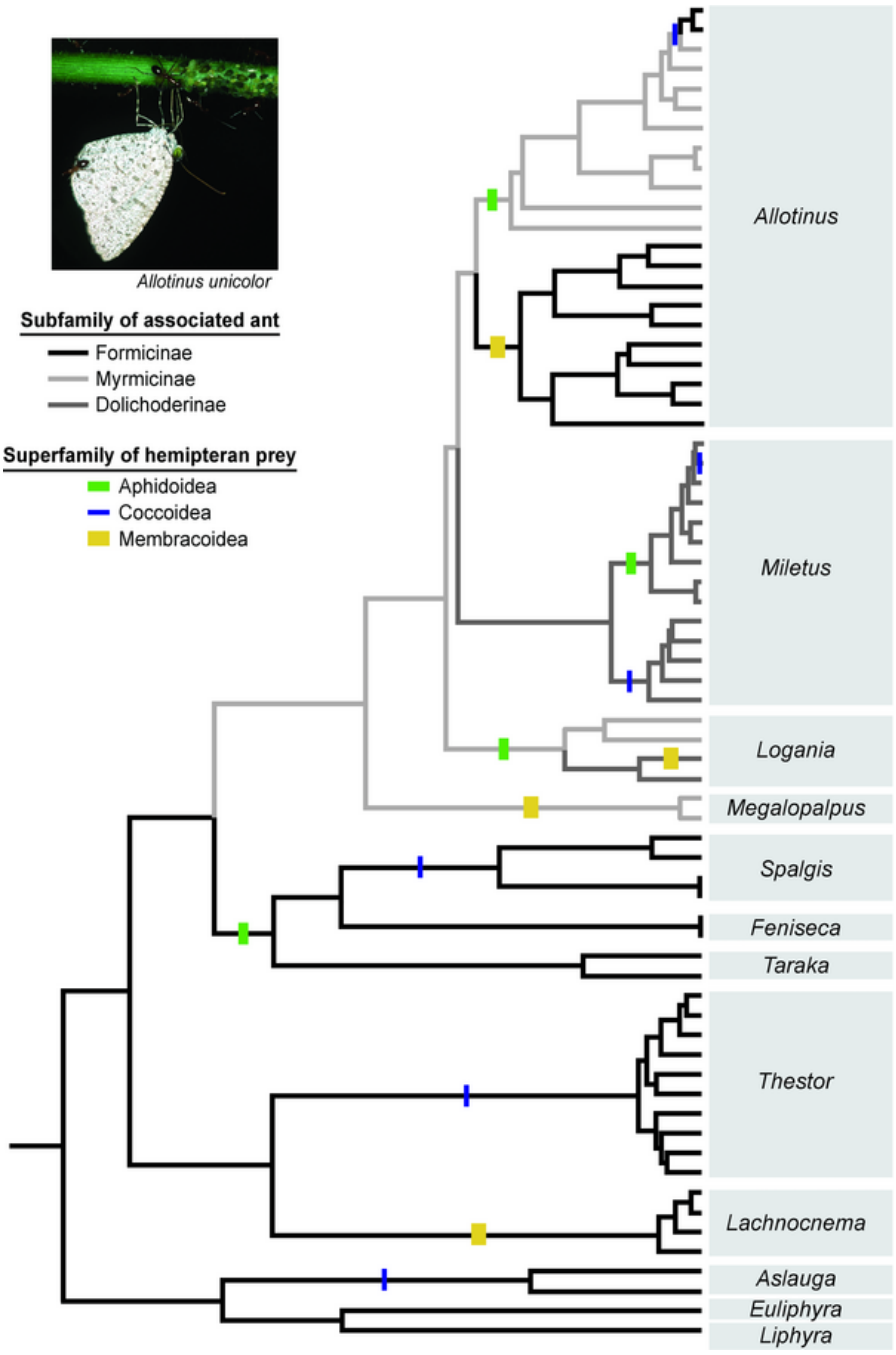

Figure 1.3 Mapping of ancestral character state reconstructions to estimate the most likely ant subfamily and Hemiptera superfamily associated with the larvae of miletine ancestors. Ant-associated subfamilies were treated as a multistate unordered character; state transitions were equally weighted. Inferred character states of the ant-associated subfamilies are indicated by branch colors and the colored bar to the right of the phylogram indicates the reconstructed Hemiptera superfamily association.

The prey taxon on which miletine larvae feed is phylogenetically conserved: the larvae of closely related butterfly species tend to feed on related prey taxa. A significant correlation exists between Miletinae phylogeny and the families of Hemiptera consumed ($P = 0.046$), as well as between Miletinae and hemipteran superfamily ($P = 0.018$; Figure 1.3). Moreover, a strong association was recovered between Miletinae phylogeny and the subfamily of ants with which they associate, either directly because the miletines consume the ants, or indirectly because the ants attend their hemipteran prey (PTP test, $P = 0.008$; Figure 1.3).

Discussion

Biogeography

Dispersals of miletines between geographic regions may have been driven by climatic changes. The Miletinae originated in Africa about 57 (95% CI, 50–64) million years ago near the beginning of the Eocene when global temperatures were higher and the Earth was covered by forests (Zachos *et al.* 2001). Even sections of Northern Africa that are currently desert were then covered by rainforest (Jacobs 2004). In the mid-Eocene, global climates and ecosystems began undergoing drastic transformations: there was significant cooling and a reduction in the prevalence of global tropical forests (Zachos *et al.* 2001). This led to mass global extinctions from around 40 to 33 million years ago (Jacobs 2004). It was during this period, specifically between 30 and 38 (23–45) million years ago, that three clades of the Miletinae dispersed out of Africa. It is possible that they shifted their ranges to cope with the

transformation of their previous ranges from warm and humid climates to the relatively harsh and dry ones of the late Eocene and early Oligocene.

Following this initial dispersal of Miletinae out of Africa, ancestors of *Spalgis lemolea* appear to have dispersed back into Africa approximately 18 (13–23) million years ago. This corresponds closely to the time when the Tethys Sea closed and the Gomphotherium land bridge formed (Harzhauser *et al.* 2007). The closure of the Tethys Sea was associated with another global cooling event. The cooler temperature reduced the atmosphere's ability to absorb moisture and as a consequence most of Africa's forests became grasslands (Zachos *et al.* 2001). After the collision of the Afro-Arabian plates with Eurasia, there was a significant faunal exchange between Africa and Eurasia. The best-known example of this is the dispersal of proboscideans that migrated from Africa to Eurasia around 19–18.5 million years ago (Harzhauser *et al.* 2007). The land bridge became a corridor not only for land mammal movement, but also for insect dispersal, including *Junonia* butterflies 19 million years ago (Kodandaramaiah and Wahlberg 2007).

Evolution of Aphytophagy

Although the Miletinae are monophyletic (Figure 1.1), we cannot determine from the phylogeny whether lichen-feeding evolved before the evolution of aphytophagy, as hypothesized by Balduf (1938). The lichen-feeding Lipteninae are sister to the Poritiinae, and this clade is sister to the Miletinae. All liptenines with known life histories feed on lichens, but the few poritiine life histories that have been described suggest that they consume the leaves of vascular plants. Maschwitz *et al.*

(1988) hypothesized that aphid-feeding (superfamily Aphidoidea) was the ancestral condition in the Miletinae, and that other feeding strategies evolved subsequently. The two earliest diverging lineages, Liphyrini and Lachnocnemini, are predominantly coccid feeders (superfamily Coccidoidea; Figure 1.3), and it thus seems likely that this was the ancestral condition. The exact role of ant association in the route to Hemiptera feeding is difficult to determine because either simple tolerance (myrmecoxeny) or mutualism (myrmeocophily, involving the production of nutritious food rewards) may have preceded or evolved concurrently with the entomophagous feeding strategy observed today. Perhaps significantly, reversal from a predatory or parasitic lifestyle to feeding once again on plants is not observed in the Miletinae. It is unclear why this transition has been unidirectional, but physiological changes associated with a shift from consuming nitrogen-poor tissue in plants to nitrogen-rich tissue in animals may be difficult to reverse.

A simplified categorization of feeding habits mapped onto a rate-smoothed version of the most likely tree (Figure 1.3) reveals that most Miletinae feed on Hemiptera, including the earliest diverging ("basal") lineages. Species that include ant brood, ant trophallaxis, insect prey within ants nests, extrafloral nectar, or hemipteran honeydew in their diets seem to be randomly distributed on the phylogeny, suggesting that these habits are evolutionarily labile and/or facultative.

Phylogenetic Conservatism of Species Interactions

Interactions between Miletinae and ants were strongly evolutionarily conserved with few transitions among ant subfamilies. Relationships with Hemiptera

were also conserved, but this pattern was less distinct. Character reconstruction suggests that feeding on Hemiptera was the ancestral state for the Miletinae. The collective prey species eaten by miletines include species from 10 families in four superfamiles that do not form a monophyletic group (von Dohlen and Moran 1995; Lee *et al.* 2009). When these hemipteran taxa are mapped onto the miletine tree, a relationship between Miletinae phylogeny and prey taxon is recovered (PTP test, $P = 0.018$; Figure 1.3): the prey eaten by the larvae of a miletine species are usually related to the prey eaten by a related miletine species.

Although closely related miletines may associate with a variety of ant species and genera, the subfamilies to which those ants belong are highly constrained across the phylogeny: sister miletines are more likely to associate with ants from the same subfamily (PTP test, $P = 0.008$; Figure 1.3). This is true both for ants that are consumed directly by caterpillars (such as *Liphyra* and *Thestor* species), or those that attend a caterpillar's prey species. The reason for this is not immediately clear, but could result from historical contingency, or possibly because adaptation to associating with a novel ant subfamily requires adaptation to a new suite of pheromones or recognition chemicals characteristic of that subfamily (Morgan 2008). Nevertheless, transitions between ant subfamilies have occurred, and the frequency of these shifts between ant subfamilies appears to be constant (BayesTraits reversible jump analysis; number of parameters = 1.2 ± 0.4). To date, Miletinae caterpillars have only been found associating with ants in the subfamilies Formicinae, Myrmicinae, and Dolichoderinae. Switching between certain ant subfamilies are equally probable: Myrmicinae to Dolichoderinae; Myrmicinae to Formicinae; Dolichoderinae to

Myrmicinae; Dolichoderinae to Formicinae; and Formicinae to Myrmicinae. However, switching from Formicinae to Dolichoderinae is not likely to have occurred.

The phylogenetic conservatism in ant association appears counterintuitive because most miletine caterpillars have a direct interaction with Hemiptera (most species eat them), but only an indirect association with ants that attend the Hemiptera. However, the significant conservatism of ant subfamily in associations recorded across the miletine family, in concert with a number of other behavioral observations, suggests that ants are a more important participant in these interactions than previously appreciated. Maschwitz *et al.* (1988) observed that fluttering female butterflies seem to be able to detect ants, even when they are not readily visible (e.g., behind a leaf). Moreover, miletine adults may be able to detect aggregations of the appropriate attending ant species even when there are no Hemiptera present (e.g., at sap flows; Fiedler and Maschwitz 1989; Lohman and Samarita 2009). Aggregations of Hemiptera are liable to be ephemeral in space and time, and an ovipositing female butterfly is challenged with locating sites with adequate numbers of hemipteran prey where she can deposit her eggs. Many aphids are known to produce alarm pheromones under duress (Nault and Montgomery 1979), but these chemical signals are not produced without provocation, and would therefore be an unreliable cue for ovipositing females to use in locating hemipteran colonies. Ants, however, produce a wide variety of different semiochemicals in different contexts (Vander Meer *et al.* 1998), and some compounds, such as trail pheromones, are released with sufficient frequency to be a reliable cue indicating the presence of ants. Because of their semiochemical and visual apparency, ants might thus act as homing beacons for

ovipositing female miletine butterflies that can smell and see the ants and use them to find Hemiptera that are frequently in the company of ants. Because different ant taxa communicate with different suites of chemical compounds, particular miletine species or genera may be adapted to detect some but not all ant taxa. These specialized ant associations suggest the possibility of a "ghost of ant association past" through which associations with specific ants may have facilitated the evolution and/or maintenance of a parasitic or predatory lifestyle.

Ants normally protect the Hemiptera that they attend, and fend off predatory insects, but miletine caterpillars can employ chemical camouflage to avoid detection by semiochemically resembling their surroundings. Ants use a mixture of cuticular hydrocarbons (CHCs) in their epicuticular wax as recognition cues (Van Zweden and d'Ettorre 2010). These identifying labels can be species-, caste-, or colony-specific, and ants appear to use CHCs to identify other insects as well. Lohman *et al.* (2006) showed that larvae of the North American miletine, *Feniseca tarquinius*, resemble the CHC profile of their woolly aphid prey rather than that of the attendant ants, thereby avoiding attack by the ants and detection by their prey. These and additional studies suggest that CHCs are used as recognition cues by ants to discriminate trophobionts from invaders and that predacious, hemipteran-feeding miletine larvae are able to produce or acquire a sufficient subset of semiochemicals to dupe ants (and possibly also aphids) to avoid detection (Lohman 2004; Youngsteadt and DeVries 2005; Lohman *et al.* 2006).

A large proportion of lycaenids that are recognized as endangered species have predatory or parasitic lifestyles. This demographic and phylogenetic pattern is similar

in other insects. Weigmann *et al.* (1993) observed that insect lineages with highly specialized carnivorous and parasitic lifestyles tend to be less diverse than their relatives with more general feeding behaviors, and suggested that one explanation for the evolutionary success of phytophagous compared to aphytophagous insects is simply the trophic pyramid, with its differences in the quantity and availability of resources at each level. Aphytophagy has arisen multiple times within the Lepidoptera, but has rarely resulted in radiation (Pierce 1995). Miletinae are a conspicuous exception to this general pattern, and it seems that their limited success as aphytophagous Lepidoptera is likely to be due to their adaptations for finding prey. The ability to use ants as cues in locating ephemeral hemipteran prey may have been especially important. Both ant and hemipteran resources must have been sufficiently abundant, predictable, and ecologically apparent to have enabled the persistence and diversification of this unusual group.

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Data Archiving

Data Archival Location: Specimens and extracted genomic DNA are vouchered in the Museum of Comparative Zoology DNA and Tissues Collection. DNA sequences are archived in GenBank (accession numbers AF279218, AF279223, DQ018905-DQ018909, DQ018938-DQ018942, KF787151, KF787166, KF787171, KF787173, KF787184, KF787191, KF787203, KF787206, KF787209, KF787214, KF787220-KF787222, KF787237, KF787245, KF787273, KF787285, KF787288, KF787291, KF787296, KF787409-KF787411, KF787426, KF787432, KF787434, KF787449, KF787462, KF787473, KF787476, KF787479, KF787484, KF787490-KF787492, KF787507, KF787513, KF787515, KF787530, KF787543, KF787555, KF787558, KF787561, KF787566, KP215665-KP215816, KP215818-KP215828, and KP215906-KP216198), the DNA sequence alignment is provided as online Supporting Information, and phylogenetic trees are archived on treebase.org (submission 17026).

CHAPTER 2

Extinction risk in parasitic butterflies may be driven by small effective population size: A case study comparing two species in South Africa

Coauthors: Alan Heath, and Naomi E. Pierce

Abstract

Aim

Approximately 30% of the lycaenid butterflies recognized by the IUCN as threatened or endangered have aphytophagous caterpillars even though such parasitic or predaceous species comprise less than 3% of species with described life histories in the Lycaenidae (the blues, coppers and hairstreaks). This raises an important question as to how diet might influence the evolutionary success of a species. To investigate why aphytophagous butterflies might be more vulnerable to extinction, we examined the biogeography and population genetics of two South African butterfly species in the family Lycaenidae: *Chrysoritis chrysaor*, whose members are strictly herbivorous, and *Thestor protumnus*, whose caterpillars induce workers of their host ants, a species of *Anoplolepis*, to engage in trophallaxis and feed them like cuckoos in the nest.

Location

Southern Africa

Methods

We sequenced c. 1200 bp of mitochondrial DNA from COI and 750 bp from nuclear DNA from ITS2 for up to 20 specimens from each of 20 localities for *C. chrysaor* and 7 localities for *T. protumnus* from throughout their respective ranges in

Southern Africa. Bayesian inference, maximum likelihood and maximum parsimony methods were used to reconstruct phylogenies, and haplotype networks were constructed using Bayesian methods. Fixation indices, effective population sizes, and other population genetic statistics were obtained using *Arlequin*.

Results

The herbivorous *C. chrysaor* has dramatically higher levels of within-population variation than the parasitic *T. protumnus*. *T. protumnus* is also more constrained by geographic distance and has effective population sizes several orders of magnitude smaller than those of *C. chrysaor*.

Main conclusions

In contrast to *C. chrysaor*, *T. protumnus* have small effective population sizes and disperse poorly. With its aphytophagous life history, *T. protumnus* appears to exhibit a higher degree of host dependence and specialization, potentially acting as a biological barrier to dispersal. Such specialization may also make populations of *T. protumnus* more susceptible to disturbance and prone to extinction.

Introduction

Butterflies whose caterpillars are predaceous or parasitic have historically succumbed to extinction at a much higher rate than their herbivorous counterparts. Less than 3% of described lycaenid butterflies are “aphytophagous” (defined as having at least one life stage obligately dependent on animal rather than plant tissues or

secretions for nutrition), but they comprise approximately 30% of the IUCN Red List of Threatened Species of lycaenid butterflies (IUCN 2013). Aphytophagous life histories have arisen numerous times within the Lepidoptera, and particularly the butterfly family Lycaenidae, but species with predaceous caterpillars seldom persist or radiate (Pierce 1995, Pierce *et al.* 2002). The behavioral and ecological diversity of the Lycaenidae makes it an ideal system for studying why some species are more prone to extinction than others. By comparing the population structure of closely related herbivorous and aphytophagous butterflies, we can gain insights into why predaceous and parasitic species appear to be evolutionary "dead ends" that go extinct more often.

Several ecological mechanisms can account for increased extinction rates: decreased speciation rate (effectively a lower "birth rate" of new species) resulting from a lack of new niches, higher extinction rates (or higher "death rate") from increased competition between species whose niche differences may be incidental to speciation, and decreased speciation opportunity because of range fragmentation (again, effectively lowering birth rate, or perhaps constraining carrying capacity) (Mitter *et al.* 1988; Barraclough *et al.* 1998; Barraclough and Vogler 2000). The genetic patterns underlying these ecological mechanisms are still not fully understood, but they can include low genetic diversity, low effective population sizes and high population fragmentation.

To identify genetic factors that could contribute to higher extinction risks in predaceous butterflies or lower extinction risks in herbivorous butterflies, we selected two closely related species based on similarities of age, habitat and geographic ranges.

Chrysoritis chrysaor (Trimen 1864) and *Thestor protumnus* (Linnaeus 1764) are both endemic species of Lycaenidae found in Southern Africa, and both have large, overlapping ranges.

The larvae of both of these species are also obligately associated with ants. Pierce (1984; 1987) hypothesized that obligate ant associates can be instrumental in fragmenting and structuring populations, potentially leading to greater diversification. Two subsequent studies failed to support this hypothesis (Costa *et al.* 1996; Eastwood *et al.* 2006), and found instead that obligate ant association can select for greater vagility in adult butterflies, resulting in larger population sizes distributed over a broader range. However, a recent study found more intra-specific Single Nucleotide Polymorphisms (SNPs) within species of lycaenids that have strong mutualistic associations with ants than within species with little or no ant association (Pellissier 2012). This suggested that in some situations, strong ant associations are indeed correlated with higher diversification rates and more pronounced genetic differentiation between populations (Pellissier 2012). While *Chrysoritis chrysaor* and *Thestor protumnus* both have obligate associations with ants, larvae of *C. chrysaor* appear to have a mutualistic relationship with *Crematogaster* ants, providing them with food secretions in exchange for defense, whereas larvae of species of *T. protumnus* parasitize their associated *Anoplolepis* ants by being fed mouth-to-mouth by workers inside the nest.

Although similar in many respects, *C. chrysaor* and *T. protumnus* have very different feeding preferences. The larvae of *Chrysoritis chrysaor*, like most Lepidoptera, are phytophagous (herbivorous). They feed on leaves from five families

of plants: Zygophyllaceae, Crassulaceae, Asteraceae, Anacardiaceae, and Fabaceae (Heath *et al.* 2008). Those of *Thestor protumnus*, by contrast, are aphytophagous: they have never been observed to feed on plants (Heath and Pringle 2004). Their caterpillars are specialists that parasitize one genus of ants. The caterpillars live in *Anoplolepsis* ant nests, where they solicit food regurgitations from their host ants via trophallaxis by mimicking host ant begging signals. Ants feed them just as they would conspecific adults and/or their own brood.

Because these two species are similar in many respects but differ greatly in their diets, they provide a potentially informative comparison of herbivorous and parasitic butterflies. We analyzed their phylogeography and population genetics for differences that might shed light on why aphytophagous species are more vulnerable than their phytophagous counterparts.

Materials and Methods

Specimen collection and taxon sampling

To investigate how life history might influence the population structure of these two species, we spent two field seasons in South Africa collecting *Chrysoritis chrysaor* and *Thestor protumnus*. In all, we sequenced two genes for 88 specimens of *C. chrysaor* and 77 specimens of *T. protumnus* collected from 26 localities throughout their respective ranges. Wings were removed from wild-caught specimens and stored in paper envelopes as vouchers. Bodies were immediately transferred into 100% ethanol and stored at -80° C upon return to the lab. All specimens were deposited in the collection of the Museum of Comparative Zoology at Harvard University in

Cambridge, Massachusetts. The specimens sequenced for this study include 2 to 22 butterflies from 7 localities for *Thestor protumnus* and 1 to 19 butterflies from 20 localities for *Chrysoritis chrysaor* (Appendix Table 3.1). This sampling spans most of the known ranges for both species, although we could only find specimens of *T. protumnus* from one locality along the Eastern Cape despite our best collecting efforts. 28 specimens of C*hrysoritis midas*, 9 specimens of *Chrysoritis natalensis* and 6 specimens of *Thestor dryburghi* were sequenced as outgroups.

DNA extraction and sequencing

Genomic DNA was extracted from three legs or a small piece of abdominal tissue using a Chloroform Extraction on the AutoGenprep 965 robot (AutoGen, Holliston, MA) or DNeasy Tissue Kit (Qiagen Inc., qiagen.com). One mitochondrial (1,197bp *cytochrome c oxidase* subunit I) and one nuclear (750bp *internal transcribed spacer-2*) gene were amplified using complementary primer pairs (Appendix Table 3.2).

Polymerase chain reaction (PCR) amplification was conducted using a Bio-Rad DNA Engine Dyad Peltier thermal cycler. All reactions were prepared in 25µL volume reactions with 16.65 µL ultra pure water, 1µL 25mM $MgCl_2$, 2.5µL 10X PCR buffer, 1µL 10mg/mL bovine serum albumin, 0.25µL 100mM dNTPs, 0.2µL 5U/µL Taq polymerase (Qiagen Inc., qiagen.com), and 1.2µL of each primer (10mM). The reactions were run with a touchdown cycling profile. Typical reaction conditions were: 2 min at 94° C, followed by 20 cycles of 50 s at 94° C, 40 s at 48° C (decreasing by 0.5° C per cycle) and 80 s at 70° C, followed by 20 similar cycles with the

annealing temperature constant at 50° C and ending with a final annealing step of 73° C for 5 min. These conditions worked 90% of the time, if they did not then annealing temperatures were lowered or raised and extension times were shortened or lengthened in small increments until the reactions worked. PCR products were purified through incubation at 37° C for 35 minutes with *Escherichia coli* enzyme Exonuclease I and Antarctic Phosphatase (EXO-AP), then the enzymes were deactivated by raising the temperature to 80° C for 20 minutes. BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kits were used for cycle-sequencing. The samples were then sequenced using Applied Biosystems 3130xl and 3470 automated sequencers. The resulting chromatograms were assembled and edited in Sequencher 4.8 (Gene Codes Corp., genecodes.com).

Alignment and Phylogenetic Analysis

All genes were aligned using MAFFT 5 (Katoh *et al.* 2005) and concatenated with MacClade 4.06 (Maddison and Maddison 2003). Several portions of ITS2 could not be aligned unambiguously. Consequently, approximately 150 bp of ITS2 were excised manually from the alignment in MacClade and excluded from the analyses, for a total of about 600 bp of ITS2 sequence.

The phylogenetic trees of *Thestor* and *Chrysoritis* were inferred using maximum likelihood, Bayesian, and maximum parsimony methods. Maximum likelihood trees were inferred for individual genes and the full data set using GARLI 0.951 (Zwickl 2006). These methods were implemented on the CIPRES portal (Miller *et al.* 2009) using the GTR+I+G model and 1000 bootstrap replicates. Akaike

Information Criterion (implemented in Modeltest 3.7, Posada and Crandall 1998) determined that the GTR+I+G model of sequence evolution was the optimal model for each individual gene and for the concatenated dataset for both genera. All model parameters were estimated from the data. Nodal support in the most likely tree based on all genes was assessed with 1,000 bootstrap replicates performed in GARLI. Each replicate automatically terminated after the search algorithm progressed 10,000 generations without improving the tree topology by a log likelihood of 0.01 or better. A majority-rule consensus tree was calculated with PAUP* 4.0b10 (Swofford 2002).

Bayesian phylogenetic analyses were performed with MrBayes 3.1.1 (Ronquist and Huelsenbeck 2003). The data were partitioned by gene, using the GTR+I+G model for each gene. The substitution rates, character state frequencies, gamma shape parameters, and proportions of invariant sites were unlinked among each of the partitions. Each analysis of 10 million generations consisted of two independent runs of four chains each with the heating temperature (temp) constrained to 0.2. Trees were sampled every 100 generations, resulting in 100,001 trees. The first 50,000 trees were discarded as burnin. The remaining trees were used to calculate a majority rule consensus tree and posterior probability branch support values. To assess whether the chains had converged by the end of the analysis, changes in the posterior probabilities of up to twenty splits were plotted over the generations of the analysis with the computer program "Are We There Yet?" (Nylander *et al.* 2008).

PAUP* was used to find the most parsimonious trees. One hundred random addition replicates were conducted using heuristic search methods with TBR branch

swapping, collapsing of zero-length branches and equal weighting of all characters. Nodal support was assessed using 1,000 bootstrap replicates.

Haplotype networks were produced using Network 4.1 (Bandelt *et al.* 1999) under the standard settings.

Statistical Analyses

We used Arlequin 3.5.1.3 (Excoffier and Lischer 2010) to calculate estimates of genetic diversity for each species. Our calculations used both COI and ITS2 sequences to estimate parameters such as the number of haplotypes (*h*), haplotypic diversity (*Hd*), number of polymorphic sites (*S*), nucleotide diversity (*p*), and theta(*S*).

The diversity among localities was tested using an AMOVA in Arlequin 4.5.1.3 (Excoffier and Lischer 2010). To test for significant correlations between genetic (linearized Φ_{ST}) and geographical distances between sites we performed Mantel Tests for each species in Arlequin. Effective population sizes were calculated in both Migrate-N (Beerli 2006) and Arlequin 3.5.1.3 (Excoffier and Lischer 2010).

Results

Gene Trees

Eighty-eight *C. chrysaor* and 77 *T. protumnus* specimens were collected from 26 locations throughout South Africa and subjected to maximum likelihood tests using two genes, COI and ITS2.

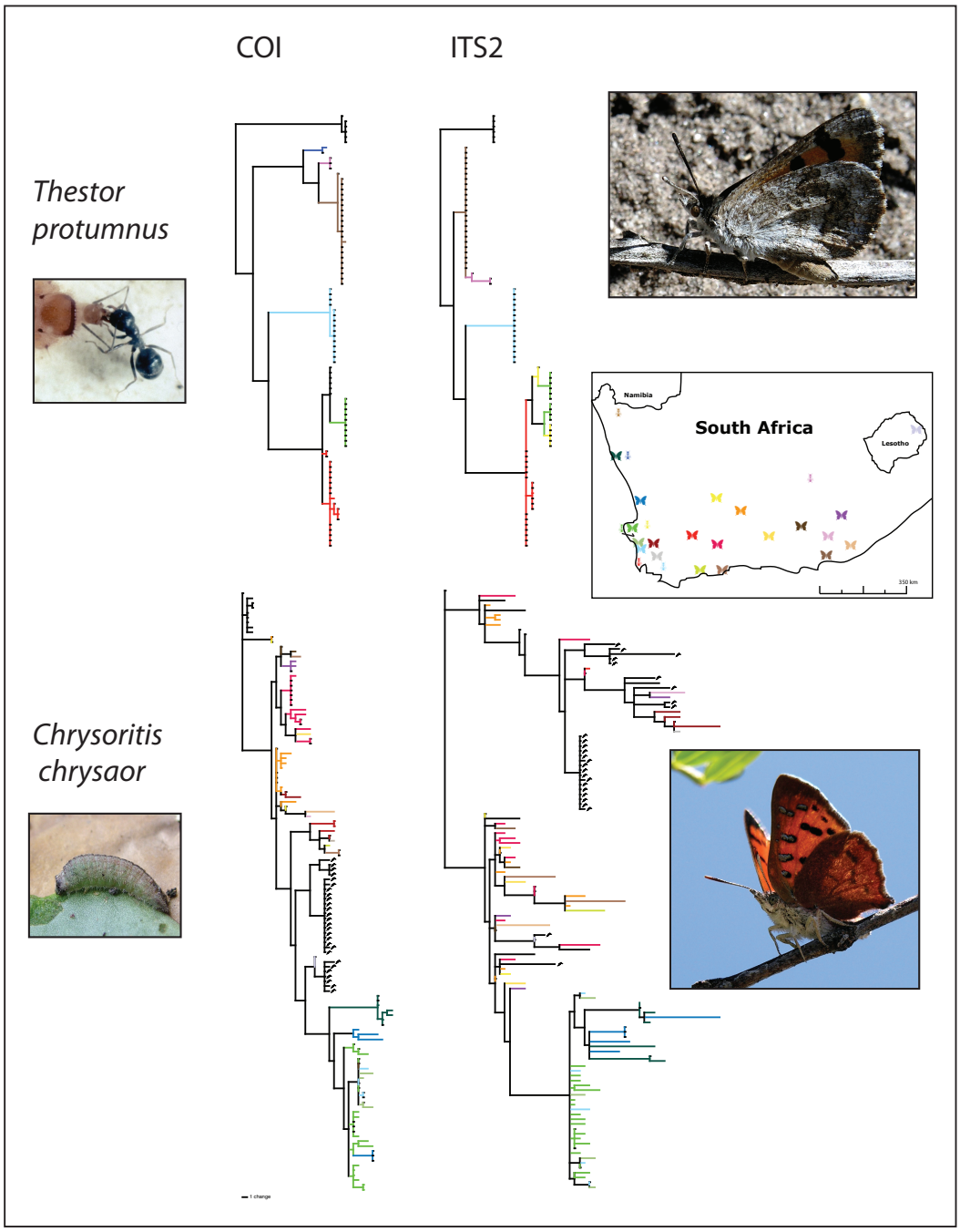

Figure 2.1 COI and ITS2 maximum likelihood trees for *C. chrysaor* and for *T. protumnus*. Tips are colored by locality. The leaves on the map represent *C. chrysaor*. The ants on the map represent *T. protumnus*. Outgroups are left black. Dots represent *C. midas*.

For the herbivorous *C. chrysaor,* both COI and ITS2 show significant variation within and between localities: For COI, the 88 individuals sampled generated 60 unique haplotypes, with up to 14 haplotypes (out of 19 individuals) in any one locality. For ITS2, the 88 individuals sampled yielded 83 unique haplotypes, with up to 18 haplotypes (out of 19 individuals) in any one locality (Figures 2.1 and 2.2). Of the few shared haplotypes, a small percentage are found in two locations (Figures 2.1 and 2.2). The variation is not completely random; there is some structure, especially a division of the coastal vs. the inland butterflies.

In the genes for the predaceous *T. protumnus*, a different pattern emerges. The COI maximum likelihood tree and haplotype network of *T. protumnus* show almost no variation within localities: an average of 2.3 haplotypes per locality, and a maximum of 5 haplotypes out of 19 individuals at Redhill (Figures 2.1 and 2.2). Of the 77 individuals and 7 localities sampled, only 14 haplotypes are unique, and no haplotypes overlap between localities. The ITS2 data show a similar trend. Of the 77 individuals sampled, only 10 haplotypes are unique with a maximum of 2 haplotypes per locality. A north-south divide is apparent in both genes.

Molecular diversity and population demographics

The percentage of genetic variation within populations acts as a measure of gene flow. Populations with a high level of gene flow between populations have lower *F*st values. For COI, 28% of the genetic variation in *C. chrysaor* is explained by variation within localities (*F*st=0.72). In contrast, for *T. protumnus*, only 2% of the

variation in COI is explained by variation within localities (*F*st=0.98). ITS2 shows a similar trend: an *F*st of 0.90 for *C. chrysaor* versus an *F*st of 0.96 for *T. protumnus*.

The effective population sizes (*N*e) for *C. chrysaor* are also several orders of magnitude larger than for *T. protumnus*. The average theta (θ=$2N_e\mu$) value calculated from COI per *T. protumnus* population is 0.52 while the average value for *C. chrysaor* is 3.8 (Appendix Table 3.3 and 3.4). Genetic diversity indices (*H*d, *S* and *p*) for *C. chrysaor* and *T. protumnus* are summarized in Appendix Tables 3.3 and 3.4.

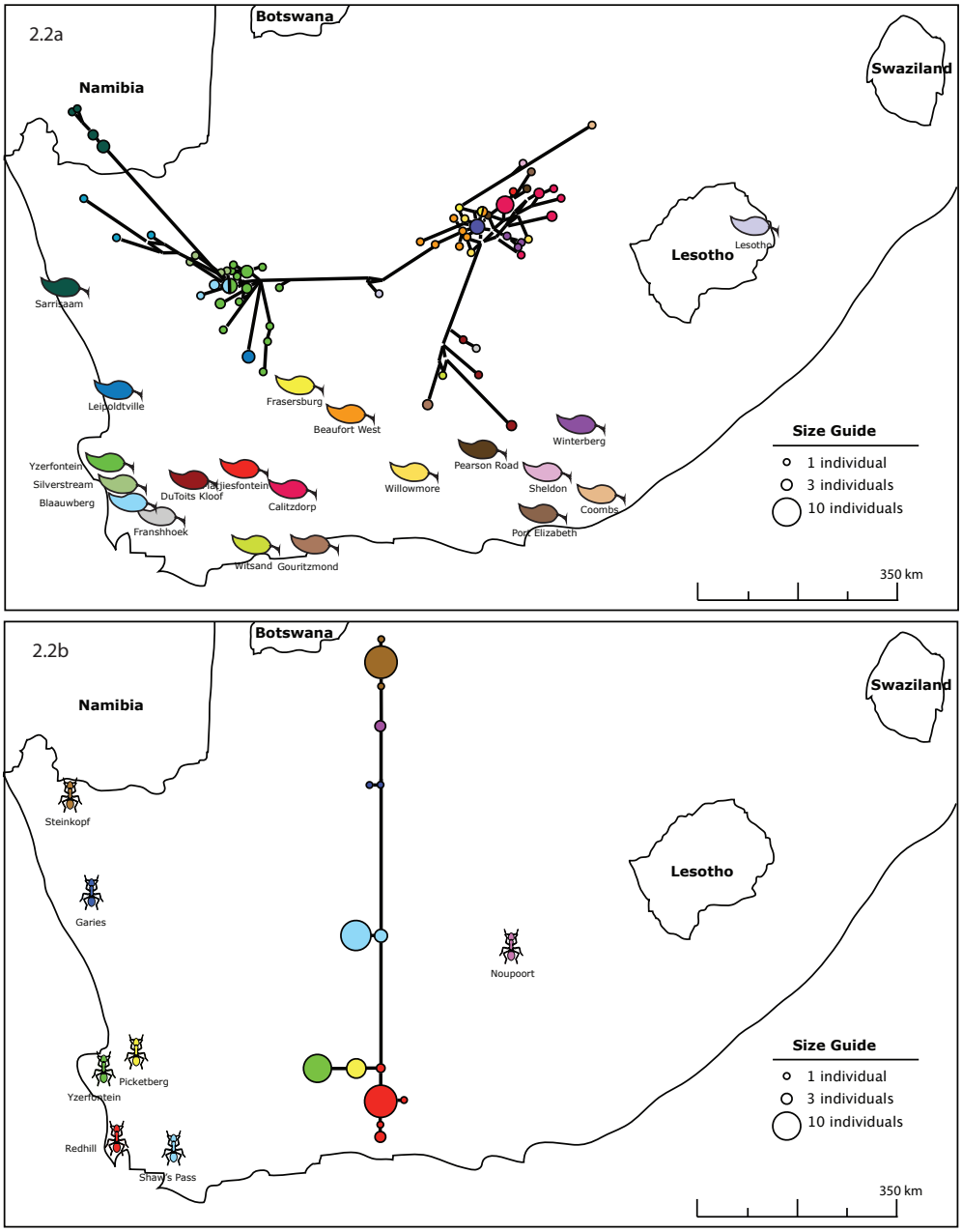

Figure 2.2 Haplotype networks for *C. chrysaor* (2.2a) and *T. protumnus* (2.2b) constructed in Network4.1. Leaf symbols correspond to localities where C. chrysaor were collected. Ant symbols correspond to localities where *T. protumnus* were collected. Colors of circles in the haplotype networks correspond to the locality where those specimens were collected, and sizes of circles correspond to the number of individuals sampled.

Isolation by Distance

Genetic isolation by geographical distance is a consequence of limited dispersal across space. Tests of isolation by distance determine how much genetic variation can be explained because two populations are in close proximity to each other or because the individuals from those populations are likely to travel long distances. The Mantel test for isolation by distance failed to detect any significant correlations between genetic and geographical distance (p=0.3) for *C. chrysaor*. Only 0.003% (ns) of their genetic structure can be ascribed to a geographical pattern of population establishment. For *T. protumnus*, however, the Mantel test detected a strong correlation between the genetic and geographical distance (p=0.15): 11.5% of the genetic structure can be ascribed to a geographical pattern of population establishment.

Discussion

Chrysoritis chrysaor and *Thestor protumnus* live in similar habitats and are relatively closely related, but they differ in at least one essential way: one is herbivorous whereas the other is aphytophagous. Despite their similarities, these two butterfly species exhibit major genetic differences in genetic structure, as reflected by the histories of both mitochondrial (COI) and nuclear (ITS2) markers. The genetics of *C. chrysaor* show significant variation both within and between localities (Figures 2.1 and 2.2), which could make them more robust to environmental perturbations. Conversely, *T. protumnus*, have almost no variation within populations (Figures 2.1

and 2.2). The lack of genetic variation within populations of *T. protumnus* could make them more susceptible to local extinction.

The genetic differences seen between *C. chrysaor* and *T. protumnus* could result from varying climatic and geological conditions encountered during the evolutionary history of each species. But if two or more species emerge at a similar point in time, evolve on a similar time scale and in the same geographical area, it seems more likely that the differences we observe are due to other factors such as life history, and not specific geological events such as mountain formation that they may have encountered during the course of their evolution. Most of the extant diversity observed in both *C. chrysaor* and *T. protumnus* appears to have arisen approximately 5-7 million years ago. We can therefore expect any differences in population genetic signatures to be driven by non-climatic and non-geological factors.

Chrysoritis chrysaor and *T. protumnus* differ not only in feeding habit, but also in the specificity of their diets. *Chrysoritis chrysaor* is a generalist and while *T. protumnus* is a specialist. They are not sister taxa, so we cannot easily take phylogenetic signal into effect. However, no other pair of species of South African Lycaenidae have these differences while still having relatively similar, overlapping, widespread ranges and a shift in diet (Woodhall 2005, Williams 2011). In South Africa as in other parts of the world, most widespread herbivorous lycaenids are generalists, and most parasitic lycaenids have small ranges (Woodhall 2005, Williams 2011). Future work replicating this study across more taxa and across diverse geographic ranges will help determine which aspect of the life histories of parasitic butterflies has the strongest impact on their ability to survive. Here we discuss how all

of these factors (being aphytophagous, parasitic, and/or specializing), individually and together, may contribute to making them more vulnerable to extinction than their herbivorous counterparts.

High estimates of gene flow and a non-significant Mantel test for isolation by distance in *C. chrysaor* suggest that they are effective dispersers compared to *T. protumnus*. *Chrysoritis chrysaor* have higher gene flow between populations than would be expected if they were severely hampered by geographical separation or barriers (Hartl and Clark 1989, Frankham *et al.* 2002); thus, they basically form two large populations within all of South Africa. In contrast, *T. protumnus* have high fixation indices and 11.5% of their genetic signature can be ascribed solely to a geographical pattern of population establishment, which implies extremely low levels of gene flow and little migration between populations. Thus *T. protumnus* either cannot disperse well, or cannot get established once they disperse and each population is highly isolated from all the others.

Host-specificity could account for the lack of within population genetic variation we see in *T. protumnus*. Species with a higher level of host-specificity are expected to have higher levels of species turnover because they usually have narrower geographical distributions than their generalist counterparts (Strong *et al.* 1984, Ødegaard 2006, Bell *et al.* 2013). Finding suitable ant nests is presumably more challenging than finding host plants, making dispersal difficult for *T. protumnus.* Aphytophagous caterpillars use chemical camouflage to escape detection by ants (e.g. Lohman *et al.* 2006 and Akino *et al.* 1999). Cuticular hydrocarbon signatures of *Thestor* caterpillars are likely to closely resemble the cuticular hydrocarbon signatures

of the ant brood in the *Anoplolepsis* ant nests, and this may restrict the range of ant nests that they can parasitize. Interestingly, suitable *Anoplolepsis* host ant nests are considerably more common in South Africa than are localities where *Thestor* species can be found, implying that there must be additional, as yet unknown constraints on which nests they can invade.

The larger the effective population size of a species, the more stable that species is and the more resistant to local environmental disturbances. *Chrysoritis chrysaor* have effective population sizes orders of magnitude larger than those of *T. protumnus*. The same haplotypes of *C. chrysaor* can occasionally be found in multiple localities, meaning that they disperse throughout their range. Thus if one population is decimated, not much genetic diversity is lost and the species as a whole can persist. In contrast, effective population sizes for *T. protumnus* are small (*N*e close to 1 per population). Small effective population sizes and small, disjointed distributions make them vulnerable to stochastic local extinction events. If all individuals in a population are closely related, they are more susceptible to all succumbing from one disease. Furthermore, if there is a natural disaster, such as a fire during the butterflies' mating season, a whole population could be wiped out, and with it a whole segment of genetic diversity for the species. Thus, small effective population sizes and the poor dispersal ability could explain why aphytophagous butterflies go extinct with a higher frequency than phytophagous butterflies.

The population size differences we observe between *C. chrysaor* and *T. protumnus* could be a consequence of food preference (phytophagy/aphytophagy), or a consequence of the diversity of their diets, or both. *Chrysoritis chrysaor* are

generalists, but phytophagous butterfly species overall can be either generalists or specialists. *Thestor protumnus* are specialists, and all the aphytophagous species of lycaenids studied so far are specialists that depend on resources that rare and not easy to locate or use. Specialists, as a general rule, have smaller effective population sizes than generalists and are more susceptible to extinction risk because they are more likely to depend on resources that are not easy to find or utilize (Kelley *et al.* 2000). Indirectly, aphytophagy may be responsible for leading to extinction because it leads to extreme specialization.

Effective population sizes could also be explained in part by the trophic (ecological) pyramid (Weigmann *et al.* 1993). *Chrysoritis chrysaor* are primary consumers and biomass is usually greater at the bottom of the trophic pyramid, so they have a higher quantity of resources available to them. By feeding on ants, some of which are themselves predaceous, *T. protumnus* are higher up the food chain. It's harder to be sustained by the same environment the higher up the trophic pyramid one is because energetic requirements are greater in apex predators given the energy loss at each level of consumption.

While higher trophic level does not imply specialization, *T. protumnus* can be considered parasitic. Parasites are more likely to be specialized because they need to accumulate adaptations to a host's defense system, to exploit a specific resource and/or to be able to live in a special habitat (Futuyma and Moreno 1988, Thompson 1994, Pierce 1995). This specialization in turn leads to smaller effective population sizes and a more fragile existence.

An unexpected result of this study places *C. midas* within *C. chrysaor*, making the latter paraphyletic (Figure 2.1). *Chrysoritis midas* may therefore be a high-altitude form of *C. chrysaor*, rather than a separate species. Morphology presents further evidence that support the hypothesis that they might be the same species. The primary morphological characteristic that separates *C. midas* from *C. chrysaor* is their darker coloring. However, increased wing melanization within a species is often associated with increasing altitude (Karl *et al.* 2009). We leave the taxonomy of *C. chrysaor* and *C. midas* for future studies. However, because of the possibility that *C. chrysaor* and *C. midas* may be the same species, we performed all analyses twice: Once using only specimens described as *C. chrysaor* and once including *C. midas*. Results for both analyses showed the same trends when compared to *T. protumnus*.

Conclusion

This study explored the population genetics and phylogeography of two South African species of butterflies, the herbivorous *Chrysoritis chrysaor* and the aphytophagous *Thestor protumnus*. Our analysis found striking differences between the genetics of these two species. *Thestor protumnus* have significantly lower levels of within population variation (COI *F*st=0.98) than *C. chrysaor* (COI *F*st=0.72). Also, *T. protumnus* have effective population sizes orders of magnitude smaller than *C. chrysaor*. The low genetic variation within localities and the small effective population sizes could be due to poor dispersal ability, high trophic position, parasitic lifestyle, and/or extreme specialization, all of which are likely to be directly related to

their diet, and may be responsible for the elevated risks of extinction observed in aphytophagous lycaenid butterflies.

CHAPTER 3

Phylogeny and life history evolution of butterflies in the genus *Thestor* (Lepidoptera, Miletinae)

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Abstract

Gause's Law of Competitive Exclusion suggests that species cannot coexist at constant populations sizes if they are all competing for the same resource, provided other ecological factors remain constant. This is the same idea that underlies the theory of the ecological niche (Hutchinson 1959). However, the genus *Thestor* (Lepdoptera: Lycaenidae), comprised of 27 recognized species, all of which are endemic to southern Africa, appears to contradict ecological theory. Almost all species of *Thestor* are believed to be parasitic, feeding on homopterans found in ant nests and/or the brood, regurgitations, and/or workers of the single ant species *Anoplolepis custodiens*. In this study, we use multiple lines of evidence to investigate whether the different species of *Thestor* are actually associated with different cryptic species within *Anoplolepis*, or whether all 27 butterfly species parasitize the nests of a single species of ant. We also test for different modes of niche partitioning between species, such as geographical separation and partitioning by flight time. It appears that all 24 of the species in the Western Cape utilize the same ant, while *T. protumnus* and *T. dryburghi* (the two species that are found in the north-western part of South Africa) use a closely related, but different species of *Anoplolepis* and *T. basutus* (the species found in the eastern part of South Africa) utilizes yet a third ant. This suggests that factors driving the diversity in the genus *Thestor* might have been ants and/or

geography when the genus originated, but that other forces may be responsible for maintaining the more recent diversity in the group. We also found that flight time may have driven the separation of the yellow and black groups of *Thestor:* The "black" group *Thestor* fly predominantly in the summer months, while the "yellow group" fly predominantly in the spring. And while species spread across the phylogeny fly in the spring and summer months, only members of the yellow group fly during the winter and fall months. Our findings show little evidence of niche partitioning, especially within the species of *Thestor* whose distributions are confined to the Western Cape, and demonstrate an extreme example of the coexistence of 24 species on a remarkably uniform resource.

Introduction

Carnivory is extremely rare in the butterflies and moths (Lepidoptera) compared with other insect orders that undergo complete metamorphosis such as flies (Diptera) and beetles (Coleoptera). Preliminary evidence suggests that even though aphytophagy (including carnivory) has evolved many times in the Lepidoptera, it has not persisted (Pierce 1995). Within the Lycaenidae, larval association with ants appears to have led in multiple instances to carnivorous lifestyles in which larvae are carried by ants into the nest where they feed, undetected, by adults, on the helpless ant brood.

The South African butterfly genus *Thestor* is a group of 27 taxa in the lycaenid subfamily Miletinae. This subfamily is remarkable in that the larvae of all the species whose life histories are known are aphytophagous, with food sources including ants and homopterans such as scale insects, including their regurgitations or sugary

secretions (Cottrell 1984; Kaliszewska *et al.* 2015). The habits of all species in the genus *Thestor* are even more unique because they reportedly parasitize only one species of ant, *Anoplolepis custodiens* (Cottrell 1984). Despite their fascinating life histories, surprisingly little is known about the biology of these insects. Details of their mating and oviposition behavior, ant adoption interactions, and the specifics of their feeding habits remain largely unstudied.

What we do know about the genus *Thestor* (Hubner 1819) (Lycaenidae: Miletinae) is that it is endemic to southern Africa, with all but one species confined to South Africa (Clark and Dickson 1971; Claassens and Dickson 1980), and in particular, the Western, Eastern and Northern Cape Provinces (Pringle *et al.* 1994). Species of *Thestor* occur in a variety of habitats and altitudes, ranging from altitudes as high as 1000 m (*T. penningtoni*) to as low as sea-level (*Thestor dicksoni malagas*) (Pringle *et al.* 1994). Females oviposit on almost any substrate (Heath and Claassens 2000), most often close to nests of *Anoplolepis custodiens* (Pringle *et al.* 1994). Adults commonly rest on the ground or on rocks. Their flight is always close to the ground, and they are seldom seen on flowers. Their mouthparts are atrophied, and thus they are unable to drink nectar. Populations are sometimes confined to areas of little more than two hundred square meters, and often densely populated (Heath and Pringle 2004). The first three caterpillar instars of *T. protumnus* and *T. basutus* can feed on Homoptera (Clark and Dickson 1960, 1971; Williams and Joannou 1996). The final two instars of *T. yildizae* and *T. pictus* live within the ant nest and feed on ant regurgitations (trophallaxis) (Heath and Claassens 2000, 2003; Heath and Pringle 2004). The final instar of *T. basutus* feeds by trophallaxis, but supplements its diet

with ant eggs and probably ant detritus (Heath and Claassens 2003; Heath and Pringle 2004). At least seven species are known to pupate in *Anoplolepis* ant nests, and no exceptions to this have been recorded (Heath and Pringle 2004).

By contrast, a great deal of research on lycaenid caterpillars feeding in ant nests has focused on *Phengaris* (*Maculinea*) a genus in the subfamily Polyommatinae. Each of the 5 European species of *Phengaris*, as well as the Japanese representative, *P. arionides*, originally appeared to have a specialized predatory association with a different species of *Mymica* ant (Thomas *et al.* 1989). An exception to this pattern is *Maculinea alcon*, a European species that has been shown to feed in the nests of three different species of *Myrmica* in separate geographic regions (Elmes *et al.* 1994). The host specificity of each *Phengaris* species has been well documented, and feeding specializations have been discovered. For example, two species of *Phengaris* solicit regurgitations from adult ants (trophallaxis) instead of feeding on brood (Thomas and Elmes 1998).

These studies of host specificity in *Phengaris* provide an interesting point of comparison for *Thestor*. In *Phengaris*, each species of butterfly has specialized on a different ant species or group of species, which represent slightly different ecological niches. As of yet, there is no comparable niche diversity that would account for the radiation of *Thestor* in the same geographic regions where its species occur.

In this study, we determine whether the different species of *Thestor* are actually associated with different cryptic species within *Anoplolepis custodiens*, or whether all 27 butterfly species parasitize the nests of a single species of ant. If so, even if evidence for niche partitioning within these colonies is found, it would

nevertheless represent an extreme case of the coexistence of 27 species on a remarkably uniform resource. Carnivory in the Lycaenidae seems to represent a rare evolutionary experiment, and a better understanding of this representative system will provide the basis for further studies designed to uncover how aphytophagous butterflies evolved, and why they have not persisted over evolutionary time.

Materials and methods

Specimen collection and taxon sampling

Butterfly specimens were collected into 90-100% ethanol and stored at -20 °C or -80 °C prior to DNA extraction. Wings were removed from the specimens while still in the field and kept separately in glassine envelopes. Ant specimens collected after 2007 were prepared in two ways: ants for one sample were collected into 90-100% ethanol for genetic studies, and workers for another were collected into vials half filled with dryerite that was plugged with a piece of kimwipe (to keep dryerite powder from coating the specimens) for stable isotope studies. Prior to 2007, all ants were collected directly into ethanol, in which case the ant specimens that were collected for genetic studies were also used for stable isotope studies. All specimens and their genomic DNA are deposited in the DNA and Tissues Collection of the Museum of Comparative Zoology at Harvard University in Cambridge, Massachusetts.

The specimens used in this study include one specimen per species of *Thestor* as designated by Heath and Pringle in 2004 for the six gene dataset, at least two specimens per species of *Thestor* for the Rad-seq dataset, and all available specimens of *Thestor* from as many populations as possible for the COI dataset. Wherever

possible, each species was sampled from multiple locations, although this was not feasible for all species of *Thestor* because some are known from a single locality. Outgroup samples for the six-gene dataset and for the COI dataset included four species from the genus *Lachnocnema*, which is the sister group to *Thestor* (Heath and Pringle 2004; Kaliszewska *et al.* 2015) plus a sampling of other Miletinae species: *Feniseca tarquinius*, *Logania malayica*, *Allotinus horsfeldi*, and *Miletus gopra*. The Rad-seq dataset was rooted based on information from the six-gene dataset.

DNA extraction

For butterflies, genomic DNA was extracted from three legs or a small piece of abdominal tissue using a DNeasy Tissue Kit (Qiagen Inc., qiagen.com) or using the AutoGenprep 965 Tissue DNA Extraction Kit (Autogen). For ants, genomic DNA was extracted from a single, whole ant per sample.

For some specimens (of both butterflies and of ants), the resulting DNA extract contained less than 7.5 ng/µL of genomic DNA: if these specimens were meant for Rad-Seq, the DNA concentration was increased by whole genome amplification using a Repli-G Mini Kit, in 20 µL reactions (Qiagen).

Sample preparation for isotopic analysis

The tissue samples of butterfly wings and ant heads and thoraxes were used for $\delta^{15}N$ and $\delta^{13}C$ isotopic analysis. All samples were oven-dried at 60°C for 24 to 48 hours once they were brought to the lab. Approximately 0.5 mg of tissue were weighed into tin boats (Bol and Pflieger 2002) combusted on a Thermo Finnigan Delta Plus XP Stable Light Isotope Ratio Mass Spectrometer (Thermo Finnigan) coupled to

a Thermo Flash EA 1112 Elemental Analyzer via a Conflo III Device. One standard of each of Choc, Mg and Val was run between every fifteen test samples.

The analytical precision (S.D.) was ± 0.2‰, as estimated from five standards analysed along with the samples. The internal working standards are related to the international standards by direct calibration with the international standards and by inter-laboratory comparison. Isotope ratios are expressed in parts per mil (‰) using the δ notation, derived from the equation: $\delta R = (R_{sample}/R_{standard} - 1) * 1000$, where R = the isotope ratio of element R ($^{x}R/^{y}R$). By convention, the δ values are measured with reference to the international standards as distributed by the IAEA of Vienna PeeDee Belemnite marine limestone (VPDB) for carbon isotopes and atmospheric N_2 (NAIR) for nitrogen isotopes.

Molecular protocols for Sanger Sequencing

Six markers comprising 4,305 bp were amplified for 52 *Thestor* and outgroups using complementary primer pairs (Table 3.1): mitochondrial *cytochrome c oxidase* subunit I (1,220 bp, COI); and five nuclear, protein-coding markers: e*longation factor* 1-alpha (1,065 bp, EF1alpha); *wingless* (369 bp, wg); *histone H3* (328 bp, H3); *carbamoyl-phosphate synthetase* 2*, aspartate transcarbamylase, and dihydroorotase* (726 bp, CAD); and *glyceraldehyde-3-phosphate dehydrogenase* (597 bp, G3PD). Only the standard 'barcode' region of mitochondrial COI (1,220 bp) was amplified for an additional 41 *Thestor* specimens (for a total of 93 COI sequences).

Table 3.1 Primers used in this study.

Locus	Direction	Sequence	Reference
COI			
LCO1490	F	GGTCAACAAATCATAAAGATATTGG	Folmer *et al.* 1994
Nancy	R	CCCGGTAAAATTAAAATATAAACTTC	Simon *et al.* 1994
TN2126	F	TTGAYCCTGCAGGTGGWGGAG	Eastwood, unpublished
Tonya	F	GAAGTTTATATTTTAATTTTACCGGG	Monteiro *et al.* 2001
Hobbes	R	AAATGTTGNGGRAAAATGTTA	Monteiro and Pierce 2001
WG			
WG1	F	GARTGYAARTGYCAYGGYATGTCTGG	Brower and DeSalle 1998
WG2E	R	ACNACGAACATGGTCTGCGT	Kaliszewska *et al.* 2015
EF			
EFM44F	F	GCYGARCGYGARCGTGGTATYAC	Cho *et al.* 1995
EF46.1F	F	GAGGAAATYAARAAGGAAG	Cho *et al.* 1995
EF51.1	R	CATGTTGTCKCCGTGCCATCC	Cho *et al.* 1995
EF51.9F	F	CARGACGTATACAAAATCGG	Cho *et al.* 1995
EFC52.6R	R	GCYTCGTGGTGCATYTCSAC	Cho *et al.* 1995
EFCM4R	R	ACAGCVACKGTYTGYCTCATRTC	Cho *et al.* 1995
H3			
H3F	F	ATGGCTCGTACCAAGCAGACACGGC	Colgan *et al.* 1998
H3R	R	ATATCCTTRGGCATRATRGTGAC	Colgan *et al.* 1998
G3PD			
G3Fa	F	TGGGGYAAGGCTGGAGCTGAATA	Kaliszewska *et al*. 2015
G3Ra	R	CCAGCCGCAGCATCAAAGA	Kaliszewska *et al*. 2015

Polymerase chain reaction (PCR) amplification was conducted using a Bio-Rad DNA Engine Dyad Peltier thermal cycler. All reactions were prepared in 25µL volume reactions with 16.65 L ultra pure water, 1L 25mM $MgCl_2$, 2.5L 10X PCR buffer, 1L 10mg/mL bovine serum albumin, 0.25L 100mM dNTPs, 0.2L 5U/L Taq polymerase (Qiagen Inc., qiagen.com) and 1.2L of each primer (10mM). The reactions were run with a touchdown cycling profile. Typical reaction conditions were: 2 min at 94° C followed by 20 cycles of 50 s at 94° C, 40 s at 48° C (decreasing by 0.5° C per cycle) and 80 s at 70° C followed by 20 similar cycles with the annealing temperature constant at 50° C and ending with a final annealing step of 73° C for 5

min. The only exception was Histone 3, in which the third phase of each cycle (the extension phase) was decreased to 60 s. PCR products were purified by incubating samples at 37° C for 35 minutes with *Escherichia coli* enzyme Exonuclease I and Antarctic Phosphatase (EXO-AP), and subsequently raising the temperature to 80° C for 20 minutes to deactivate the enzymes. Cycle-sequencing was done using BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kits, and sequencing was done on Applied Biosystems 3100 or 3470 automated sequencers. The resulting chromatograms were assembled and edited in Sequencher 4.2 (Gene Codes Corp., genecodes.com). All genes were aligned using MAFFT 5 (Katoh *et al.* 2005) and concatenated with MacClade 4.06 (Maddison and Maddison 2003).

Molecular protocols for Rad-seq

DNA markers were selected, amplified, and sequenced using the double-digest Restriction-site Associated DNA sequencing (RADseq) protocol of Peterson *et al.* (2012), modifying their Bench Protocol as follows: We began with 150 ng of genomic DNA; DNA was quantified fluorimetrically throughout the protocol using a Quant-iT dsDNA High Sensitivity Assay Kit (Life Technologies). We digested gDNA with the enzymes Eco-RI-HF (P1-end restriction enzyme) and BfaI (P2-end; both enzymes from New England Biolabs). For magnetic bead cleanups throughout the Peterson *et al.* protocol, we used the MagNA beads described in Rohland and Reich (2012), using a ratio of 1.5 bead volume:original reaction volume. Illumina adapters were ligated to 25-75 ng of digested DNA using 100 Units of T4 DNA ligase (New England Biolabs), with 10-30 nM concentration of each Illumina adapter in each reaction. Ligated DNA fragments between 264-336 bp were selected using a 2% agarose gel cassette on the

Pippin Prep (Sage Science). Size-selected samples were amplified by 10 rounds of PCR using Phusion High Fidelity DNA Polymerase (New England Biolabs). Resulting libraries were then sequenced on an Illumina HiSeq 2000, producing single-read, 100bp libraries.

Bioinformatics

We used the process_radtags program from the Stacks package (Catchen *et al*. 2011, 2013) to demultiplex the Illumina reads. We then trimmed the restriction site, as well as one more additional, often-low-quality base, from the beginning of each read using the fastx_trimmer tool from the fastx toolkit (Pearson *et al*. 1997). We then used the fastx_filter tool from the same toolkit to quality filter any read that did not have 98%+ of bases with a quality score of 25 or more. 14 low-coverage individuals were removed prior to further analyses.

Demultiplexing left a total of 45 million reads, each 89 bp long, with an average of 517,000 reads per individual. We then used the de_novo.pl pipeline of Stacks to group these reads into homologous loci across all individuals. Finally, we used the Stacks' populations program to output a list of sites that were fixed within but variable among individuals. All loci that included one or more of these potentially phylogenetically informative sites were included in our analysis, totaling 2099.

Phylogenetic analyses for Sanger Sequencing

Phylogenetic analyses for the six-gene dataset were performed using Bayesian inference and maximum likelihood-based methods. Bayesian phylogenetic analyses for the six-gene data set were done with BEAST. For the analysis the uncorrelated

relaxed clock (Drummond *et al.* 2006) and a constant population size under a coalescent model were set as priors. Two independent chains were run for 50 million generation each, sampling values every 5000 steps. A burn-in of 500,000 generations was applied after checking MCMC convergence in Tracer ver. 1.5 (Rambaut and Drummond 2007).

Bayesian phylogenetic analyses were also performed with MrBayes 3.2 (Ronquist and Huelsenbeck 2003). The data were partitioned by gene, using the GTR+I+G model for each gene. The substitution rates, character state frequencies, gamma shape parameters, and proportions of invariant sites were unlinked among each of the six partitions. Each analysis of 10 million generations consisted of two independent runs of four chains each with the heating temperature (temp) constrained to 0.2. Trees were sampled every 100 generations, resulting in 100,001 trees. The first 50,000 trees were discarded before a majority rule consensus tree and posterior probability branch support values were calculated from the remaining trees.

Maximum likelihood trees were inferred for individual genes and the full data set using GARLI 0.951 (Zwickl 2006). The GTR+I+G model of sequence evolution was selected by Modeltest 3.7 (Posada and Crandall 1998) for each gene and the concatenated dataset using the Akaike Information Criterion (AIC). Confidence in the most likely tree based on all genes was assessed with 1,000 bootstrap replicates performed in GARLI. Each replicate automatically terminated after the search algorithm progressed 10,000 generations without improving the tree topology by a log likelihood of 0.01 or better. A majority-rule consensus tree was calculated with PAUP* 4.0b10 (Swofford 2002).

COI uncorrected pairwise *p*-distances were calculated between all ingroup samples to compare genetic distances within and among species using PAUP*, and frequency distributions of *p*-distances between congeneric species were plotted along with the distribution of *p*-distances between species from different genera.

Phylogenetic analysis for Rad-Seq

Phylogenetic analyses for the Rad-seq data were performed using maximum likelihood and Bayesian inference-based methods. Maximum likelihood analysis was performed using RAxML version 7.7.5 (Stamatakis, 2006, Stamatakis *et al.*, 2008). The data were partitioned, with each 89 bp locus given its own partition. RAxML determined the tree with the highest likelihood using a GTR+I+G model of rate heterogeneity and an estimated proportion of invariant sites, and performed a rapid bootstrap analysis with 100 bootstraps.

Bayesian inference was performed using MrBayes version 3.2 (Ronquist and Huelsenbeck 2003). Molecular data were partitioned as in the maximum likelihood analysis. We used two runs, each with 4 chains, and ran the analysis for 25 million generations, sampling every 10,000 generations. Likelihoods were viewed using Tracer version 1.5.0 (Rambaut and Drummond 2007) and a burn-in set at 1 million generations before summarizing the sampled trees.

Statistical Analysis for Isotopic Analysis

Mean stable isotope values of $\delta^{15}N$ and $\delta^{13}C$ were calculated for all conspecific butterflies at a given locations and plotted along with the minimum and

maximum isotope values in R (R Core Team 2014). Beanplots were calculated for the $\delta^{15}N$ and $\delta^{13}C$ of the butterflies minus the $\delta^{15}N$ and $\delta^{13}C$ of the ants. They were created using the R package *beanplot* (Kampstra 2008). Beanplots are similar to boxplots, except that the underlying distribution is described using kernel density estimates instead of traditional descriptive, statistics such as IQR. The dotted gray line represents the grand median value and the 'bean lines' represent the median value of each 'bean'. Each value is illustrated by a line within the bean.

Results

Phylogenetic relationships of Thestor

Seventy-eight *Thestor* individuals were collected for this study. COI was sequenced for all 78 individuals plus 15 outgroup specimens. 70 specimens of *Thestor* were sequenced using the Rad-seq method, and 38 specimens of *Thestor* plus 14 outgroup specimens were sequenced for six genes each.

The Bayesian consensus trees, for both the 6-gene dataset and for the Rad-seq dataset provided well-supported and overall congruent phylogenetic hypotheses (Figures 3.1 and 3.2). The maximum likelihood trees (not shown) are very similar to the Bayesian consensus trees for both datasets. Most nodes are strongly supported; more than half of all nodes have Bayesian posterior probabilities of 1 and very few have posterior probabilities < 0.90. The relationship between *T. murrayi*, the *T. kaplani/T. compassbergae/T. camdeboo/T. pringlei* clade, and the black *Thestor* clade is unresolved in the 6 gene Bayesian consensus and is poorly supported in the Rad-seq Bayesian consensus. The COI Neighbor Joining tree (Figure 3.3) is not well

supported at the deeper nodes, but is consistent with the 6 gene and Rad-seq datasets.

The three main groups of *Thestor* (the *T. basutus/T. protumnus/T. dryburghi* group; the Yellow group (*T. braunsi, T. malagas, T. dicksoni, T. vansoni, T. pictus, T. rooibergensis, T. swanepoeli, T. rossouwi, T. strutti and T. montanus*) and the Black group (*T. murrayi, T. kaplani, T. compassbergae, T. camdeboo, T. pringlei, T. penningtoni, T. holmesi, T. stepheni, T. claassensi, T. overbergensis, T. rileyi, T. yildizae, T. barbatus, T. petra and T. brachycerous*) cluster together, but are not necessarily monophyletic. *T. basutus* is sister to all other *Thestor* according to the MrBayes Bayesian and to the COI Neighbor Joining phylogenetic hypotheses. Accoding to the BEAST Bayesian analysis it is sister to the *T. protumnus*/*T. dryburghi* clade, and according to the Maximum Likelihood analysis the relationship between them is unresolved. *T. protumnus* and *T. dryburghi* are monophyletic and sister to the Yellow and Black *Thestor*. The Black *Thestor* are each other's closest relatives and are monophyletic, but they render the Yellow *Thestor* paraphyletic. *T. murrayi* and the *T. kaplani/T. compassbergae/T. camdeboo/T. pringlei* clade have been classified as part of the Yellow group (Heath and Pringle 2004). Morphologically they are Yellow, but if phylogenetically they were classified as part of the black group (or as their own separate group), and if *T. basutus* is classified as part of its own group, then the Black group, the Yellow group and the *T. protumnus/T. dryburghi* group would each be monophyletic entities.

Most of the species delimitations corresponded to the taxonomic review of *Thestor* by Heath and Pringle in 2004. Comparison of inter- and intraspecies pairwise distances revealed that genetic distances between species are similar in magnitude, and

mostly do not overlap with distances between other species in different genera within *Thestor*. The main exception to this is *T. protumnus*. The pairwise distances between the two subspecies of *T. protumnus* (*T. protumnus protumnus* and *T. protumus aridus*) show greater pairwise distances than that found between species in two different species of *Thestor*. Two other examples are *T. dicksoni* and *T. swanepoeli,* where the genetic pairwise distances between species are greater within species than between the species. Intraspecific pairwise distances in *T. camdeboo* and *T. pringlei* were the same as interspecific distances between the two species for COI (the dataset where we had multiple individuals of each of the two species). These two species came out closely related in the multi-gene and Rad-seq phylogenetic hypothesis, and they were intermixed in the COI NJ tree. *T. kaplani* and *T. compassbergae* were recovered as closely related sister species in the Rad-seq Bayesian consensus, and *T. compassbergae* rendered *T. kaplani* paraphyletic in the COI NJ tree.

Thestor brachycerus is the only species whose placement conflicts significantly with relationships proposed by Heath and Pringle in 2004 (Figure 3.2a). If *T. brachycerus dukei* is excluded, then individuals of *T. brachycerus brachycerus* are monophyletic and come out sister to specimens of *T. overbergensis,* which are also monophyletic, and these relationships are consistent with the taxonomic designations proposed by Heath and Pringle. However, *T. brachycerus dukei* is separated into two clades: the *T. brachycerus dukei* from Stanford come out sister to *T. overbergensis*, but the *T. brachycerus dukei* from Rooiberg and Swartberg come out within the *T. petra* clade.

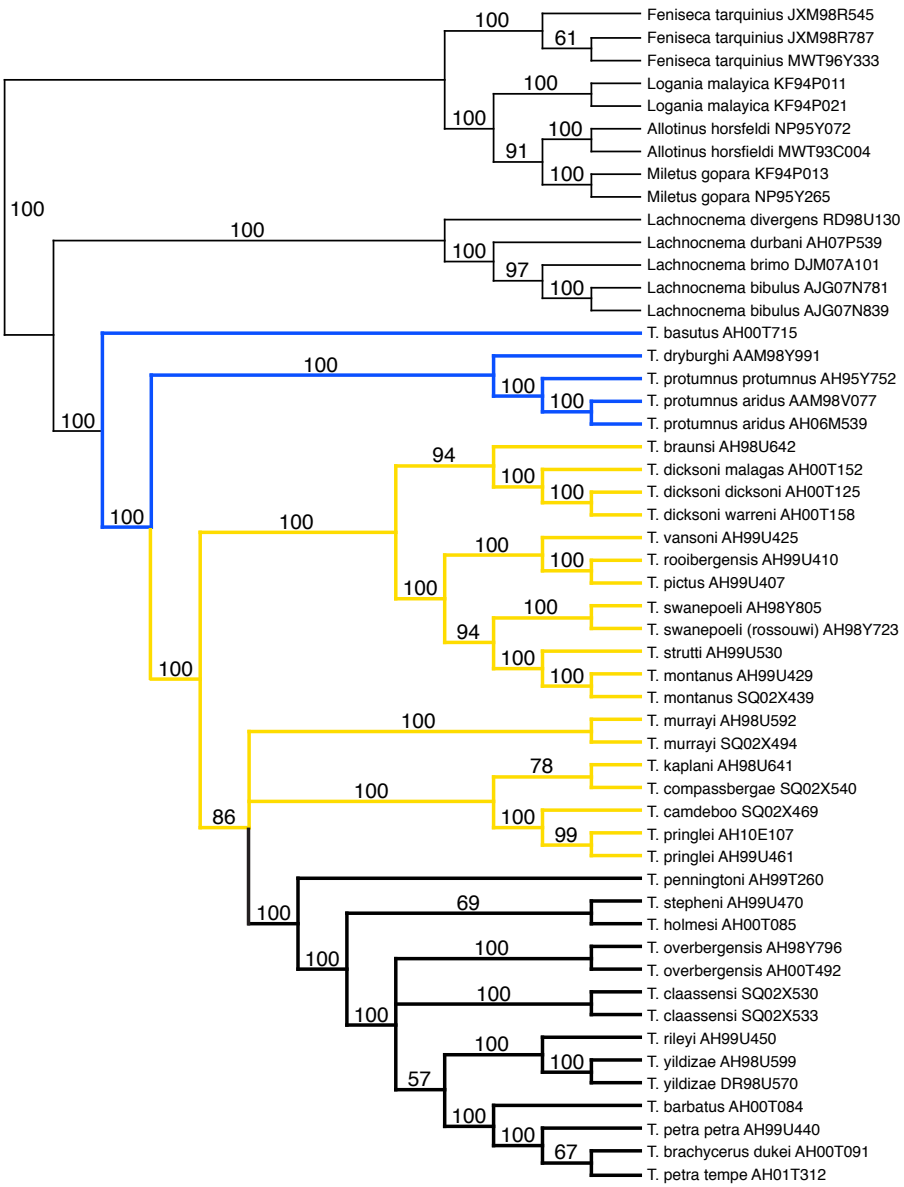

Figure 3.1 Phylogeny of the African genus *Thestor* (Lycaenidae: Miletinae): MrBayes Bayesian Consensus of the 6-gene dataset. Colors highlight the three main groups of *Thestor* as designated by Heath and Pringle in 2004.

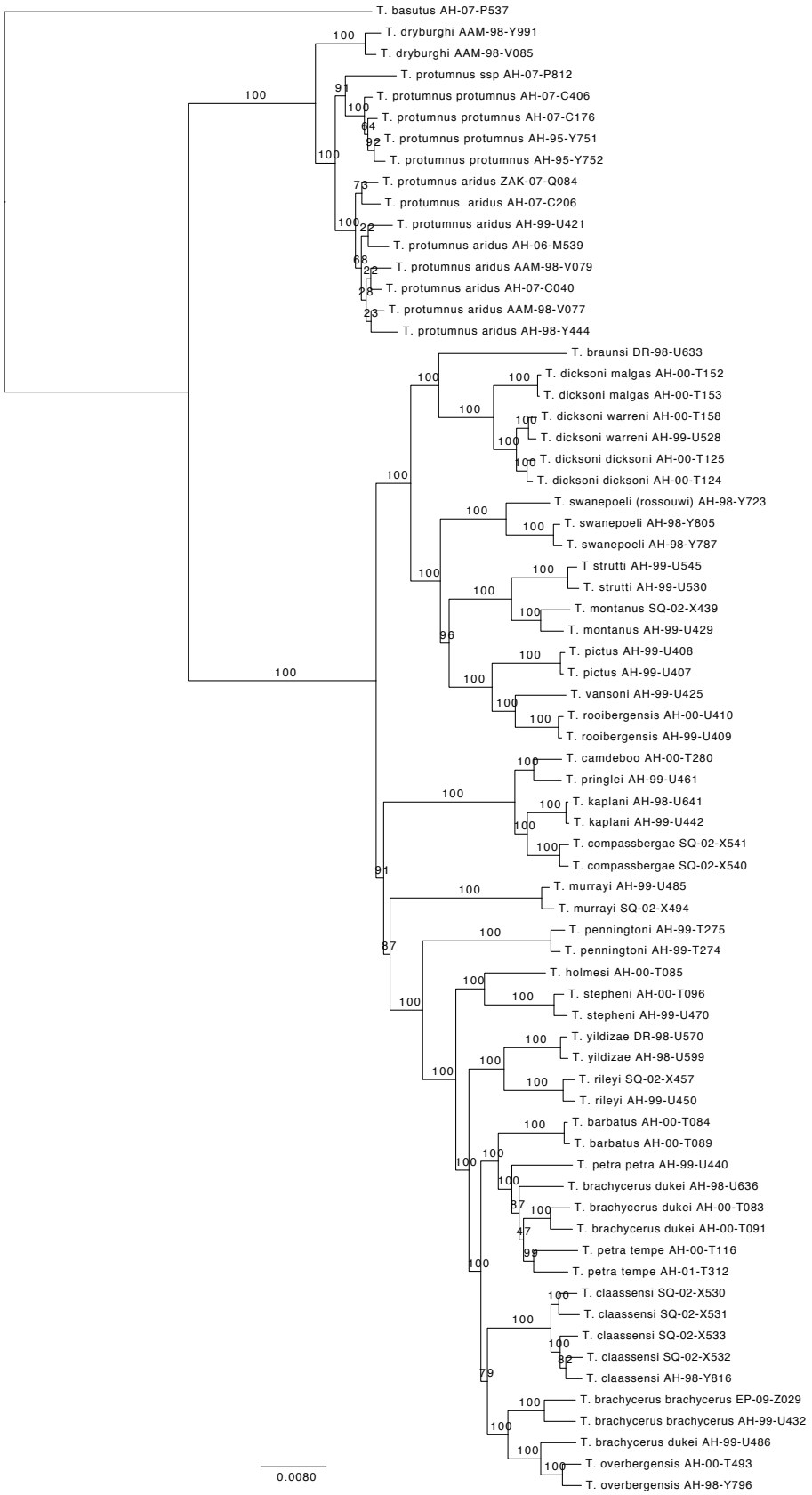

Figure 3.2 Phylogeny of the African genus *Thestor* (Lycaenidae: Miletinae): Bayesian Consensus of the Rad-seq dataset.

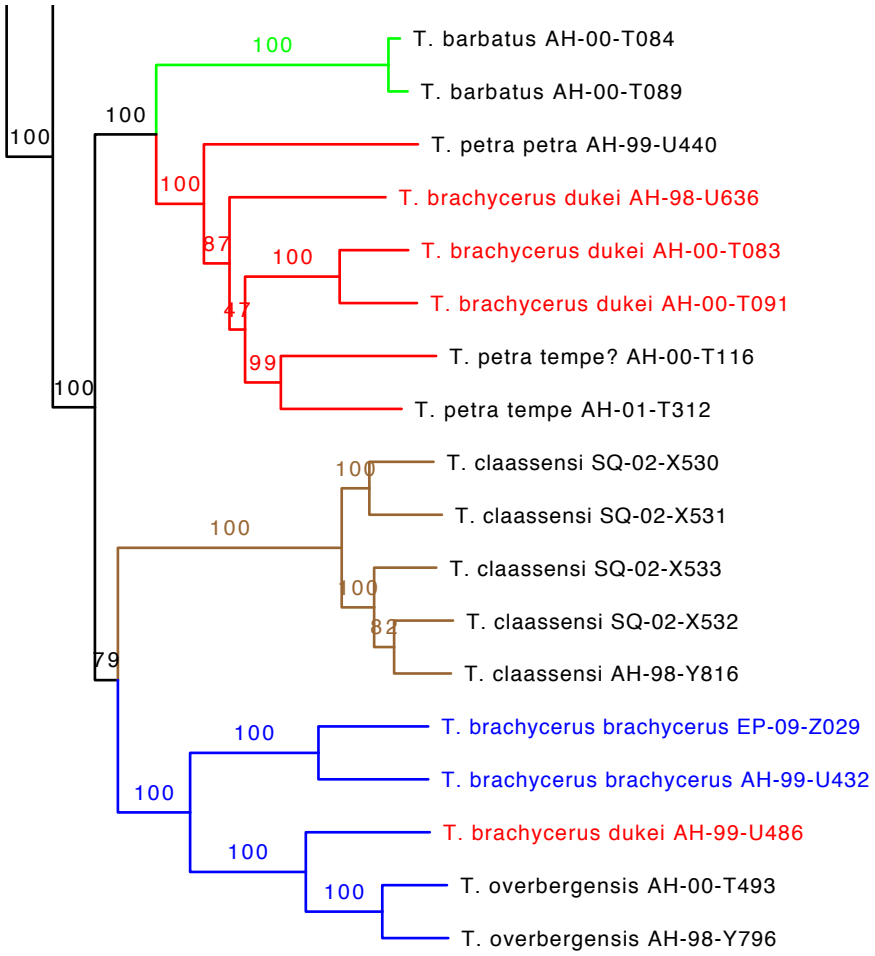

Figure 3.2a Partial highlight from the phylogeny of the African genus *Thestor* (Lycaenidae: Miletinae): The *Brachycerus* group is highlighted by color with localities labeled from the Bayesian Consensus of the Rad-seq dataset.

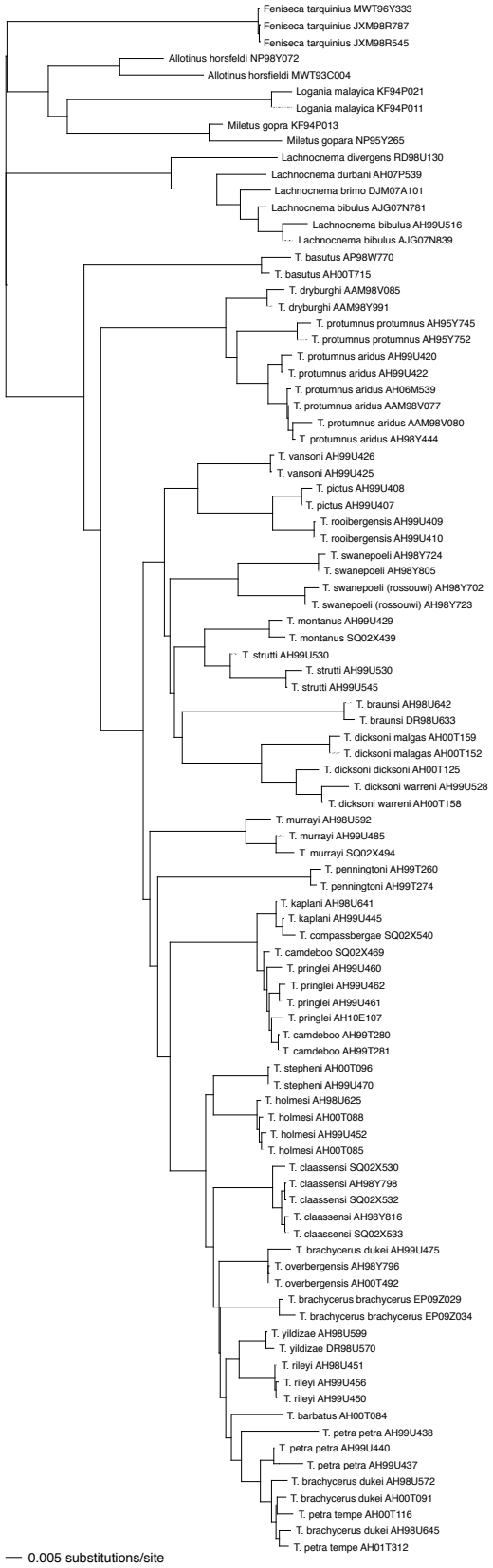

Figure 3.3 Phylogeny of the African genus *Thestor* (Lycaenidae: Miletinae): Neighbor-joining tree of the COI dataset.

Phylogenetic relationships of Anoplolepis

The Bayesian consensus tree for the Rad-seq dataset of *Anoplolepis* shows that nearly all the *Anoplolepis* ants used by *Thestor* are genetically very similar (Figure 3.4). We could not confirm these data with targeted genes because there are pseudogenes in the *Anoplolepis* mitochondrial genome. Even when we cloned them out, we found multiple copies and could not assemble them to give phylogenetic signal. When we sequenced multiple nuclear genes for a subset of the ants, they all appeared to be identical. Comparing them to the Rad-Seq consensus, it is clear that they came out this way because they are, indeed, extremely similar overall.

In the Bayesian consensus for *Anoplolepis,* only three individuals differed significantly from the rest. These were from ants that corresponded to the *Thestor* species *T. basutus*, *T. protumnus* and *T. dryburghi*. The *T. protumnus* and *T. dryburghi* ants came out sister to each other and closely related, just like their corresponding butterfly species. The split in the ants corresponds to the phylogenetic split in the butterflies. *Thestor basutus*, *T. protumnus* and *T. dryburghi* form the most ancient split from the rest of the genus. The split also corresponds to the geographic ranges of the butterflies. *Thestor basutus* is the only species that has a range along the Eastern Coast of South Africa. *T. protumnus* has a wide range through out Western and Central South Africa. The range of *T. dryburghi* is confined within the range of *T. protumus*, but is much smaller and localized in the north-west of the country. All the other *Thestor* species are confined to the Western Cape.

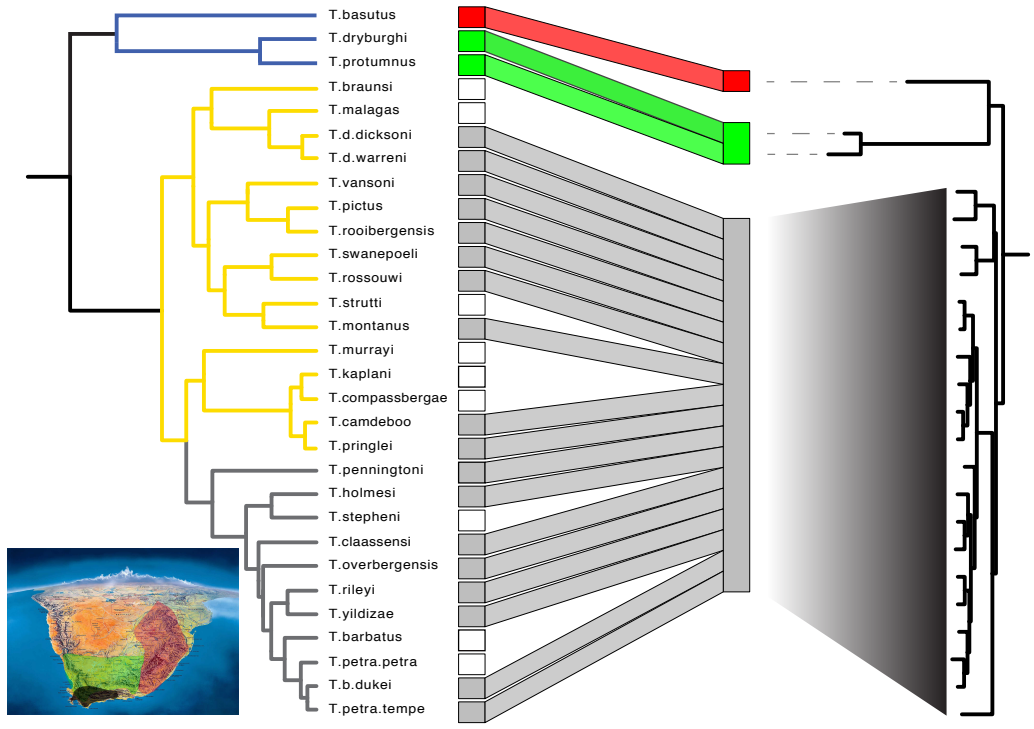

Figure 3.4 The evolutionary history of the African genus *Thestor* (Lycaenidae: Miletinae) and the *Anoplolepis* ants (Formicidae: Formicinae) that it parasitizes: 6-gene BEAST Bayesian Consensus of *Thestor* on the left, and Rad-seq Bayesian Consensus of the *Anoplolepis* on the right, compared with a bipartite plot. The map and bipartite plot are colored by locality.

Stable Isotopic Evidence

Species higher up the food chain tend to have higher $\delta^{15}N$ values (going up approximately 2-3 ‰ per trophic level). *Thestor* butterflies have, on average, 2.5 ‰ higher $\delta^{15}N$ values than do their corresponding ants (Figure 3.5). The largest difference is between *T. basutus* and its ant. The $\delta^{15}N$ difference between the two is 4.6 ‰.

$\Delta^{15}N$ signatures differ by trophic level, but they are also affected by geography. Factors such as aridity and altitude can affect $\delta^{15}N$. While there is geographic variation among $\delta^{15}N$ signatures for *Thestor*, the majority of populations/ species have similar $\delta^{15}N$ values (just over 5 ‰) and little variation per locality (Figure 3.5). The taxa that differ from this pattern are *T. protumnus*, *T. dryburghi* and *T. dicksoni malagas*.

$\Delta^{13}C$ isotopic signatures tend to reflect an organism's diet. $\delta^{13}C$ for both the butterflies and the ants was around -24 ‰ (Figure 3.5). The ant values were slightly higher than the butterfly values (on average 0.7 ‰ higher). The main exception to this was *T. basutus* and its ants, with signatures closer to -15 ‰.

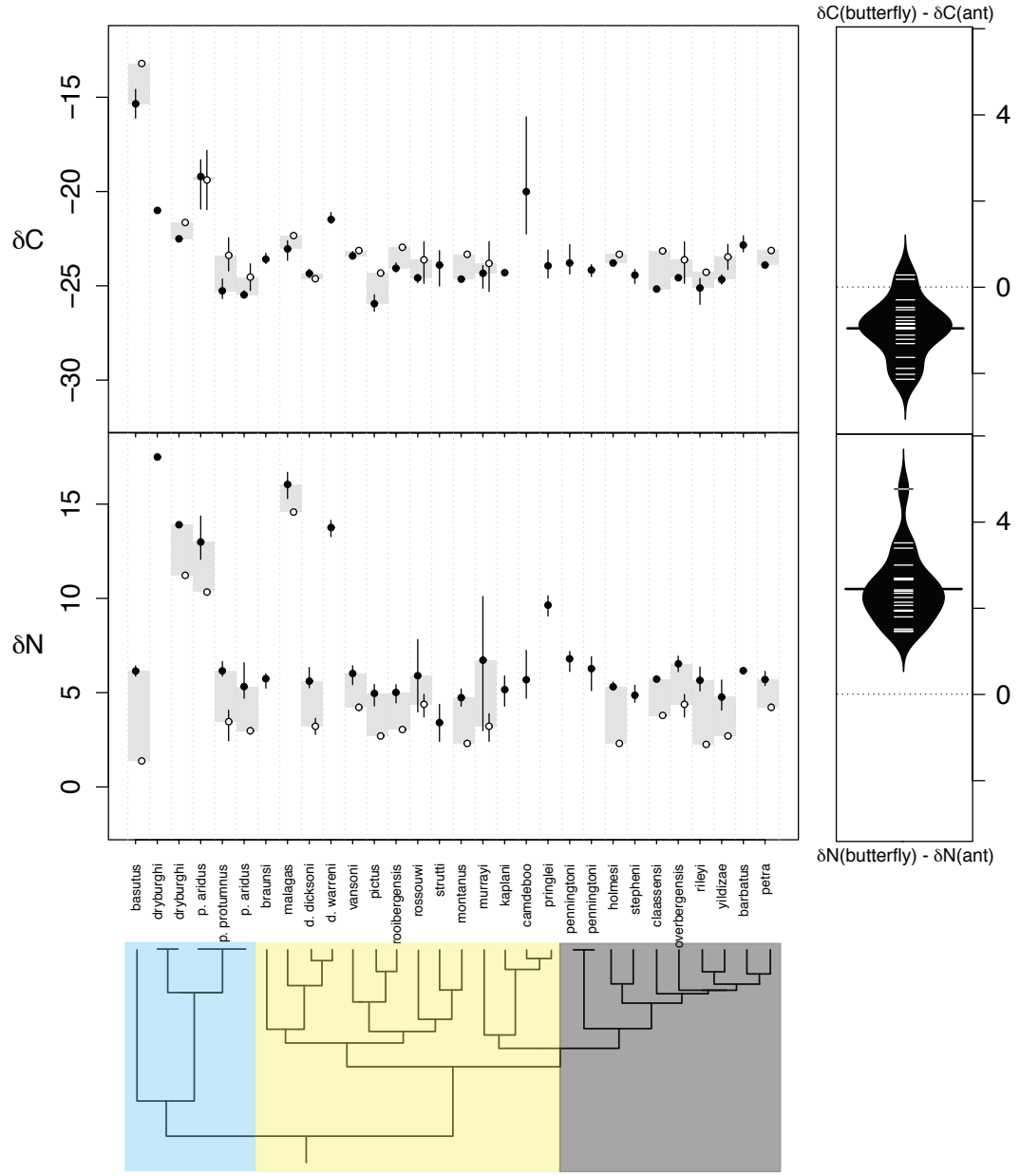

Figure 3.5 Comparison of Nitrogen and Carbon stable isotopes of species of the African genus *Thestor* (Lycaenidae: Mileatinae) and the *Anoplolepis* ants (Formicidae: Formicinae) that the caterpillars parasitize. Hollow Open dots represent average butterfly signature; solid dots represent average ant signature; grey blocks represent difference between butterfly and ant average signatures.

Flight Times

Thestor butterflies spend most of their lifecycle as caterpillars, when they are thought to live in ants' nests. Each individual lives as an adult for up to three weeks. Adult butterflies of species in the genus *Thestor* fly year round. One species, *T. strutti,* flies in late winter. But the average species/population has a flight period of about two months (Table 3.2). Adults of the *T. basutus*, *T. protumnus* and *T. dryburghi* group are active in the spring, summer and fall (Figure 3.6). Members of the black group (*T. murrayi, T. kaplani, T. compassbergae, T. camdeboo, T. pringlei, T. penningtoni, T. holmesi, T. stepheni, T. claassensi, T. overbergensis, T. rileyi, T. yildizae, T. barbatus, T. petra and T. brachycerous)* fly predominantly in the summer (Figure 3.6), but can also be found in the spring. Members of the yellow group (*T. braunsi, T. malagas, T. dicksoni, T. vansoni, T. pictus, T. rooibergensis, T. swanepoeli, T. rossouwi, T. strutti and T. montanus*) can be found predominantly in the spring, but also during summer, fall and winter (Figure 3.6). While species spread across the phylogeny fly in the spring and summer months, only members of the yellow group fly during the winter and fall months (Figure 3.7).

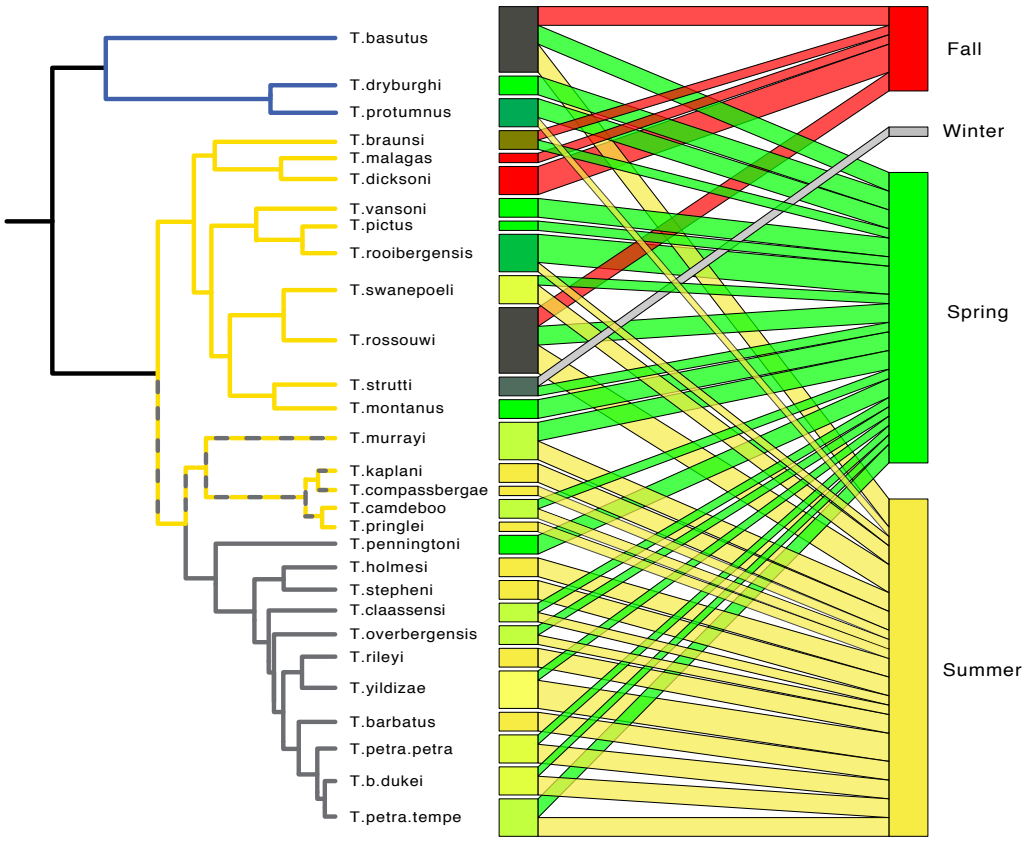

Figure 3.6 Comparison of the phylogeny of the African genus *Thestor* (Lycaenidae: Miletinae) and flight times of the adults. 6-gene BEAST Bayesian Consensus of *Thestor* on the left as compared with a bipartite plot of flight time on the right. The bipartite plot is colored by flight season. Fall, Winter, Spring and Summer represent seasons in the Southern Hemisphere.

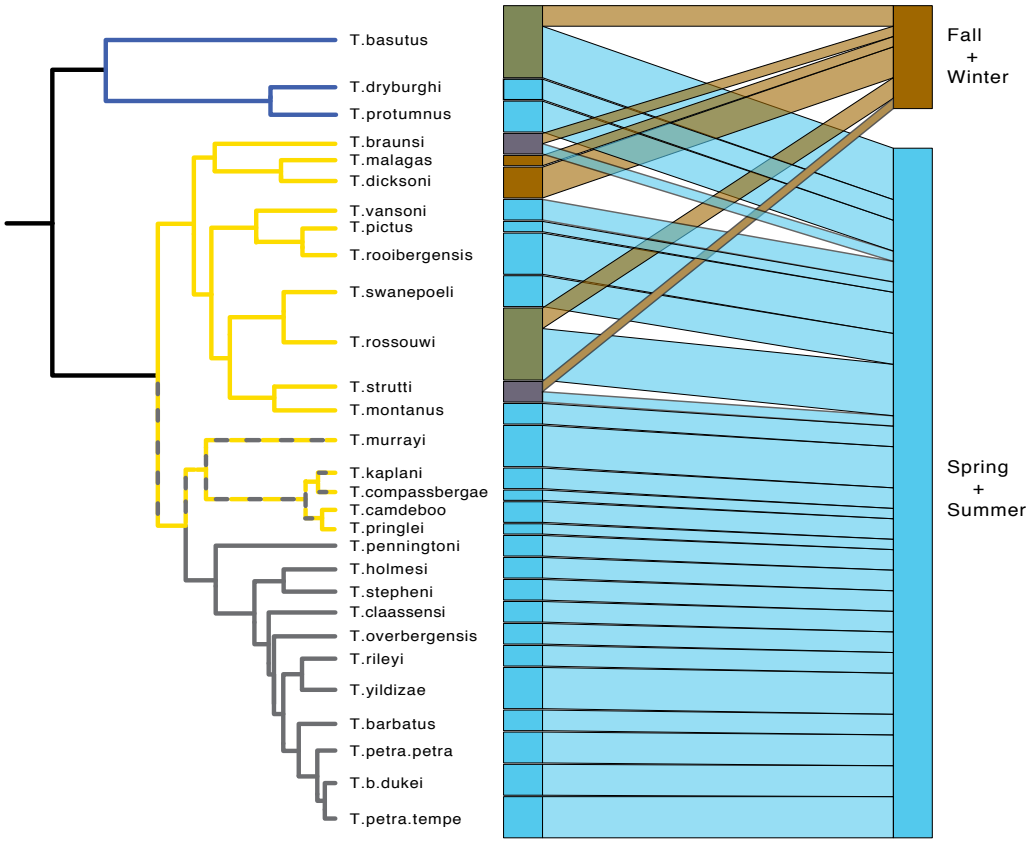

Figure 3.7 6-gene BEAST Bayesian Consensus of *Thestor* on the left compared with a Bipartite plot of flight time on the right. The Bipartite plot is colored by flight season. Fall, Winter, Spring and Summer represent seasons in the Southern Hemisphere.

Discussion

Phylogenetic relationships of Thestor

In 2004, Heath and Pringle divided *Thestor* into several groups, with two main groups based upon wing coloration. Our phylogenetic analysis recovered members of the Yellow group and the Black group clustering together, but with the Black group nested within a paraphyletic Yellow group. More specifically, the black group is placed within the clade of the yellow group that contains *T. murrayi*, *T. kaplani*, *T. compassbergae*, *T. camdeboo* and *T. pringlei.*

In other respects, our results support most of the taxonomic designations of Heath and Pringle 2004, with a few exceptions and a number of cases where our fine-scale analysis suggests further modifications to the taxonomy. For example, in some cases, two populations of *T. protumnus* show greater pairwise genetic distance than between species in two different *Thestor* species. This suggests that *T. protumnus* could potentially be split into two separate species, but further behavioral and morphological work should be done before this happens.

Two exceptions to Heath and Pringle's arrangement involve the placement *T. dicksoni* and *T. swanepoeli*. *T. malagas* was reduced from a species designation to a subspecies of *T. dicksoni* (Heath and Pringle 2004). Based on the COI genetic distance between it and the other *T. dicksoni* (T. *dicksoni dicksoni* and *T. dicksoni warreni*), we suggest that it should be restored as a good species. The same is also true for *T. swanepoeli*: *T. rossouwi* was re-assigned to *T. swanepoeli.* However, representatives of *T. rossouwi* cluster separately from those of *T. swanepoeli*, and could be assigned a unique designation, either as a subspecies of *T. swanepoeli* or as

T. rossouwi.

At the other end of the spectrum intraspecific pairwise distances in *T. camdeboo* and *T. pringlei* were the same as interspecific distances between the two species for COI. These two taxa were intermixed in the COI NJ tree, and were also recovered as extremely closely related in the multi-gene and Rad-seq phylogenetic hypothesis. We suggest that *T. pringlei* and *T. camdeboo* could be combined into one species.

Thestor kaplani and *T. compassbergae* were extremely closely related sister taxa in the Rad-seq Bayesian consensus, and *T. compassbergae* rendered *T. kaplani* paraphyletic in the COI NJ tree. However, the geographic distance separating the two discrete populations is 510km and they are distinct morphologically as well as very different biomes including winter rainfall versus summer rainfall. The intervening terrain has been reasonably well explored and contains the type locality of *T. camdeboo* but no other populations. Thus these two taxa should also be looked at more carefully to determine if they are distinct species or not.

The placement of *T. brachycerus* conflicts significantly from the relationships proposed by Heath and Pringle in 2004 (Figure 3.2a). *T. brachycerus dukei* appears in two separate clades. In one case, it is recovered as synonymous with *T. petra,* and in another, it is sister to *T. overbergensis*. We suggest a future population level study to determine where this break occurs and to explore whether the two taxa differ in their behavior.

The implications of this work for a revised classification of *Thestor* will be treated in a further paper by Heath and colleagues.

Myrmecophily - Phylogenetic relationships

All of the species within the genus *Thestor* are thought to have a close association with ants in the genus *Anoplolepis*, and mostly with just one species, *A. custodiens* (Heath and Pringle 2004). It seemed implausible to us that that so many species would share a common food resource, and we were initially motivated to investigate whether *Anoplolepis custodians* may actually be a complex of cryptic species with which the genus Thestor may have co-diversified.

However, our results support the original observation that 24 of the species in the Western Cape utilize the same ant species, while the two species that are found in the north-western part of South Africa use a closely related, but different ant species, and a single species of *Thestor* found in the eastern part of South Africa utilizes yet a third ant. Thus there must be other explanations for how so many closely related species of butterflies can coexist on a single food resource. although factors driving the diversity in the genus *Thestor* might have been ants and/or geography when the genus originated, but that other forces may be responsible for generating the more recent diversity in the group.

Myrmecophily - Stable Isotopic Evidence

Although all known life history records of *Thestor* have found that they are closely associated with *Anoplolepis* ants, these records are rather sparce: partial life histories are only known for only 4 of the 27 species. *T. protumnus* and *T. basutus* have been observed to feed on Hemiptera during their first three instars (Clark and Dickson 1960, 1971; Williams and Joannou 1996). Observed in captivity, the final

instar of *T. basutus* feeds mainly by trophallaxis and occasionally consumes ant eggs and detritus (Heath and Claassens 2003; Claassens and Heath 2003). *T. yildizae* and *T. pictus* feed by trophallaxis (Heath and Claassens 2000, 2003). Thus, while available collecting data indicate that all *Thestor* caterpillars are aphytophagous within the nests of *A. custodiens*, complete life histories of the majority of the species have not been confirmed.

We used C and N stable isotopic analysis to determine whether any *Thestor* species deviate from the prediction that all are aphytophagous. The average differences between almost all of the species of *Thestor* and their corresponding ants are 2.5 ‰, which is the amount expected per standard trophic level (Figure 3.5). Thus our findings are consistent with the idea that final instars of *Thestor* caterpillars feed either via trophallaxis or directly on ant brood. The only exception to this is *T. basutus*. The $\delta^{15}N$ difference between adults of *T. basutus* and its ants is 4.6 ‰, which is considerably higher than the differences seen in its congeners. A possible explanation for this is that since the first three instars of *T. basutus* feed on Hemiptera (Williams and Joannou 1996, Heath and Claassens 2003; Heath and Pringle 2004), perhaps the final instars do as well. An alternative explanation is that the ants feed the caterpillars of this species in a different manner than those of other species.

While they exhibit geographic variation, individuals measured from the majority of *Thestor* populations/species have similar $\delta^{15}N$ values (just over 5 ‰) and little variation per locality. The main deviations from this can be explained by geography and ant feeding habits. Localities with three high $\delta^{15}N$ values correspond to those where we collected adults of *T. protumnus* and *T. dryburghi*, both from localities far

north along the west coast of South Africa. This region is very arid, which can sometimes elevate $\delta^{15}N$ values. It is also likely that the ants in those harsh conditions are scavengers further up the food chain than their conspecifics in the Western Cape. The second main deviation can be seen in adult $\delta^{15}N$ values for *T. dicksoni malagas*. The only known locality for *T. dicksoni malagas* is very close the shore, and its host ants likely feed on debris that is washed up from the ocean. Ocean food chains are longer than land food chains, and thus we can expect the ants that feed on debris washed up from the ocean to have higher $\delta^{15}N$ values than ants feeding inland. The butterflies feeding on the ant brood or regurgitations are one trophic level up from that.

$\delta^{13}C$ isotopic signatures for both the butterflies and the ants were around -24 ‰, suggesting the ants feed on C3 plants. The ant values were slightly higher than the butterfly values (on average 0.7 ‰ higher). The main exception to this was found with adults of *T. basutus* and their ants. Their signatures are close to -15 , which would correspond to those exhibited by an insect foraging on C4 plants. These individuals of *T. basutus* were collected from KwaZulu-Natal, where they fly on grassy hilltops. The ants possibly eat grass seeds or scavenge on prey that feed on grasses.

Our data support the hypothesis that with the possible exception of *T. basutus*, all the *Thestor* species for which we have corresponding ant samples are utilizing the ants in a similar manner. For two localities where we have samples of *A. custodiens*, we also collected silverfish from inside the ant nests (Zygentoma, Nicoletiidae). Silverfish are generally scavengers and detritus feeders, but some species have been

known to be cleptoparasites of ants engaging in trophallaxis. The silverfish $\delta^{15}N$ signatures differ from the ant signatures by 2.5 ‰, just as the *Thestor* butterfly signatures do.

Table 3.2 *Thestor* species, common names and group designations, distributions and flight times. Reprinted from Mathew 2003.

Species	**Common Name**	**Group**	**Heath's Species Groups**	**Distribution**	**Flight Time**
T. basutus basutus	Basuto Skolly	Basutus	basutus	Eastern Cape Free State, KwaZulu Natal, Gauteng, Mpumalanga, Northern Province, Southern Bostwana Lesotho, Zimbabwe	October - April
T. basutus capeneri	Basuto Skolly	Basutus	basutus	Gauteng	October – April
T. protumnus aridus	Boland Skolly	Yellow	protumnus	Northern Cape, Free State	September - December
T. protumnus protumnus	Boland Skolly	Yellow	protumnus	Northern, Eastern, Western Cape	October - December
T. dryburghi	Dryburgh's Skolly	Yellow	protumnus	Western Cape, restricted to Namaqualand	September - October
T. rossouwi	Rossouw's Skolly	Yellow	rossouwi	Southern Western Cape	October - April
T. swanepoeli	Swanepoel's Skolly	Yellow	rossouwi	Southern Western Cape	November - January
T. murrayi	Murray's Skolly	Yellow	rossouwi	Western Cape	October - January
T. strutti	Stutt's Skolly	Yellow	rossouwi	Western Cape	August - September
T. braunsi	Braun's Skolly	Yellow	dicksoni	Western Cape	October and March (double brooded)
T. dicksoni dicksoni	Dickson's Skolly	Yellow	dicksoni	Western Cape	March - April

T. dicksoni warreni	Dickson's Skolly	Yellow	dicksoni	Western Cape	March - April

Table 3.2 (Continued)

Species	**Common Name**	**Group**	**Heath's Species Groups**	**Distribution**	**Flight Time**
T. calvaniae	Dickson's Skolly	Yellow	dicksoni	North Western Cape	December - February
T. dicksoni malagas	Atlantic Skolly	Yellow	dicksoni	Western Cape, restricted to Saldanha Bay	March
T. montanus	Mountain Skolly	Yellow	montanus	Western Cape	October to November
T. vansoni	Van Son's Skolly	Yellow	montanus	Western Cape	October to November
T. rooibergensis	Rooiberg Skolly	Yellow	montanus	SouthernWestern Cape, restricted to the Rooiberg Moutains.	September - December
T. pictus	Langeberg Skolly	Yellow	montanus	Southern Western Cape	November
T. compassberga e	Compassberg Skolly	Yellow	kaplani	Western Cape	December
T. kaplani	Kaplan's Skolly	Yellow	kaplani	Southern Western Cape	December - January
T. pringlei	Pringle's Skolly	Yellow	kaplani	Western, North-western Cape	December
T. camdeboo	Camdeboo Skolly	Yellow	kaplani	Eastern Cape	November – December
T. stepheni	Stephen's Skolly	Black	black	Western Cape	December - January
T. holmesi	Holmes's Skolly	Black	black	Western Cape	December - January
T. brachycerus	Knysna	Black	black	Western Cape	October –

	Skolly				February
T. tempe	Tempe's Skolly	Black	black	Western Cape	October - January
T. yildizae	Peninsula Skolly	Black	black	Western Cape (restricted to Table Mountain range)	November to February

Table 3.2 (Continued)

Species	**Common Name**	**Group**	**Heath's Species Groups**	**Distribution**	**Flight Time**
T. rileyi	Riley's Skolly	Black	black	Southern Western Cape	December – January
T. petra	Rock Skolly	Black	black	Western Cape	November - January
T. dukei	Duke's Skolly	Black	black	Southern-Western Cape	November - January
T. penningtoni	Pennington's Skolly	Black	black	Western Cape	October - November
T. barbatus	Hairy Skolly	Black	black	Western Cape	December-January

Flight Times

The Western Cape of South Africa, where most of the *Thestor* species live, has a Mediterranean climate, consisting of warm summers, relatively short transition periods, and mild, wet winters. *Thestor* butterflies spend most of their lives as caterpillars, and since these have never been observed in the open, despite considerable efforts made by generations of avid lepidopterists to find them, they are likely to live hidden in ants' nests. Each individual lives as an adult for approximately three weeks (Heath, personal communication), but the average species/population has a flight period of about two months (Table 3.2). Thus while the caterpillars are mostly

protected from the weather and the differences in the seasons, the flight time of the adult butterflies could be greatly influenced by factors such as temperature and rainfall.

Since the *Thestor* species that live only in the Western Cape do not seem to partition their ants phylogenetically, we thought that they might partition them temporally. The fact that there are species that fly in the same areas, but at different times provides some evidence in favor of this theory: *T. penningtoni* and *T. brachycerus dukei* both fly at the Swartberg Pass, but while *T. penningtoni* adults fly from October through November, *T. brachycerus dukei* adults fly from November through January. *T. kaplani* and *T. braunsi* both occur in the Greyton area, but while *T. kaplani* fly from December through January, *T. braunsi* appear to be double-brooded and fly in October and in March.

Overall, members of the black group fly predominantly in the summer months and members of the yellow group fly predominantly in the spring months. And while species spread across the phylogeny fly in the spring and summer months, only members of the yellow group fly during the winter and fall months (Figure 3.7). Thus flight time may help to account for the separation of the yellow and black groups of *Thestor.*

Conclusion

As ant parasites, the species of *Thestor* are strikingly different from those of the genus *Phengaris* (*Maculinea*), where each species of butterfly has largely

specialized on a different ant species or group of species, which represent slightly different ecological niches (Thomas *et al.* 1989). Thus far, there is no comparable niche diversity that can account for the radiation of *Thestor* in Southern Africa. It is possible that *Thestor* initially diverged with three species of *Anoplolepis* ants: the ancestor of *Thestor basutus* with one, the ancestor of *T. protumnus/T. dryburghi* with another and the ancestor of the rest of the *Thestor* species with a third. Without knowing more about the phylogeny of the genus *Anoplolepis*, it is also possible that these different groups of *Thestor* have colonized the three different species of *Anoplolepis* because they live in different geographical locations. The split between Yellow and Black *Thestor* may have been driven in part by phonological differences in flight time. However, the reason for the majority of the recent diversity of *Thestor* remains unknown, and possible differences in resources/ ecological niches that we have explored thus far cannot explain how so many species can coexist on the same resource. The ecology of species of *Thestor* may represent a unique case where isolated (largely montane) populations with small ranges and low dispersal were able to diversify while utilizing the same abundant resource (colonies of *Anoplolepis custodiens*). The ranges of these isolated, nascent species may have been sufficiently non-overlapping that individuals never had the opportunity to compete for their common resource. The ants might not show this same pattern of diversification if they have larger populations sizes and are able to disperse more readily than the butterflies. This seems a plausible hypothesis, but we would need to know much more about many factors, including the phylogeography of both ants and butterflies and the geological history of the region.

CHAPTER 4

Estimation of diet using 13C and 15N analysis: A survey of the isotopic variability in butterfly species of the Cape region, South Africa

Coauthors: Mark Cornwall, Alan Heath, Jeremy Midgley, and Naomi E. Pierce

Abstract

We used 15N and 13C analyses of Cape butterflies to determine the diets of nearly 150 species of poorly studied South African lycaenids. A controlled feeding trial indicated fractionation levels likely to occur during metabolism and metamorphosis. Analysis of field collected butterfly samples of 13C values reflected C3, CAM, C4 and lichen food plants. 15N values provided a useful index of the spread from herbivorous to insectivorous diets. We noted high intra-specific variability in isotopes of some species, consistent with the diet of a generalist feeder. We found exceptionally high 15 N values in some species.

Introduction

The Lycaenidae are a large family of with approximately 5200 species world-wide, the majority of which have caterpillars that associate with ants (van Nieukerken *et al.* 2011-12; Hinton 1951; Pierce *et al.* 2002). Their diets range from being generalists on many different plants, to specializing on foods such as lichen, Hemiptera, ant brood and/or even ant regurgitations via trophallaxis (Cottrell 1984; Pierce *et al.* 2002). Although most lepidopteran larvae feed on plant tissue (more than

99% of the some 160,000 species), shifts to predation and/or parasitism have occurred and are thought to have evolved partly in response to harsh environmental conditions (Fiedler 1988), and/or because of associations with ants and the insects that they tend (Pierce 1995).

Nowhere is the diversity of diet more marked than among the lycaenid species of southern Africa, with its varied landscape and corresponding floral diversity (REF).

Like most butterflies, the habits of Lycaenidae are well known compared with other insects, with thousands of life histories documented. However, many of these species have proved to be difficult to study. For example it is difficult to find the larvae of many South African taxa, particularly when they are hidden in rock crevices or underground being cared for by ant hosts.

Stable Isotope Analysis can be used to study trophic interactions between taxa by quantifying the abundance of heavy and light isotopes of nitrogen ($^{15}N/^{14}N$, calculated as $\delta^{15}N$) and carbon (^{13}C /^{12}C , calculated as $\delta^{13}C$) found in sample tissues expressed as a ratio relative to a standard. For example, stable isotopes were used to show that larvae of the African species *Anthene usamba* were parasitizing an ant-plant mutualism not only by being able to chemically fool the phytoecious ants inhabiting the ant plant into tending the caterpillars rather than attacking them, but also by entering the domatia of the ants and either being fed mouth-to-mouth or consuming the ant brood directly (Martins *et al.* 2013).

Here we have surveyed a large number of lycaenid butterflies from South Africa and their potential food sources. We have confirmed some known or suspected life histories, and established that in any given area, a species can have a highly

variable diet. Some of the nitrogen stable isotope values are much higher than expected for land animals.

Materials and methods

Rearing Experiment

To determine the degree of isotope enrichment from one trophic level to the next, as well as during development from caterpillars to adult butterflies, we raised two species from early instars through to adults. *Vanessa cardui* are medium-sized butterflies (6cm average wingspan), and in nature their caterpillars are herbivorous and feed on a great many host plant species, primarily composites in the Asteraceae, but also species of Malvaceae and Boraginaceae (HOSTS: The host plant and caterpillars database, Natural History Museum, London). They have a cosmopolitan distribution, and are famous for their impressive long-distance migrations. *Feniseca tarquinius* are small (3cm average wingspan) aphytophagous butterflies. They are the only North American representatives of the subfamily Miletinae, with closest relatives in Japan and China, and they are recorded to feed on woolly aphids, *Paraprociphilus tessellatus* (Aphidoidea: Pemphigidae) feeding on alder, *Alnus rugosa* (Betulaceae) (Mathew *et al.* 2008).

Vanessa cardui caterpillars feeding on an artificial diet were ordered from Carolina Biological Supply. Four small caterpillars were frozen immediately, and the rest were placed in individual plastic cups filled with medium. One week later, five caterpillars were frozen. Once the caterpillars pupated, three pupa were frozen. The remaining five butterflies were frozen within 24 hours of eclosion.

Feniseca tarquinius caterpillars were collected feeding on aphids on alders growing in Harvard Forest, near Petersham, MA, along with alder leaves, bark and woolly aphids. A sample of alder bark and four samples of leaves from the stems on which the caterpillars were found were frozen. Five *F. tarquinius* caterpillars were frozen within days of being collected and six caterpillars were reared through to adults. Once the caterpillars pupated, the remaining woolly aphids (some small and some large) were also frozen.

All samples were initially frozen and then dried at 50 degrees C. All the samples except the adult butterflies were then ground in liquid nitrogen, and samples of approximately 1 mg of insect matter or 4 mg of plant matter were weighed, placed individually into tin capsules and processed using standard methods for stable isotope analyses (Tieszen and Boutton 1988; Lajtha and Michener 1994; Webb 1997). Samples were then sent to the UC Davis Stable Isotope facility and processed for both $\delta^{15}N$ and $\delta^{13}C$.

Taxon sampling

To investigate whether caterpillar trophic levels can be distinguished based on $\delta^{15}N$ and $\delta^{13}C$ stable isotopic signatures, we collected samples from localities throughout South Africa. Wherever possible, we collected the presumed food plant for herbivorous butterflies, the presumed host ant for aphytophagous butterflies and the lichen for lichen feeding butterflies. When the life history was unknown, we collected either likely candidates for food plants or host ants if we could find them. We collected a total of 424 specimens of butterflies representing 142 species in 33

genera; 103 samples of ants in 5 genera; 199 samples of plants representing 35 genera; and 13 samples of lichen.

Specimen collection

Lichen samples were scraped from rocks and stored in plastic containers while in the field, then they were dried and frozen at -20 ºC once they were brought to the lab.

Plant samples were photographed, and then put in a plant press. At the end of each field excursion, they were dried at a low temperature in an oven for 1 hour. Identifications were made at the Herbarium at the University of Cape Town.

Hemiptera, Zygentoma and ant specimens were collected into vials half-filled with an anhydrous calcium sulfate dessicant (W A Hammond Drierite Co Ltd) that were plugged with a piece of kimwipe (to keep dryerite powder from coating the specimens). Several ant specimens were collected prior to 2007, and these were collected into 90-100% ethanol.

Adult butterfly specimens were collected into 90-100% ethanol. Wings were removed from the specimens while still in the field and kept separately in glassine envelopes.

All butterfly and ant specimens are deposited in the DNA and Tissues Collection of the Museum of Comparative Zoology at Harvard University in Cambridge, Massachusetts.

Sample preparation

All samples were oven-dried for 24-48 hours at 55 degrees Celsius prior to processing.

For plant samples, unless otherwise noted, only leaves were used. Plant samples were ground up in a mini wood chipper to homogenize the samples. For ants, heads and thoraxes were used. For butterflies, only wings were used. Approximately 0.5 mg of animal tissue or 2.5 mg of plant tissue was weighed into tin capsules (Bol and Pflieger 2002) combusted on a Thermo Finnigan Delta Plus XP Stable Light Isotope Ratio Mass Spectrometer (Thermo Finnigan) coupled to a Thermo Flash EA 1112 Elemental Analyzer via a Conflo III Device. For the animal tissue samples, one standard of each of Choc, Mg and Val was run between every fifteen test samples. For the plant tissue samples, one standard of each of Acacia, Anu Sucrose and New Nastd was run between every fifteen test samples.

The analytical precision (S.D.) was ± 0.2‰, as estimated from five standards analysed along with the samples. The internal working standards are related to the international standards by direct calibration with the international standards and by inter-laboratory comparison. Isotope ratios are expressed in parts per mil (‰) using the δ notation, derived from the equation: $\delta R = (R_{sample}/R_{standard} - 1) * 1000$, where R = the isotope ratio of element R ($^{x}R/^{y}R$). By convention, the δ values are measured with reference to the international standards as distributed by the IAEA of Vienna PeeDee Belemnite marine limestone (VPDB) for carbon isotopes and atmospheric N_2 (NAIR) for nitrogen isotopes.

Statistical Analysis

(1) **Stable isotope comparisons ofAfrican genera of lycaenid butterflies feeding on different food sources.**

Mean stable isotope values of N and C were calculated for all conspecific butterflies (diamond symbols) at a given location (species x location) along with the minimum and maximum isotope values. A single species can be represented by multiple rows if it was collected at multiple locations. Butterfly trophic level is indicated by the color of the diamonds: herbivorous (green), aphytophagous (red), trophollaxis (orange), herbivorous->aphytophagous (yellow). Species are ordered from lowest to highest average isotope value for N. If host plants (green circles) or ant associates (red circles) were collected at the same location, their values are plotted beside the butterfly values; if multiple host plant species (or ant associates) were collected at the same location, they are represented as multiple circles. The shaded regions represent the difference between the butterfly and host isotope values when both butterfly and host were collected.

(2) **Isotope values indicating trophic level as measured using *boxplot.***

The two 'hinges' are versions of the first and third quartile, i.e., close to quantile(x, c(1,3)/4). The hinges equal the quartiles for odd *n* (where n <- length(x)) and differ for even *n*. Whereas the quartiles only equal observations for n %% 4 == 1 (*n = 1 mod 4*), the hinges do so *additionally* for n %% 4 == 2 (*n = 2 mod 4*), and are in the middle of two observations otherwise.

The notches extend to +/-1.58 IQR/sqrt(n), and appear to rely on the same calculations

as the formula with 1.57 in Chambers *et al.* (1983), given in McGill *et al.* (1978). They are based on asymptotic normality of the median and roughly equal sample sizes for the two medians being compared, and are largely insensitive to the underlying distributions of the samples. This provides an indication of 95% confidence intervals for the difference in two medians.

There was a significant difference in values of both N (ANOVA, $F(5,309) = 25.26$, $P < 0.001$) (Fig (a)) and C (ANOVA, $F(5,309) = 23$, $P < 0.001$) (Fig (b)). Pairwise differences were tested using Tukey's Post-hoc test, and pairwise differences with p-values less than 0.05 are indicated by different letters. Box plots with different letters have statistically different values.

(3) **Trophic differences created using the R package *beanplot***

Beanplots are similar to boxplots, except that the underlying distribution is described using kernel density estimates instead of traditional descriptive, statistics such as IQR. The dotted gray line represents the grand median value and the 'bean lines' represent the median value of each 'bean'. Each value is illustrated by a line within the bean.

Results

Rearing Experiment

Species higher up the food chain tend to have higher $\delta^{15}N$ values (with a difference of approximately 2-3 ‰ per trophic level). We expect to see an enrichment in $\delta^{15}N$ both for the trophic level shift between the butterflies and their food sources

as well as for the process of metamorphosis. The enrichment between the artificial diet and the adult *V. cardui* wings is 3 ‰ for $\delta^{15}N$ and -0.1 ‰ for $\delta^{13}C$ (Figure 4.1). For *F. tarquinius* it is 2.8 ‰ for $\delta^{15}N$ and -0.3 ‰ for $\delta^{13}C$ (Figure 4.1).

For *V. cardui,* the $\delta^{15}N$ is enriched slightly as the juveniles mature, with the highest values obtained for the adults. $\Delta^{13}C$ is enriched slightly from the food source to the caterpillar, and then decreases with age until pupation, when again the value increases slightly between the pupa and the adult (Figure 4.1).

For *F. tarquinius,* we do not have samples for caterpillars of different instars or for pupae, but the general trend with age appears to be similar to *V. cardui*. The $\delta^{15}N$ is enriched from the food source (small Woolly Aphids) to the caterpillars to the adults. The $\delta^{13}C$ trend is also similar. Initially the $\delta^{13}C$ value increases slightly between the food source and the caterpillar, and then it decreases slightly between the caterpillar and the adult stage (Figure 4.1).

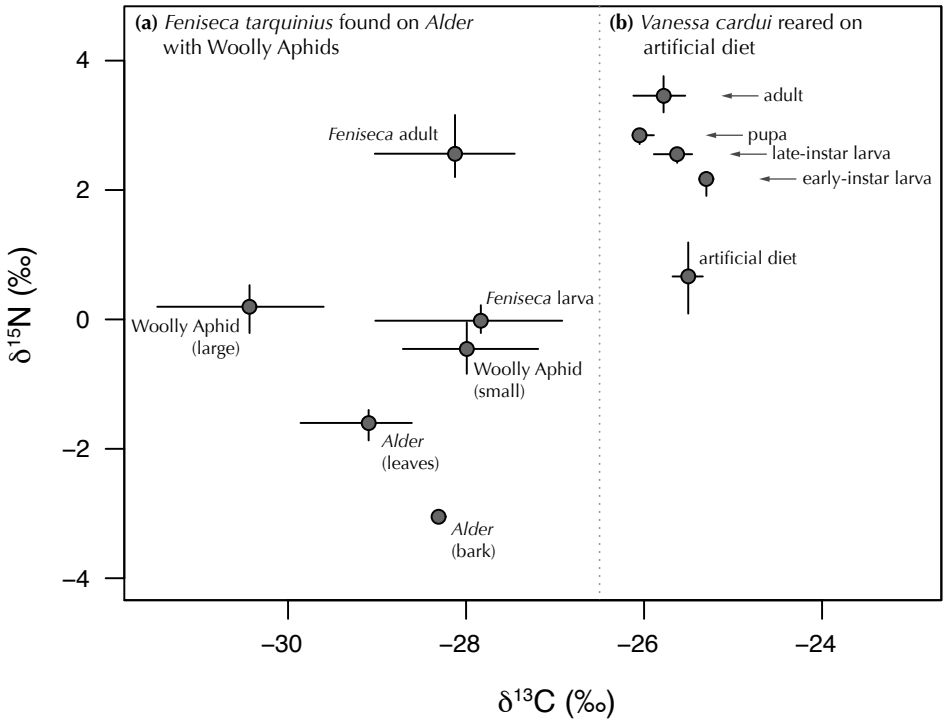

Figure 4.1 Rearing experiment results. $\delta^{15}N$ values are on the y-axis, $\delta^{13}C$ are on the x-axis. Gray dots represent the median values for the given category and the bars represent the range (min/max).

Description of Stable Isotopic Data from the Field

Extreme ranges of stable isotopic data

We found an impressive range of $\delta^{15}N$ values for the butterflies in our study spanning 26.33 ‰ (Figure 4.2). The lowest value, -7.29 ‰, was for the lichen-feeding butterfly, *Durban amakosa,* from Woodrige, in the KuaZulu Natal (KZN) Province. The highest value, 10.04 ‰, was for *Crudaria capensis,* whose pupae have been found in nests of the ant, *Anoplolepis custodiens*, and whose caterpillars presumably feed on ant brood or regurgitations.

We also found a large range of $\delta^{13}C$ values (Figure 4.2) from -31.06 ‰ for *Chrysoritis zeuxo*, which feeds on *Chrysanthemoides* plants at Redhill, in the Western Cape, to -11.01 ‰ for *Orachrysops subravus* from near Howick in KZN. The second highest value was -11.26 for *Leptomyrina lara* from Cathcart, which feeds on *Cotyledon*, a plant known to have Crassulacean acid metabolism (CAM photosynthesis).

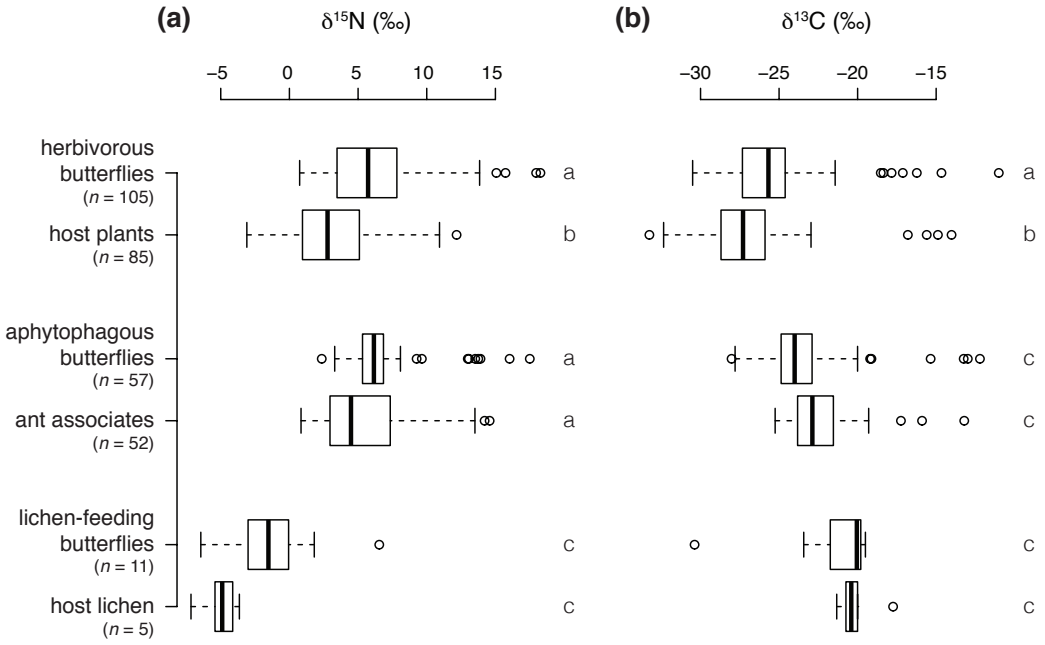

Figure 4.2 Boxplots for the different food categories and for the butterflies that feed on each. The black bars represent the median values and the boxes the quartiles. Significant differences are marked with the letters a-c.

Variation between localities

We also found large intra-specific differences between localities, especially for $\delta^{15}N$. For example, for *Thestor protumnus*, which is presumed to feed on ant regurgitations in their final instars, the $\delta^{15}N$ values range from 4.70 for an individual from Shaw's Pass in the Western Cape to 14.37 for an individual from a locality 30km north of Steinkopf in the Northern Cape.

Enrichment between food sources and butterflies

The enrichment between $\delta^{15}N$ for butterflies and their food source was on

average about 2.3 ‰. This was the same whether the butterflies were suspected or known to be herbivorous, aphytophagous or lichen-feeding (Figure 4.3). For $\delta^{13}C$, the difference between the butterfly signature and the food source signature was close to zero, although it was just below zero for aphytophagous butterflies, and just above zero for herbivorous butterflies and lichen-feeding butterflies (Figure 4.3).

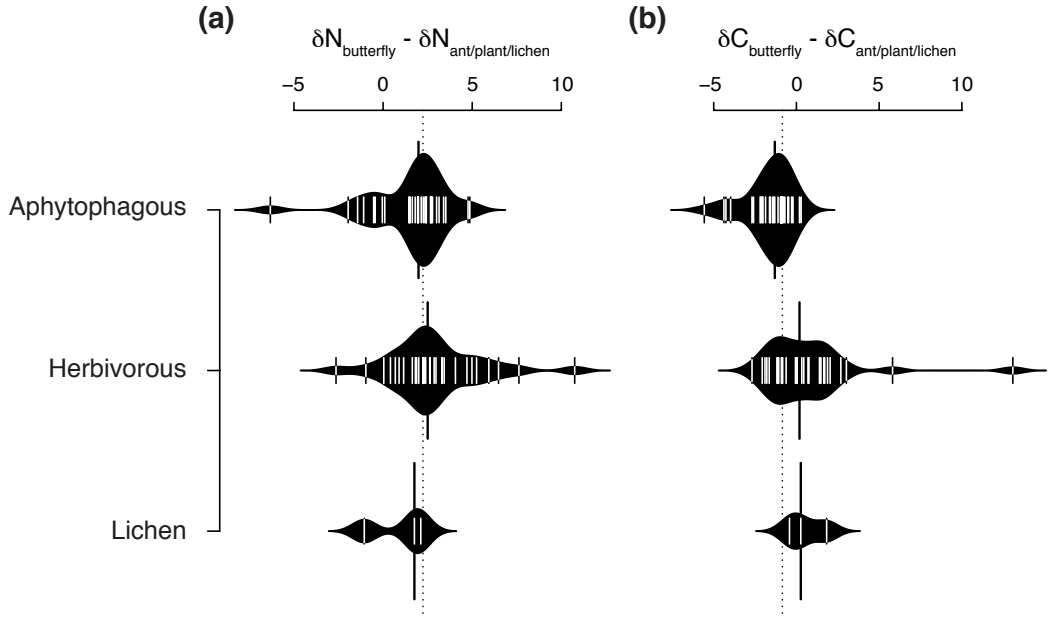

Figure 4.3 Beanplots of the $\delta^{15}N$ differences between the butterflies and the food sources in part a. And $\delta^{13}C$ differences between the butterflies and the food sources in part b.

When the butterflies were separated by food source, the median $\delta^{15}N$ ‰ value for herbivorous butterflies was 5.2 ‰, and 50 percent of the butterflies fell between 3.3 and 7.8 ‰. For the plants they consumed, the median value was 2.8 ‰, and 50 percent of the plants sampled fell between 1.0 and 5.1 ‰.

The values for the aphytophagous butterflies and their corresponding ants are

very similar to the plant values (median of 6.1 ‰ for butterflies, and 4.5 ‰ for ants).

The lichen-feeding butterflies have a median $\delta^{15}N$ value of -1.5 ‰ and 50 percent of them fall between -3.0 and 0.1 ‰. The lichen values are even lower, with a median of -4.9 ‰ and most of them falling between -5.4 and -4.1 ‰ (Figure 4.2).

Despite the large differences between localities, the plant, ant and lichen $\delta^{15}N$ signatures differ significantly from each other. And while the plant feeding butterflies and the aphytophagous butterflies do not differ significantly from each other, the $\delta^{15}N$ signatures for the lichen-feeding butterflies are significantly less enriched than the others.

A surprising result is that there are nearly as pronounced differences in $\delta^{13}C$ between the different categories. Plants have a median value of -27.3 ‰; ants have a median value of -22.9 ‰; and lichen has a median value of -20.4 ‰ (Figure 4.2). The plant $\delta^{13}C$ signature is significantly different from the ant and lichen $\delta^{13}C$ signatures, and the plant-feeding butterflies differ significantly from both the plants that they consume and from aphytophagous and lichen-feeding butterflies.

Thus when we look at a combined $\delta^{15}N$ and $\delta^{13}C$ signature of a butterfly, we can be relatively certain whether they feed on plants or lichen, or whether they are aphytophagous.

Specialists vs. Generalists

Pooled variances represent the weighted average of the variance in $\delta^{15}N$ or $\delta^{13}C$ values for conspecifics from the same localities. The variances between the

specialist herbivores and the aphytophagous butterflies were small and very similar: 1.4 for $\delta^{13}C$ in specialist herbivores, and 1.1 for $\delta^{13}C$ in aphytophagous butterflies. However, the pooled variance of the generalist herbivores was 4.5. The trend for pooled variances for $\delta^{15}N$ was similar, though not as strong: 1.8 for specialist herbivores, 2.5 for aphytophagous butterflies and 4.1 for generalist herbivores.

Specific Examples

Thestor, Chrysoritis and Lichen Feeders

Our most complete sampling is from the genera *Thestor* and *Chrysoritis* (Figure 4.4). The $\delta^{15}N$ values differ more by locality and species for these butterflies than by trophic level. However, the difference between the butterflies and what they eat is about the same for all three feeding styles (Figure 4.3 and Figure 4.4), and the five genera of lichen -butterflies included in this sample have $\delta^{15}N$ values around or below zero. The ants that tend *Chrysoritis* have equal or higher $\delta^{15}N$ values that the butterflies, while the *Thestor* species are enriched by approximately a trophic level from the *Anoplolepis* ants they are thought to parasitize.

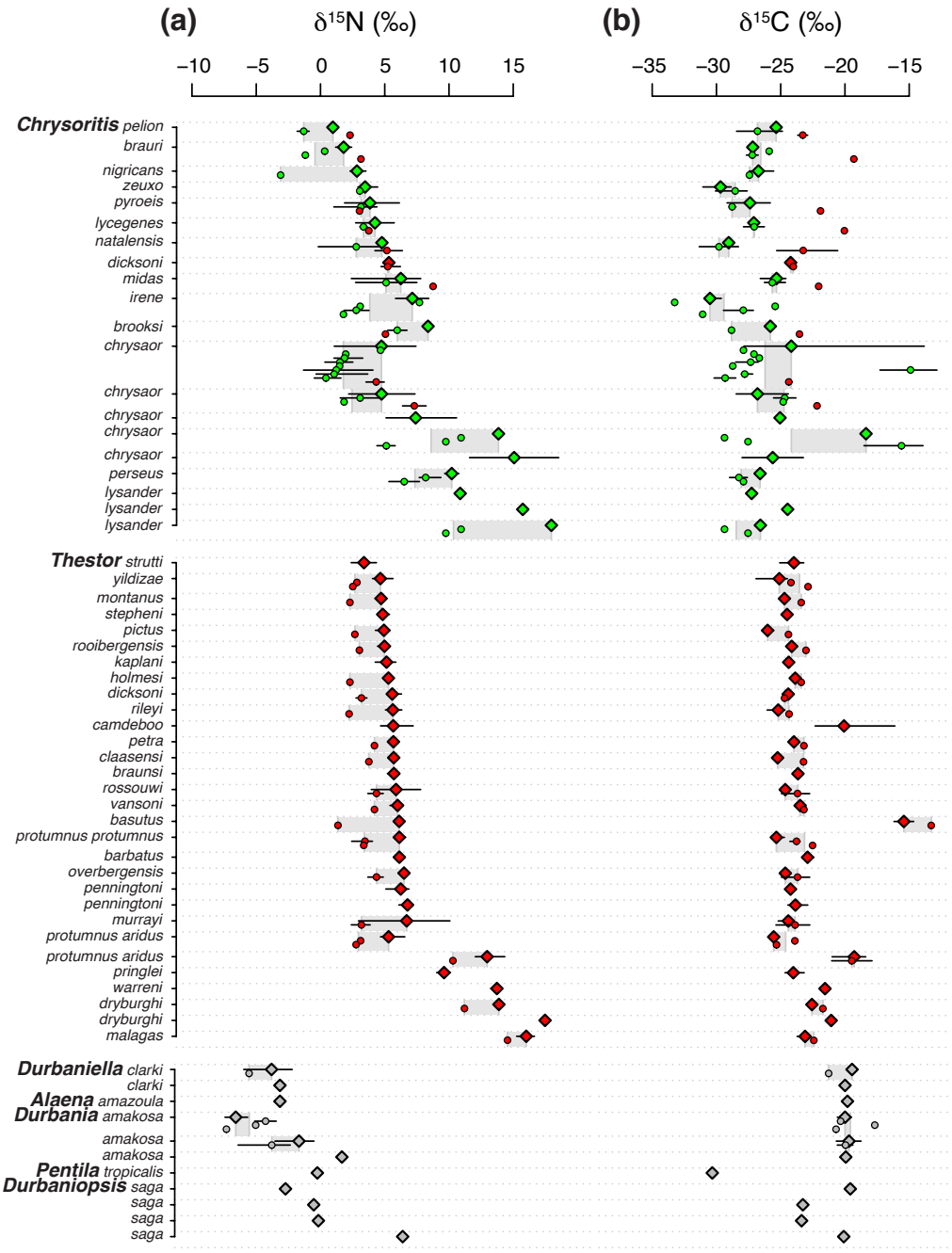

Figure 4.4 $\delta^{15}N$ and $\delta^{13}C$ values for the two best sampled genera, *Chrysoritis* and *Thestor*, and for five genera of lichen feeding butterflies ordered by genus and then by $\delta^{15}N$. The diamonds are butterflies, the small circles are plants or ants. Green is for plant feeding and for plants. Red is for aphytophagous or for ants. Clear is for unknown feeding style. The gray bars in between are the average difference between butterfly and what they feed on.

Aloeides, Lepidochrysorps and Crudaria

Aloeides $\delta^{15}N$ values range from below zero to 15 ‰ (Figure 4.5). The variances per locality range from small to large, and most of their $\delta^{13}C$ values are between -30 and -25 ‰. Thus species of *Aloeides* are likely to be feeding on C3 food sources. *Lepidochrysops* $\delta^{15}N$ signatures are remarkably uniform considering their presumed phytopredaceous life histories (starting out feeding on plants but switching to ants and/or ant regurgitations during the final larval instar) and the different localities in which they fly. Their $\delta^{13}C$ signatures are mostly around -27 ‰, but four species have $\delta^{13}C$ signatures that are between -20 and -15 ‰ (Figure 4.5), suggesting that while most of them feed on C3 food sources, at least some of them feed on C4 or CAM food sources.

Crudaria $\delta^{15}N$ values are mostly around 5 ‰ for *C. leroma*, on average 10 ‰ for *C. wykehami* and 15 ‰ for *C. capensis* (Figure 4.5). Their $\delta^{13}C$ signatures are around -25 ‰. The $\delta^{13}C$ are closer to the plant $\delta^{13}C$ values for *C. leroma* and closer to ant values for *C. wykehami* (Figure 4.5).

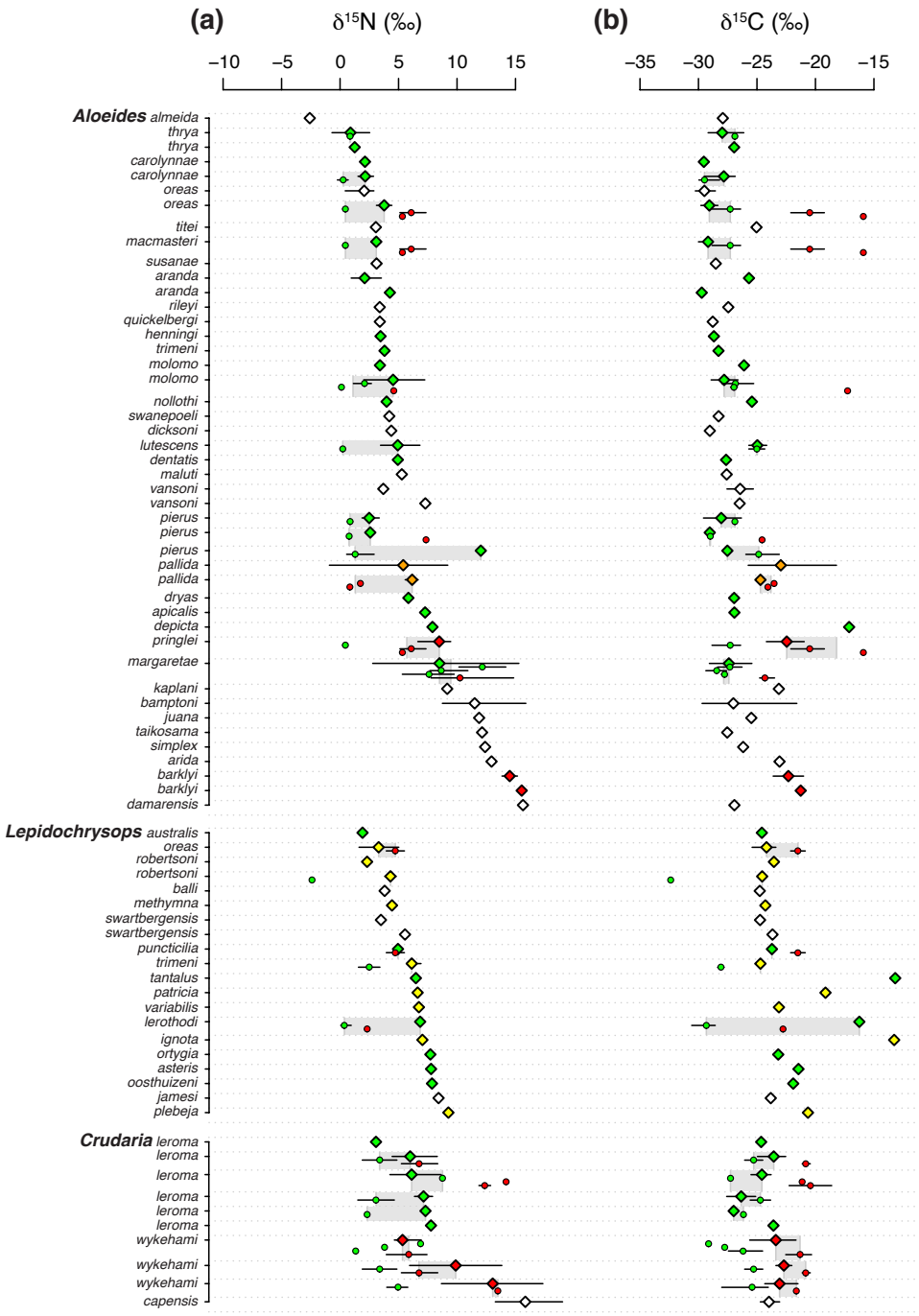

Figure 4.5 $\delta^{15}N$ and $\delta^{13}C$ values for the two best sampled genera, *Chrysoritis* and *Thestor* and for five genera of lichen feeding butterflies ordered by genus and then by $\delta^{15}N$. The diamonds are butterflies, the small circles are plants or ants. Green is for plant feeding and for plants. Red is for aphytophagous or for ants. Orange is for ant brood feeding. Yellow if for butterflies whose caterpillars feed on plants for their first three instars and on ants for the final instars. Clear is for unknown feeding style. The gray bars in between are the average difference between butterfly and food source.

Winterberg Aloeides

A case study of four species of *Aloeides* that all fly on the Winterberg in the Eastern Cape shows that *A. pringlei* has a median $\delta^{15}N$ value of 8.3 ‰ and $\delta^{13}C$ value of -23 ‰ (Figure 4.6), which is approximately a trophic level above the workers of *Lepisiota* ants with which they are associated (6 ‰ $\delta^{15}N$ -21 ‰ $\delta^{13}C$). Since lycaenid caterpillars are likely to feed on ant brood, not adult workers, we also tested the *Lepisiota* ant brood for $\delta^{15}N$ and $\delta^{13}C$, but the differences are small compared to the variation within each locality and thus adult ants are a good approximation for ant brood.

A. macmasteri, *A. dicksoni* and *A. oreas* have $\delta^{15}N$ of between 3 and 4 ‰ and $\delta^{13}C$ values of approximately -29 ‰. Their presumed food plant, *Indigastrum*, has a $\delta^{15}N$ signature of 1 ‰ and $\delta^{13}C$ signature of -27 ‰, which is approximately a trophic level below the signatures of the butterflies (Figure 4.6).

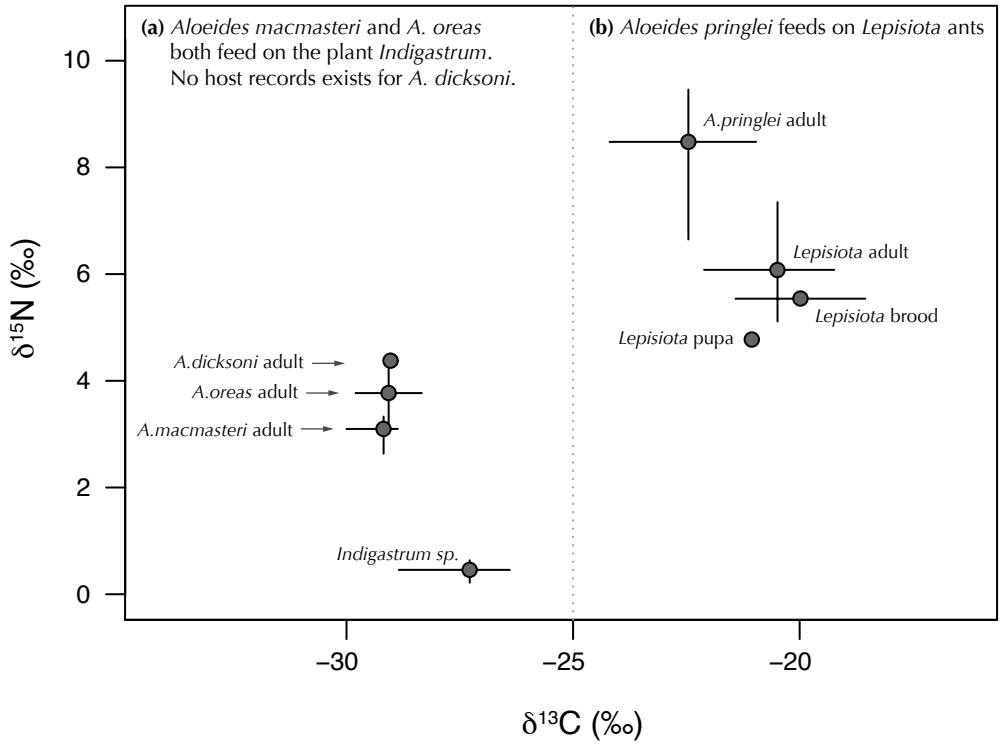

Figure 4.6 Case study of four species of *Aloeides* from the Winterberg (Eastern Cape, South Africa). $\delta^{15}N$ values are on the y-axis, $\delta^{13}C$ are on the x-axis. Gray dots represent the median values for the given category and the bars represent the range (min/max).

Discussion

Rearing Experiment

During metamorphosis, the internal concentrations of stable isotopes change by approximately half of the average trophic level shift for $\delta^{15}N$ (Doi *et al.* 2007, Spence and Rosenheim 2005). For both *V. cardui* and *F. tarquinius,* the difference between the food source (artificial diet for *V. cardui* and small Woolly Aphids for *F. tarquinius*) are approximately 3 ‰ for $\delta^{15}N$ and -0.2 ‰ $\delta^{13}C$ (Figure 4.1). Because the enrichment between the larval food source and the adult butterfly wings appears

similar for both a large, herbivorous butterfly and for a small aphytophagous butterfly, we can assume the isotopic signatures found in adult butterfly wings reflect those of larval diets in a predictable way.

Even though the *F. tarquinius* caterpillars can be found among both the small and larger Woolly Aphids, based on the more negative $\delta^{13}C$ values of the large Woolly Aphids and on the high $\delta^{15}N$ values of the large Woolly Aphids, the *F. tarquinius* caterpillars appear to feed only on the young Woolly Aphids and not on the larger, mature ones.

Thestor, Chrysoritis and Lichen Feeders

Our most complete sampling of a range of species is for two genera that are endemic to southern Africa, *Thestor* and *Chrysoritis* (Figure 4.4). *Chrysoritis* is a genus of well-studied mostly herbivorous generalists; only one species, *C. dicksoni* is known to feed on ant regurgitations (Rand *et al.* 2000). *Thestor* is a highly specialized, aphytophagous genus whose species are thought to feed mostly on ant regurgitations of *Anoplolepis* ants (Heath and Pringle 2004). These two genera, along with the African lichen-feeding butterflies, members of the Lipteninae (Lycaenidae), represent the extremes of feeding styles among lycaenid butterflies.

Even though we commonly assume that individuals with higher $\delta^{15}N$ values are at higher trophic levels, this trend does not hold up for *Thestor* and *Chrysoritis*. The $\delta^{15}N$ values differ more by locality and species than by trophic level. The difference between the butterflies and what they eat is roughly equivalent for all three feeding

styles (Figure 4.3 and Figure 4.4). And the five genera of lichen-feeding butterflies have $\delta^{15}N$ values around or below zero, which is consistent with he idea that lichen are nitrogen fixers, and so "zero out" the $\delta^{15}N$.

Both *Chrysoritis* and *Thestor* have close associations with ants. But while *Thestor* feed on ant brood or ant regurgitations, *Chrysoritis* feed on a wide range of plants (including *Thesium* in the Santalaceae, *Chrysanthemoides* in the Asteraceae, *Rhus* in the Anacardiaceae, and *Zygophyllum* in the Zygophyllaceae), and are tended by ants (Figure 4.4). The ants that tend *Chrysoritis* have equal or higher $\delta^{15}N$ values than the butterflies, so the butterflies are unlikely to be feeding on them. On the other hand, *Thestor* species are enriched by approximately a trophic level from the *Anoplolepis* ants, suggesting that they do indeed parasitize them.

Chrysoritis is a genus with many generalist species: *C. chrysaor*, for example feeds on five families of plants. However, *Thestor* is a genus where almost every species is thought to parasitize only one species of ant (Chapter 3, this thesis). This is reflected in both the $\delta^{15}N$ and the $\delta^{13}C$ signatures. The variances in stable isotopic signatures within the localities for species of *Thestor* (aphytophagous specialists) are much smaller than the variances seen among species of *Chrysoritis* (herbivorous generalist).

Aloeides, Lepidochrysorps and Crudaria

Aloeides are a diverse group of butterflies whose caterpillar diets range from feeding on plants (most often, though not uniquely, *Aspalathus* in the Fabaceae) to feeding on ant brood and/or ant regurgitations. Some species are presumed to be

specialists on one food source, while others are known to be generalists. The enrichment between the presumed food sources and the butterflies, as well as the variances within populations is consistent with this diversity in diet preferences of different species of *Aloeides* (Figure 4.5).

The some 120 species of *Lepidochrysops* are all thought to be phyto-predaceous: caterpillars initially feed on flowers of *Becium* and other Lamicaeae, but eventually drop to the ground where they are carried by ants into the brood chamber of the nest. Here they either eat the ant brood directly, or are fed mouth-to-mouth like cuckoos for the remainder of their development. As in the Palearctic genus *Maculinea* (now considered a junior synonym of *Phengaris*), many *Lepidochrysops* species are listed as endangered, vulnerable or rare (20 out of 21 assessed species) and one is even recorded as extinct (Henning and Henning 1989). All species of *Lepidochrysops* are presumed to be predators of *Camponotus* ants (Formicidae: Formicinae) or feed by trophallaxis, and are almost certainly highly specialized (Cottrell 1984) but diet has only been documented in 11 of the species (Pierce 1995). We do not have sufficient comparisons with their presumed food sources to infer life histories for different species, but the $\delta^{15}N$ signatures are remarkably uniform considering the different localities where they fly (Figure 4.5).

Crudaria is a small endemic genus with only three known species. *C. leroma* have been reared out on acacia (*Vachellia*) plants and are thus herbivorous. *C. wykehami* lay their eggs on acacia plants, but their final instar caterpillars and their pupae can be found in *Anoplolepis* ant nests, and thus they might be aphytophagous for at least part of their life history. The life history of *C. capensis* is not well known,

but a pupa of this species has also been found in an ant nest (Heath pers. comm.). Thus it might also be aphytophagous for all or part of its life history. For the *C. leroma* where we have plant and ant samples, $\delta^{15}N$ is enriched by about a trophic level from the plants, and they are below the ant values (Figure 4.5). Their $\delta^{13}C$ are similar to the plant values and lower than the ant values. This is consistent with the hypothesis that *C. leroma* are phytophagous and only tended by ants. *C. wykehami* $\delta^{13}C$ values are much more similar to those of the ants they are thought to be parasitizing than to the plants, but for two populations, the $\delta^{15}N$ values are similar for the butterflies and the ants and not enriched by a trophic level (Figure 4.5). Thus it is possible that while they live in ant nests, they do not feed on the ants, but workers may feed the caterpillars the same food that the ants are eating.

Winterberg Aloeides

$\delta^{15}N$ studies can give strong impications regarding the specific feeding habits of the caterpillars. One such example is of the four species of *Aloeides* that all fly on the Winterberg in the Eastern Cape.

A. pringlei and *A. macmasteri* can be seen flying at the same time on one saddle of the Winterberg mountain. While *A. macmasteri* only fly on one side of the saddle, *A. pringlei* fly mostly on the other side, but also overlap on the *A. macmasteri* side. It is unusual to have two such closely related species flying at the same time, in the same place but to not intermix. It turns out that they are separated by food source. *A. pringlei* $\delta^{15}N$ values are approximately one trophic level above the *Lepisiota* ants

found in the same habitat (Formicidae: Formicinae). Values of *A. macmasteri* $\delta^{15}N$ are approximately one trophic level above *Indigastrum* plants (Fabaceae), which are significantly less enriched in $\delta^{15}N$ than *Lepisiota* ants. The *A. pringlei* butterflies also have much more similar $\delta^{13}C$ values to *Lepisiota* ants than they do the *Indigastrum* plants, and vice versa for *A. macmasteri* butterflies (Figure 4.6). Thus even though these two species fly at the same time and in the same place, we can assume that they partition their habitat by food source.

A. oreas and *A. dicksoni* fly close by to the saddle where *A. macmasteri* and *A. pringlei* fly, but they they are never seen flying in the same places (they are usually on different hilltops). *A. oreas* and *A. dicksoni* have overlapping $\delta^{15}N$ and $\delta^{13}C$ signatures with *A. macmasteri* (Figure 4.6). They probably feed on the same food source (*Indigastrum* plants), but they are separated from *A. macmasteri* spatially and temporally.

APPENDIX 1

Chapter 1 Supplemental

Taxonomy and Systematics of the Miletinae (Lepidoptera: Lycaenidae)

Introduction

Early hypotheses of relationships within the Miletinae relied on morphological and life history characters (Figure A1.1; Eliot 1973, 1986; Corbet *et al.* 1994). The taxonomic work of Eliot (1961, 1973, 1986) and Libert (1994a, b) have provided a stable taxonomic foundation for the Miletinae, but none of these publications provides a character matrix that can be used for cladistic analysis.

The position of the Miletinae within the Lycaenidae has also been uncertain. Although Balduf (1938) inferred that miletines were sister to the Lipteninae because of their unusual dietary habits, Ackery and colleagues (1999) and Scott and Wright (1990) used morphological evidence to support a sister-group relationship between Poritiinae and Miletinae. Robbins (1988) also used morphological evidence to include these two subfamilies in a polytomy with Curetinae. Eliot (1973; Corbet *et al.* 1994) added Lycaeninae to this unresolved polytomy, which he placed as sister to the Riodininae.

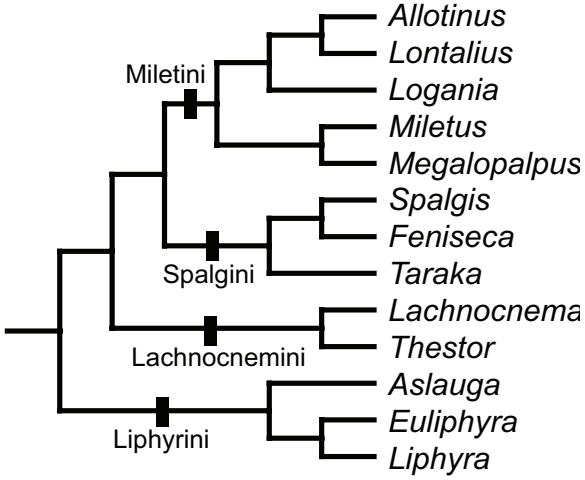

Figure A1.1 Synthesis of morphological phylogenetic hypotheses of generic relationships within the Miletinae by (Eliot 1973, 1986; Corbet *et al.* 1994; Libert 1994b, a).

Results

Our molecular phylogeny (Figure 1.1) serves as an independent evaluation of the morphology-based systematic conclusions of Eliot (1973, 1986; Corbet *et al.* 1994) and Libert (1994a, b) (Figure A1.1). Although these authors did not use optimality criteria in delineating their morphologically defined subfamilies, tribes, and genera, their taxonomic designations were meant to designate monophyletic groups. The four tribes of the Miletinae are all monophyletic, and their relationships to one another are identical to those suggested by Eliot: (Liphyrini (Lachnocnemini (Spalgini+Miletini))) (Figures 1.1, A1.1). The placement of the African genus *Megalopalpus* within the Miletini is the only difference between our phylogenetic reconstruction and Eliot's, who suggested that the genus was sister to *Miletus*. We find that *Megalopalpus* is sister to the other genera in the Miletini, which are all Oriental. Eliot (1973) included the genera *Paraslauga,*

Euliphyrodes, and *Egumbia* in the subfamily Liphyrinae, but Libert (1994a, b) subsequently sank these genera into *Aslauga*, and Eliot subsequently designated the Liphyrinae as the tribe Liphyrini within the Miletinae (Corbet *et al.* 1994). Our analysis suggests that the Liphyrini is sister to the rest of the Miletinae and thus could be considered either a subfamily or a tribe; we prefer the latter to maintain a stable taxonomy. However, our sample does not include species that would be placed in Eliot's (1973) genera *Paraslauga, Euliphyrodes*, and *Egumbia*, and we therefore cannot evaluate the taxonomic changes of Libert (1994a, b, 1997).

The genus *Allotinus* is not monophyletic because the monotypic genus *Lontalius* is strongly supported as a member of the *Allotinus* clade. Few morphological characters distinguish *Lontalius* from *Allotinus*, and Eliot recognized their close affinity when formulating the genus name as an anagram of *Allotinus* (Eliot 1986). *Lontalius eltus* is therefore more appropriately called *Allotinus eltus* (Eliot). Eliot most likely described this species in a new genus because its distinctive, mottled wing patterns differ markedly from the remarkably uniform *Allotinus sensu strictu*. Wing patterns among different species of *Allotinus* are so similar that genitalic dissection of males is frequently required for confident identification. However, this morphological uniformity masks considerable genetic diversity. The genus *Allotinus* is more genetically divergent than any other miletine genus; COI pairwise distances between congeneric *Allotinus* species were as large as 12.5%, which is greater than the genetic divergence between species in different genera. *Logania marmorata* and *Allotinus strigatus*, for example, differed by only 7.4%. There is a well-supported, basal split in the genus, and each of Eliot's (1986) subgenera (Table A1.1) is monophyletic except that *Allotinus* (*Allotinus*) *nicholsi* comes out in a clade with the subgenus *Paragerydus*. Eliot (1961) also subdivided *Miletus* into species groups based primarily on the shape of the clasp of the male genitalia. While the *symethus* group appears to be monophyletic, the *boisduvali* and *chinensis* groups are not (Table A1.1, Figure 1.1).

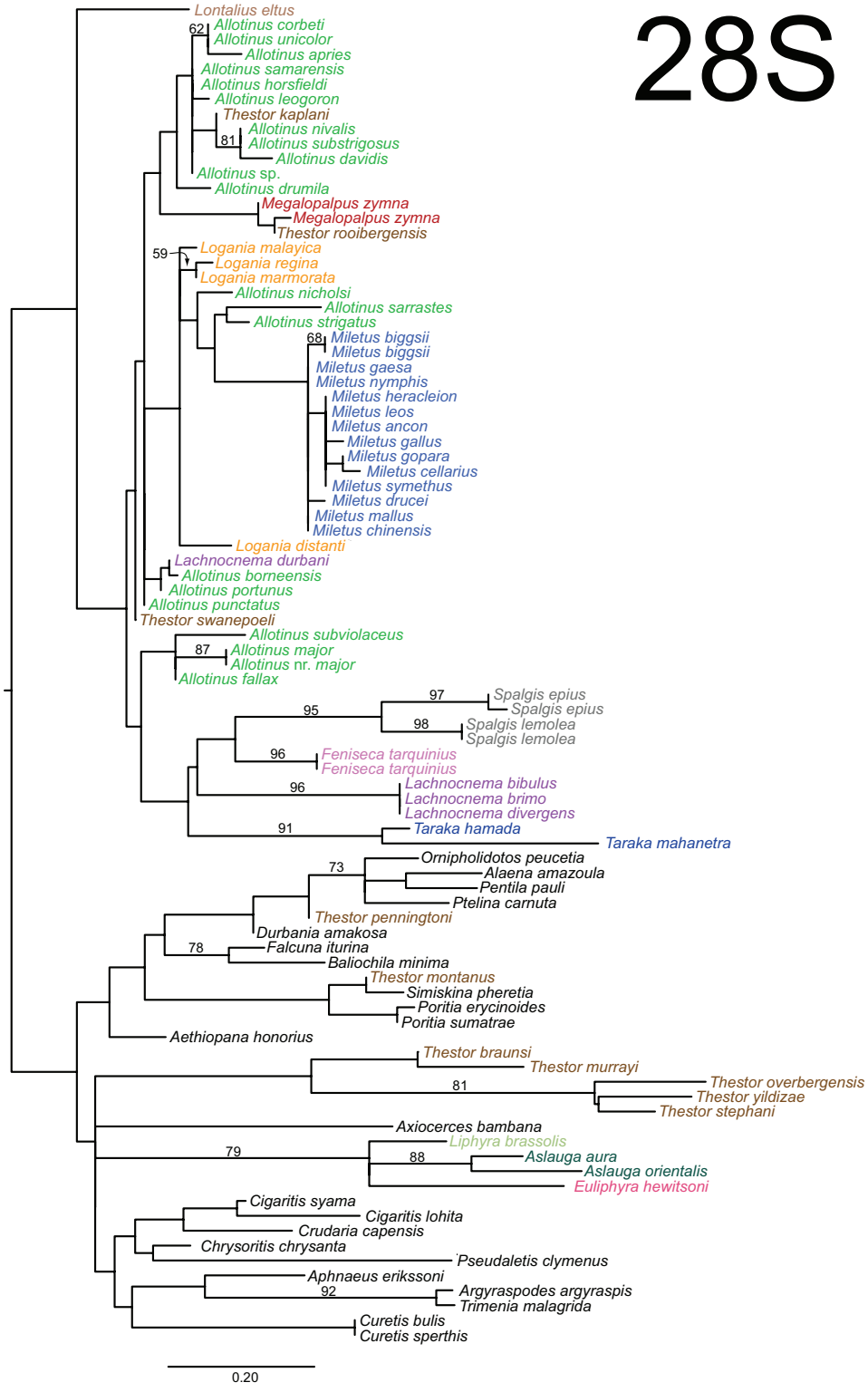

Figure A1.2 Maximum likelihood phylogenies based on single genes do not recover the topology of the tree based on the full data set. Branch support values from 100 bootstrap replicates are provided on most likely trees found by GARLI 0.95.

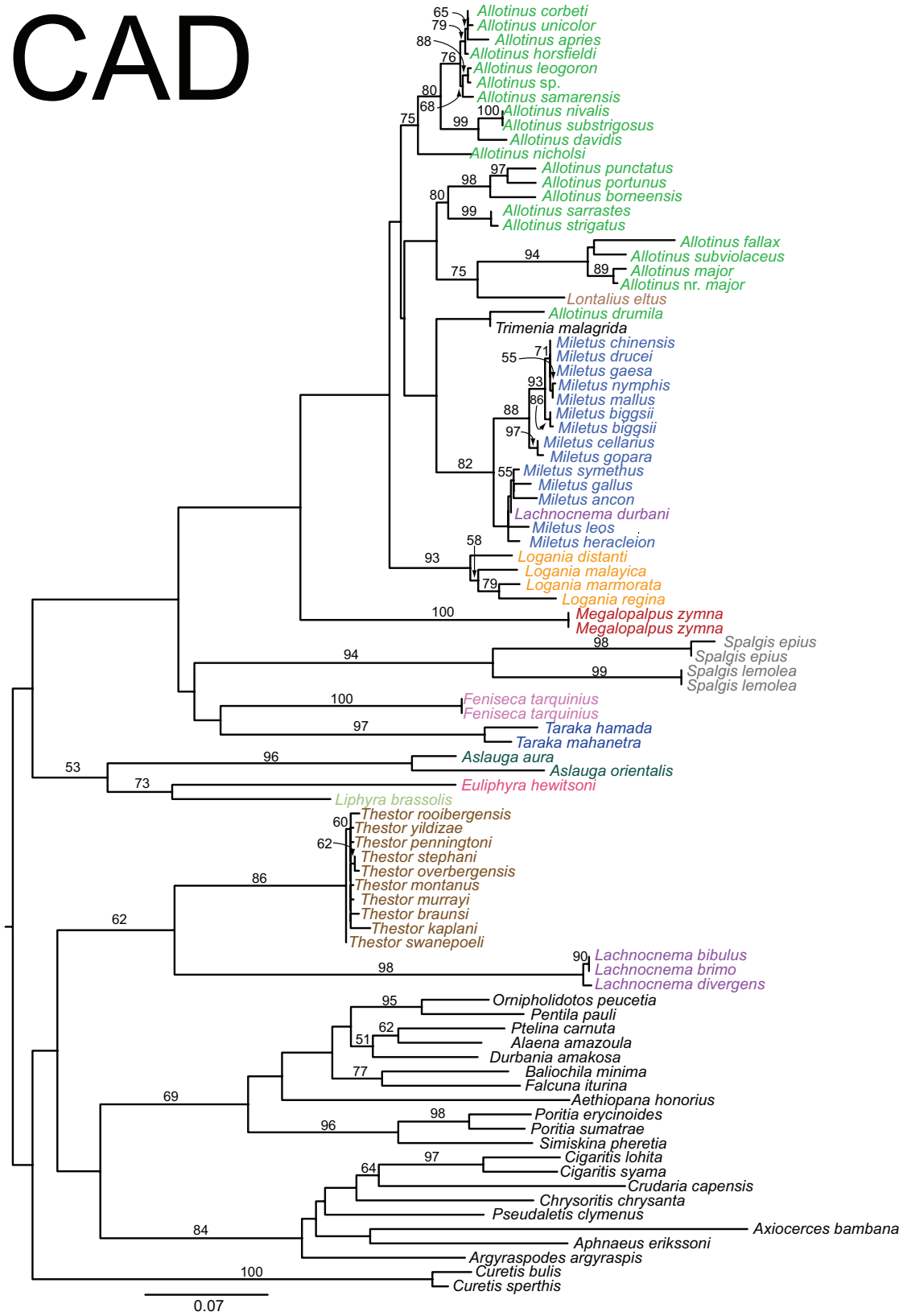

Figure A1.2 (Continued)

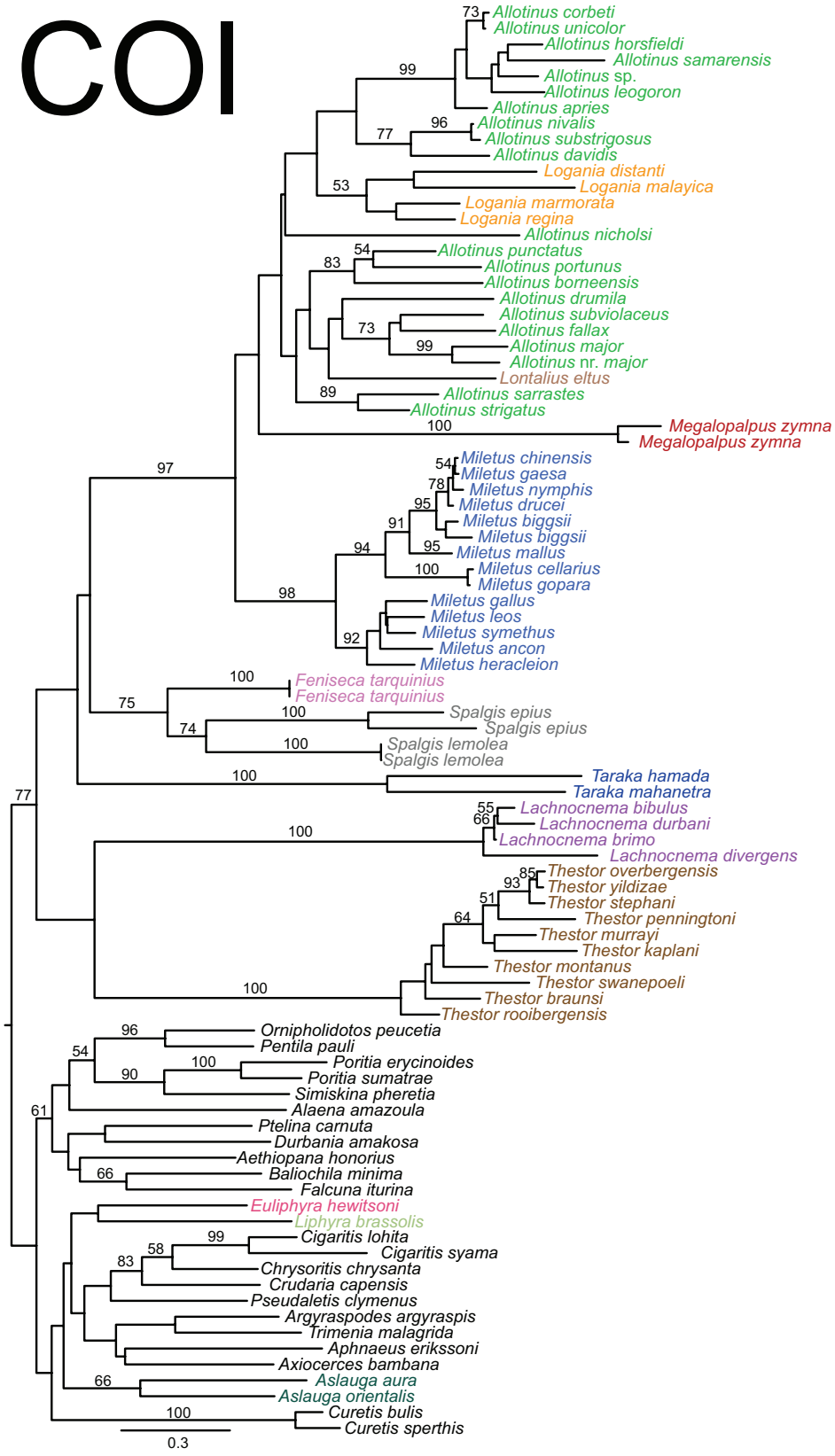

Figure A1.2 (Continued)

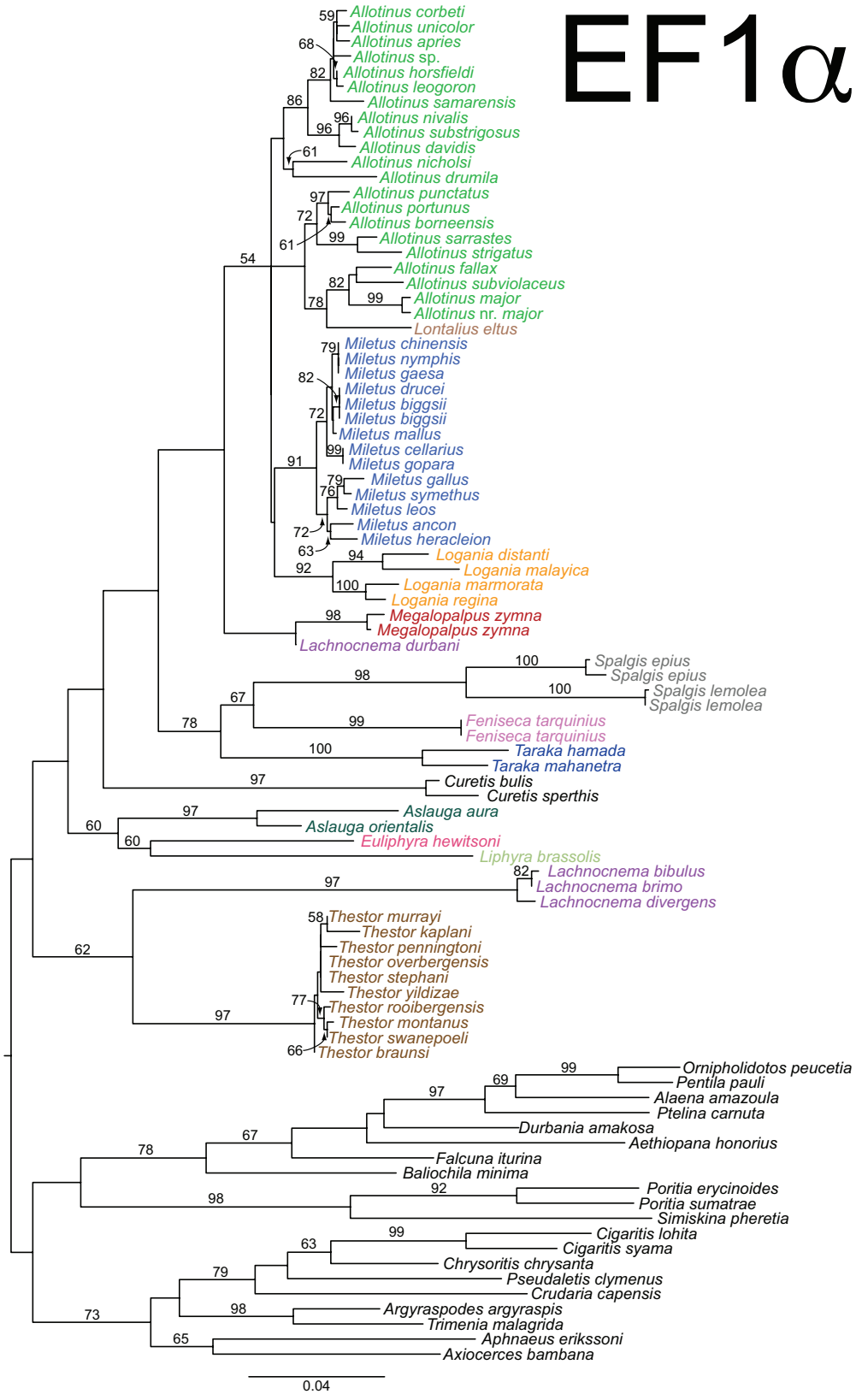

Figure A1.2 (Continued)

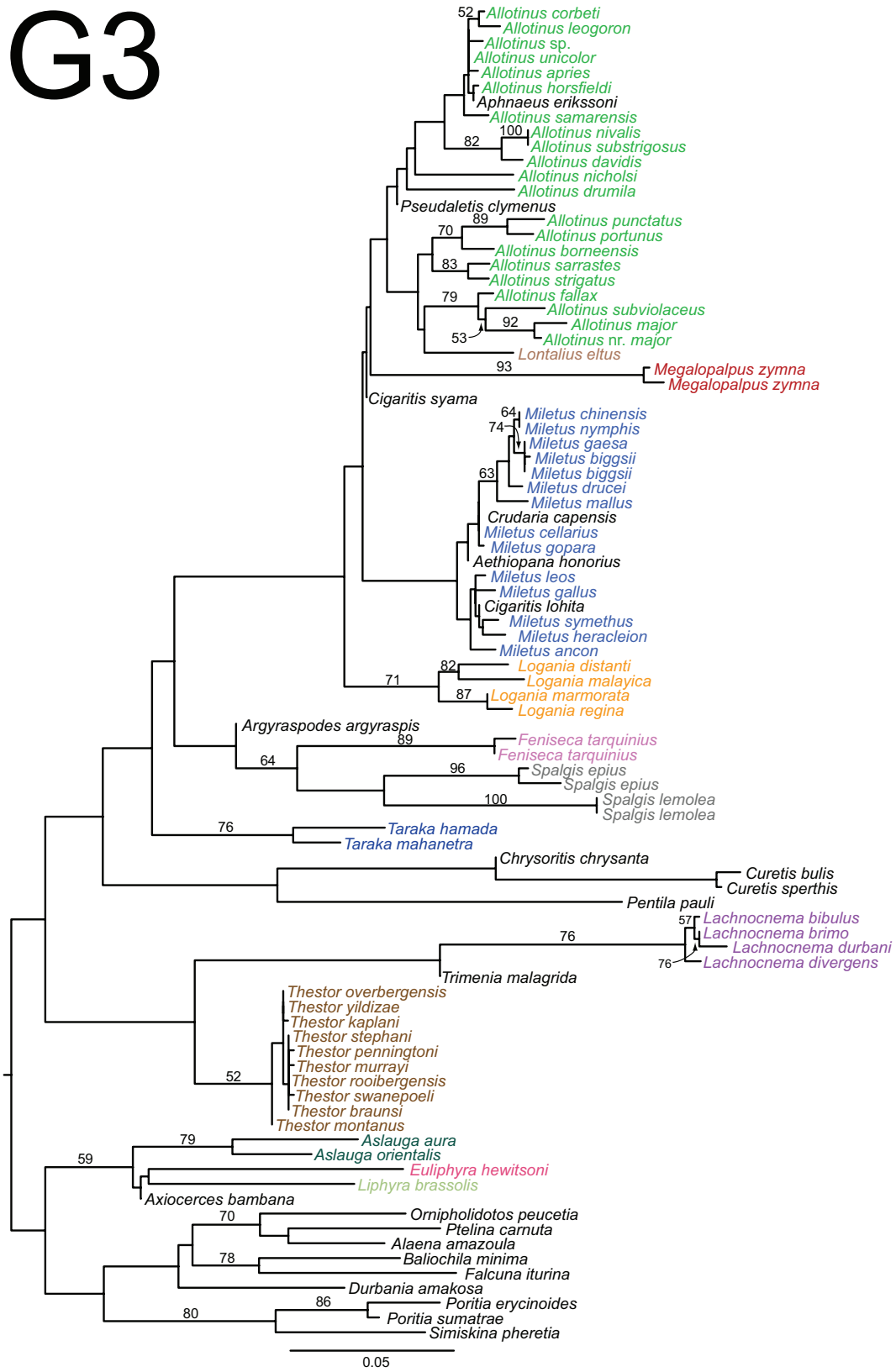

Figure A1.2 (Continued)

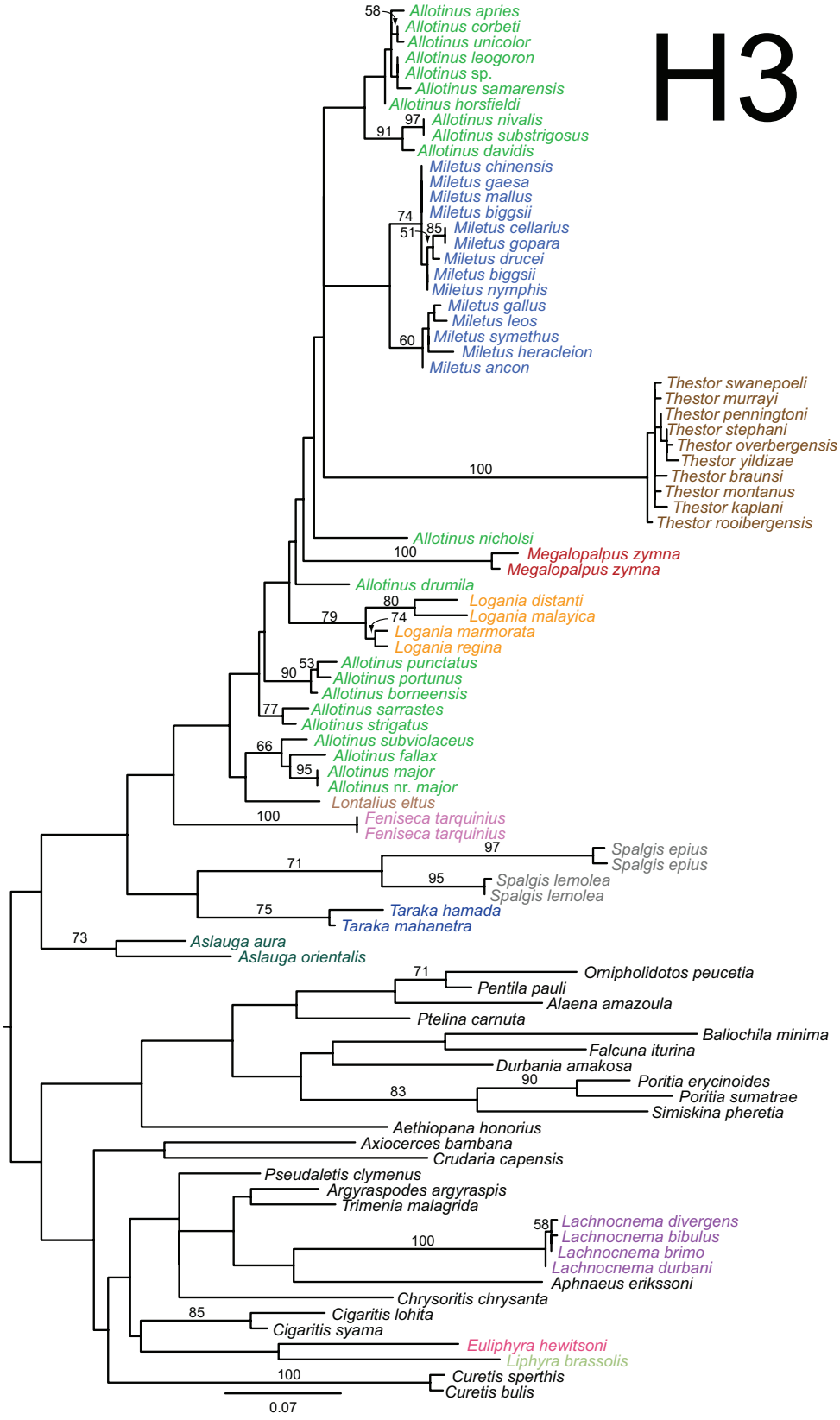

Figure A1.2 (Continued)

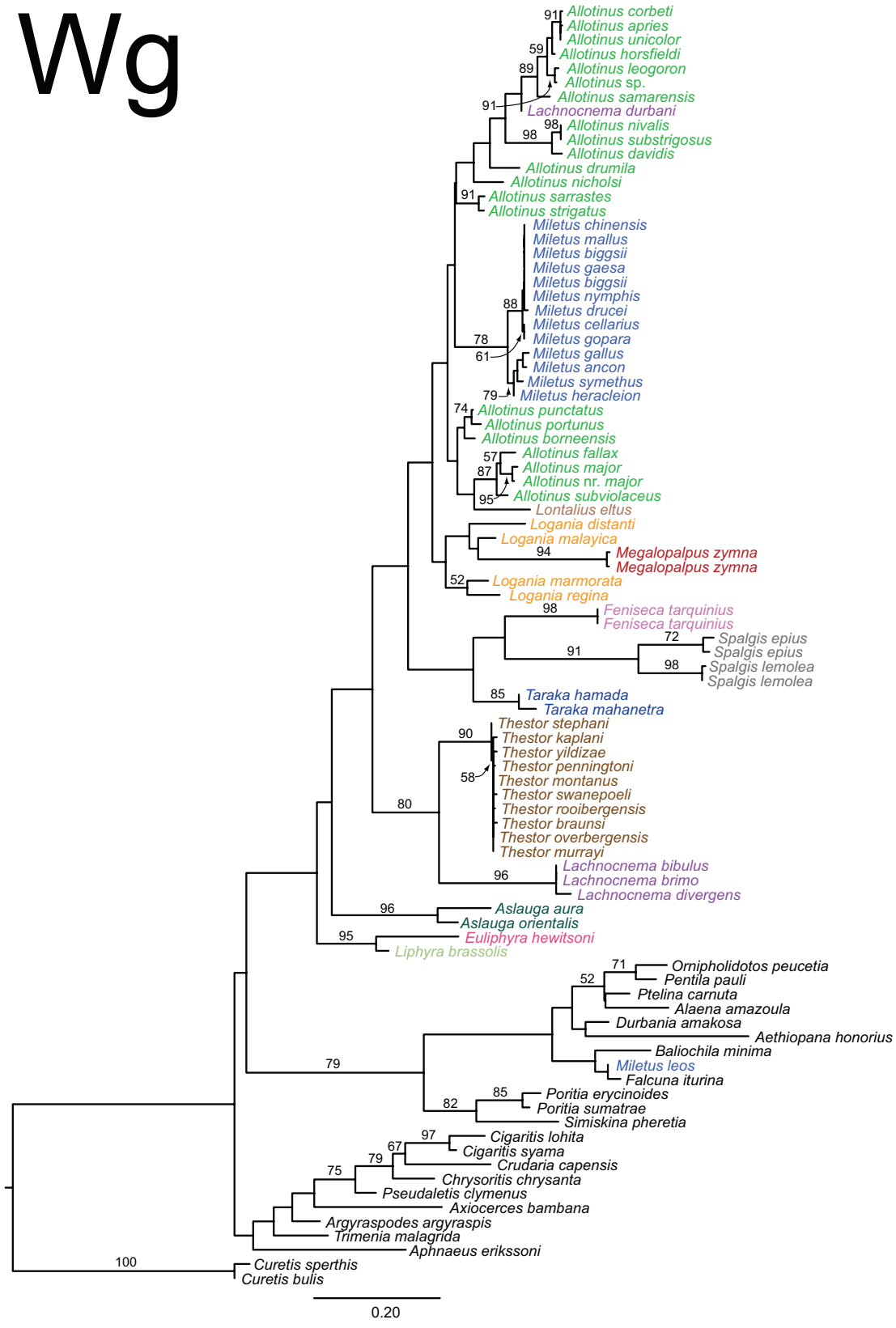

Figure A1.2 (Continued)

Table A1.1 Specimen information and GenBank accession numbers for species included in this study. The 68 Miletinae species in the ingroup include all 13 currently recognized genera, and approximately half of all described species. Genomic DNA was extracted from imagos, with the exception of two samples of immature stages marked with an asterisk (*). Taxonomy follows Eliot (1986), except for Miletus species group designations, which are from Eliot (1961). Vouchers are deposited in the DNA and Tissues Collection of the Museum of Comparative Zoology, Harvard University.

Taxon	Voucher	Collection Locality	COI	EF1a	Wg	H3	CAD	G3PD	28s
Miletinae: Lachnocnemini									
Lachnocnema									
Lachnocnema bibulus	AJG-07-N839	Harare, Zimbabwe	KP216082	KP216010	KP215939	KP215864	KP216157	KP215701	KP215814
Lachnocnema brimo	DJM-07-A101	Kajiado North, Kenya	KP216083	KP216011	KP215940	KP215865	KP216158	KP215702	KP215827
Lachnocnema divergens	RD-98-U130	Beni, Congo	KP216084	KP216012	KP215941	KP215866	KP216159	KP215703	KP215779
Lachnocnema durbani	AH-07-P539	KwaZulu-Natal, South Africa	KP216085			KP215867		KP215704	
Thestor									
Thestor braunsi	AH-98-U642	Western Cape, South Africa	KP216112	KP216039	KP215967	KP215896	KP216188	KP215733	
Thestor kaplani	AH-98-U641	Western Cape, South Africa	KP216114	KP216041	KP215969	KP215898	KP216190	KP215735	
Thestor montanus	AH-99-U429	Western Cape, South Africa	KP216115	KP216042	KP215970	KP215899	KP216191	KP215736	
Thestor murrayi	AH-98-U592	Western Cape, South Africa	KP216113	KP216040	KP215968	KP215897	KP216189	KP215734	KP215754
Thestor overbergensis	AH-98-Y796	Western Cape, South Africa	KP216111	KP216038	KP215966	KP215895	KP216187	KP215732	KP215811
Thestor penningtoni	AH-99-T260	Western Cape, South Africa	KP216116	KP216043	KP215971	KP215900	KP216192	KP215737	
Thestor rooibergensis	AH-99-U410	Western Cape, South Africa	KP216117	KP216044	KP215972	KP215901	KP216193	KP215738	
Thestor stepheni	AH-99-U470	Western Cape, South Africa	KP216118	KP216045	KP215973	KP215902	KP216194	KP215739	KP215812
Thestor swanepoeli	AH-98-Y805	Western Cape, South Africa	KP216119	KP216046	KP215974	KP215903	KP216195	KP215740	
Thestor yildizae	AH-98-U599	Western Cape, South Africa	KP216120	KP216047	KP215975	KP215904	KP216196	KP215741	KP215755
Miletinae: Liphyrini									
Aslauga									
Aslauga aura	RD-00-T111	Beni, Congo	KP216078	KP216006	KP215935	KP215860	KP216153	KP215696	KP215780
Aslauga orientalis	SC-99-T553	Shimba Hills, Kenya	KP216079	KP216007	KP215936	KP215861	KP216154	KP215697	KP215781
Euliphyra									
*Euliphyra hewitsoni**	SC-99-T536	Banco, Ivory Coast	KP216080	KP216008	KP215937	KP215862	KP216155	KP215698	KP215782
Liphyra									
*Liphyra brassolis**	KD-94-T063	Queensland, Australia	DQ018940	DQ018907	AF233551	KP215868	KP216160	KP215705	KP215783
Milentinae: Miletini									
Allotinus									
Allotinus (*Allotinus*) *fallax*	DL-07-B157	Quezon, Philippines	KP216061	KP215989	KP215919	KP215843	KP216136	KP215679	KP215822

Table A1.1 (Continued)

Taxon	Voucher	Collection Locality	COI	EF1a	Wg	H3	CAD	G3PD	28s
Allotinus (*A.*) *major*	DL-07-B359	Southeast Sulawesi, Indonesia	KP216065	KP215993	KP215922	KP215847	KP216140	KP215683	KP215826
Allotinus (*A.*) nr. *major*	DL-07-Z020	South Sulawesi, Indonesia	KP216068	KP215996	KP215925	KP215850	KP216143	KP215686	KP215818
Allotinus (*A.*) *nicholsi*	MWT-93-E039	Sarawak, Malaysia	KP216066	KP215994	KP215923	KP215848	KP216141	KP215684	KP215785
Allotinus (*A.*) *subviolaceus*	MWT-93-D011	Sabah, Malaysia	KP216076	KP216004	KP215933	KP215858	KP216151	KP215694	KP215772
Allotinus (*Fabitaras*) *borneensis*	DXC-99-S325	Central Kalimantan, Indonesia	KP216057	KP215985	KP215915	KP215839	KP216132	KP215675	KP215810
Allotinus (*F.*) *portunus*	KF-94-P024	Sabah, Malaysia	KP216070	KP215998	KP215927	KP215852	KP216145	KP215688	KP215765
Allotinus (*F.*) *punctatus*	DL-07-Z012	Mindanao, Philippines	KP216069	KP215997	KP215926	KP215851	KP216144	KP215687	KP215815
Allotinus (*F.*) *sarrastes*	KF-94-P016	Sabah, Malaysia	KP216072	KP216000	KP215929	KP215854	KP216147	KP215690	KP215763
Allotinus (*F.*) *strigatus*	DXC-99-S310	Central Kalimantan, Indonesia	KP216074	KP216002	KP215931	KP215856	KP216149	KP215692	KP215809
Allotinus (*Paragerydus*) *apries*	KF-94-P015	Sabah, Malaysia	KP216056	KP215984	KP215914	KP215838	KP216131	KP215674	KP215762
Allotinus (*P.*) *corbeti*	NP-95-Z261	Sarawak, Malaysia	KP216058	KP215986	KP215916	KP215840	KP216133	KP215676	KP215778
Allotinus (*P.*) *drumila*	DL-02-P759	Chiang Mai, Thailand	KP216060	KP215988	KP215918	KP215842	KP216135	KP215678	KP215757
Allotinus (*P.*) *davidis*	MWT-93-A069	Selangor, Malaysia	KP216059	KP215987	KP215917	KP215841	KP216134	KP215677	KP215806
Allotinus (*P.*) *horsfieldi*	MWT-93-C004	Perak, Malaysia	KP216062	KP215990	KP215920	KP215844	KP216137	KP215680	KP215771
Allotinus (*P.*) *leogoron*	MWT-93-B034	Pahang, Malaysia	KP216063	KP215991	KP215921	KP215845	KP216138	KP215681	KP215770
Allotinus (*P.*) *nivalis*	DXC-99-S312	Central Kalimantan, Indonesia	KP216067	KP215995	KP215924	KP215849	KP216142	KP215685	KP215807
Allotinus (*P.*) *samarensis*	DL-07-B358	Southeast Sulawesi, Indonesia	KP216071	KP215999	KP215928	KP215853	KP216146	KP215689	KP215825
Allotinus (*P.*) *substrigosus*	MWT-93-A030	Kuala Lumpur, Malaysia	KP216075	KP216003	KP215932	KP215857	KP216150	KP215693	KP215766
Allotinus (*P.*) *unicolor*	MWT-93-A042	Kuala Lumpur, Malaysia	KP216077	KP216005	KP215934	KP215859	KP216152	KP215695	KP215767
Allotinus sp.	DL-07-B206	West Sumatra, Indonesia	KP216073	KP216001	KP215930	KP215855	KP216148	KP215691	KP215824
Logania									
Logania distanti	MWT-93-B021	Selangor, Malaysia	KP216087	KP216014	KP215943	KP215870	KP216162	KP215707	KP215769
Logania malayica	KF-94-P011	Sabah, Malaysia	KP216088	KP216015	KP215944	KP215871	KP216163	KP215708	KP215786
Logania marmorata	DL-06-B054	Singapore	KP216086	KP216013	KP215942	KP215869	KP216161	KP215706	KP215819
Logania regina	DXC-99-S318	East Kalimantan, Indonesia	KP216089	KP216016	KP215945	KP215872	KP216164	KP215709	KP215759
Lontalius									
Lontalius eltus	DXC-99-S322	Central Kalimantan, Indonesia	KP216090	KP216017	KP215946	KP215873	KP216165	KP215710	KP215787
Megalopalpus									
Megalopalpus zymna	TL-96-W950	Prah-Suhien, Ghana	KP216092	KP216019	KP215948	KP215875	KP216167	KP215712	KP215784
Megalopalpus zymna	RD-98-U079	Beni, Congo	KP216091	KP216018	KP215947	KP215874	KP216166	KP215711	KP215823
Miletus									
***boisduvali* group**									
Miletus biggsii	MWT-93-A068	Selangor, Malaysia	KP216094	KP216021	KP215950	KP215878	KP216170	KP215715	KP215768
Miletus biggsii	DL-06-B055	Singapore	KP216093	KP216020	KP215949	KP215876	KP216168	KP215713	KP215820
Miletus cellarius	NP-95-Z260	Sarawak, Malaysia	KP216095	KP216022	KP215951	KP215879	KP216171	KP215716	KP215777
Miletus drucei	DL-07-B094	Quezon, Philippines	KP216097	KP216024	KP215953	KP215881	KP216173	KP215718	KP215821

Table A1.1 (Continued)

Taxon	Voucher	Collection Locality	COI	EF1a	Wg	H3	CAD	G3PD	28s
***chinensis* group**									
Miletus chinensis	NP-95-Y274	Perak, Malaysia	KP216096	KP216023	KP215952	KP215880	KP216172	KP215717	KP215776
Miletus gaesa	NP-95-Y201	Pahang, Malaysia	KP216098	KP216025	KP215954	KP215882	KP216174	KP215719	KP215774
Miletus mallus	DL-01-Q670	Chiang Mai, Thailand	KP216102	KP216029	KP215958	KP215886	KP216178	KP215723	KP215756
Miletus nymphis	DL-02-Q785	Chumpon, Thailand	KP216103	KP216030	KP215959	KP215887	KP216179	KP215724	KP215758
***symethus* group**									
Miletus ancon	KF-94-P022	Sabah, Malaysia	DQ018941	DQ018908	AF233550	KP215877	KP216169	KP215714	KP215764
Miletus gallus	MWT-93-A072	Kuala Lumpur, Malaysia	KP216099	KP216026	KP215955	KP215883	KP216175	KP215720	KP215788
Miletus heracleion	DXC-99-S326	Central Kalimantan, Indonesia	KP216101	KP216028	KP215957	KP215885	KP216177	KP215722	KP215808
Miletus leos	DL-07-B364	Maluku, Indonesia	KP216064	KP215992		KP215846	KP216139	KP215682	KP215828
Miletus symethus	NP-95-Y244	Pahang, Malaysia	KP216104	KP216031	KP215960	KP215888	KP216180	KP215725	KP215775
***zinckenii* group**									
Miletus gopara	KF-94-P013	Sabah, Malaysia	KP216100	KP216027	KP215956	KP215884	KP216176	KP215721	KP215761
Miletinae: Spalgini									
Feniseca									
*Feniseca tarquinius**	JXM-98-R545	Massachusetts, USA	KP216081	KP216009	KP215938	KP215863	KP216156	KP215699	KP215760
Feniseca tarquinius	MWT-96-Y333	Massachusetts, USA	KF787220	KF787409	KF787393	KF787149	KF787490	KP215700	KP215789
Spalgis									
Spalgis epius	MWT-93-C079	Pahang, Malaysia	KP216105	KP216032	AF233552	KP215889	KP216181	KP215726	KP215791
Spalgis epius	PDV-93-E009	Hainan, China	KP216106	KP216033	KP215961	KP215890	KP216182	KP215727	KP215816
Spalgis lemolea	RD-98-U155	Beni, Congo	KP216108	KP216035	KP215963	KP215892	KP216184	KP215729	KP215790
Spalgis lemolea	AJG-07-N803	Mozambique	KP216107	KP216034	KP215962	KP215891	KP216183	KP215728	KP215813
Taraka									
Taraka hamada	UA-95-N001	Rissho, Japan	KP216109	KP216036	KP215964	KP215893	KP216185	KP215730	KP215792
Taraka mahanetra	NP-95-Y171	Pahang, Malaysia	KP216110	KP216037	KP215965	KP215894	KP216186	KP215731	KP215773
Curetinae									
Curetis bulis	MWT-93-A028	Kuala Lumpur, Malaysia	DQ018942	DQ018909	AF233549	KP215829	KP216122	KP215665	KP215793
Curetis sperthis	MWT-93-E013	Sabah, Malaysia	KP216049	KP215977	KP215907	KP215830	KP216123	KP215666	KP215794
Poritiinae: Liptenini									
Aethiopana honorius	TL-96-W905	Kibi, Ghana	KP216050	KP215978	KP215908	KP215831	KP216124		KP215796
Baliochila minima	SP-93-P006	North Coast, Kenya	DQ018938	DQ018905	DQ018879	KP215833	KP216126	KP215668	KP215798
Durbania amakosa	SJ-97-Y839	KwaZulu-Natal, South Africa	KP216052	KP215980	KP215910	KP215834	KP216127	KP215669	KP215795
Falcuna iturina	RD-98-U085	Beni, Congo	KP216053	KP215981	KP215911	KP215835	KP216128	KP215670	KP215797

Table A1.1 (Continued)

Taxon	Voucher	Collection Locality	COI	EF1a	Wg	H3	CAD	G3PD	28s
Poritiinae: Pentilini									
Alaena amazoula	AH-95-Y843	Buumba Cloudlands, South Africa	KP216051	KP215979	KP215909	KP215832	KP216125	KP215667	KP215799
Ornipholidotos peucetia	SJ-97-Y842	KwaZulu-Natal, South Africa	KF787221	KF787410	KF787396	KF787150	KF787491	KP215671	KP215802
Pentila pauli	TL-96-X010	Aburi, Ghana	KP216054	KP215982	KP215912	KP215836	KP216129	KP215672	KP215801
Ptelina carnuta	TL-96-W931	Swedru, Ghana	KP216055	KP215983	KP215913	KP215837	KP216130	KP215673	KP215800
Poritiinae: Poritiini									
Poritia erycinoides	MWT-93-B007	Selangor, Malaysia	DQ018939	DQ018906	DQ018880	KP215905	KP216197	KP215742	KP215803
Poritia sumatrae	MWT-93-D004	Kokol, Sabah, Malaysia	KP216121	KP216048	KP215976	KP215906	KP216198	KP215743	KP215804
Simiskina pheretia	NP-95-Y170	Pahang, Malaysia	KF787222	KF787411	AF233547	KF787151	KF787492	KP215744	KP215805
Aphnaeinae									
Aphnaeus erikssoni	AH-99-U517	Harare, Zimbabwe	KF787237	KF787426	KF787359	KF787166	KF787507		KP215745
Argyraspodes argyraspis	AH-95-Z422	Northern Cape, South Africa	AF279223	KF787432	KF787363	KF787171	KF787513		KP215746
Axiocerces bambana	RD-98-U072	Beni, Congo	KF787245	KF787434	KF787364	KF787173	KF787515		KP215747
Chrysoritis chrysantas	AH-95-Z431	Northern Cape, South Africa	AF279218	KF787449	KF787370	KF787184	KF787530		KP215748
Cigaritis lohita	MWT-93-A022	Kuala Lumpur, Malaysia	KF787288	KF787476	KF787380	KF787206	KF787558		KP215749
Cigaritis syama	MWT-93-A039	Kuala Lumpur, Malaysia	KF787291	KF787479	KF787385	KF787209	KF787561		KP215750
Crudaria capensis	AAM-98-W799	Western Cape, South Africa	KF787273	KF787462	KF787388	KF787191	KF787543		KP215751
Pseudaletis clymenus	RD-98-U087	Beni, Congo	KF787285	KF787473	KF787401	KF787203	KF787555		KP215752
Trimenia malagrida	AH-98-U487	Western Cape, South Africa	KF787296	KF787484	KF787405	KF787214	KF787566		KP215753

Table A1.2 Primers used in this study. Primers marked in bold were used for initial amplification attempts and primers marked in plain text were used on recalcitrant samples.

Locus	Direction	Sequence	Short Reference
COI			
LCO1490	F	GGTCAACAAATCATAAAGATATTGG	(Folmer *et al.* 1994)
Nancy	R	CCCGGTAAAATTAAAATATAAACTTC	(Simon *et al.* 1994)
TN2126	F	TTGAYCCTGCAGGTGGWGGAG	Eastwood, unpublished
Hobbes	R	AAATGTTGNGGRAAAATGTTA	(Monteiro and Pierce 2001)
EF			
EFM44F	F	GCYGARCGYGARCGTGGTATYAC	(Cho *et al.* 1995)
EF46.1F	F	GAGGAAATYAARAAGGAAG	(Cho *et al.* 1995)
EF51.1	R	CATGTTGTCKCCGTGCCATCC	(Cho *et al.* 1995)
EF51.9F	F	CARGACGTATACAAAATCGG	(Cho *et al.* 1995)
EFC52.6R	R	GCYTCGTGGTGCATYTCSAC	(Cho *et al.* 1995)
EFCM4R	R	ACAGCVACKGTYTGYCTCATRTC	(Cho *et al.* 1995)
Wingless			
Wg1	F	GARTGYAARTGYCAYGGYATGTCTGG	(Brower and DeSalle 1998)
Wg1E	F	CATGGYATGTCTGGTTCCTG	this study
Wg2E	R	ACNACGAACATGGTCTGCGT	this study
Wg2	R	ACTICGCRCACCARTGGAATGTRCA	(Brower and DeSalle 1998)
28s			
S3660		GAGAGTTMAASAGTACGTGAAAC	(Sequeira *et al.* 2000)
S1		GACCCGTCTTGAAMCAMGGA	(Sequeira *et al.* 2000)
A1		TCCKGTKTTCAAGACGGGGTC	(Sequeira *et al.* 2000)
A335		TCGGARGGAACCAGCTACTA	(Sequeira *et al.* 2000)
H3			
H3F	F	ATGGCTCGTACCAAGCAGACACGGC	(Colgan *et al.* 1998)
H3R	R	ATATCCTTRGGCATRATRGTGAC	(Colgan *et al.* 1998)
CAD			
CAD787F	F	GGDGTNACNACNGCNTGYTTYGARCC	(Moulton and Wiegmann 2004)
CADFa	F	GDATGGTYGATGAAAATGTTAA	this study
CADRa	R	CTCATRTCGTAATCYGTRCT	this study
CADRb	R	ACRGTTTCRGGGTTGTARTT	this study
G3PD			
G3Fa	F	TGGGGYAAGGCTGGAGCTGAATA	this study
G3Ra	R	CCAGCCGCAGCATCAAAGA	this study

Table A1.3 Calibration points. Five dates extracted from Heikkilä *et al.* (2012) were used to calibrate the Miletinae tree in BEAST. Normally distributed priors within the 95% HPD were assumed.

Clade	Age	95% HPD
(Poritinae + Lipteninae) + Miletinae	60.1025	48.1793 – 72.2161
Curetinae + (Aphnaeinae + ((Lipteninae+Poritinae)+Miletinae))	71.7373	58.944 – 84.3686
Liphyrini + (Lachnocnemini + (Spalgini + Miletini))	50.1915	38.7481 – 61.8201
Poritinae + Lipteninae	42.8681	30.599 – 55.1154
Miletus + *Allotinus*	29.854	21.3673 – 32.0825

APPENDIX 2

Chapter 2 Supplemental

Table A2.1 Specimen information and GenBank accession numbers for species included in this study. The 124 *Chrysoritis* and 88 *Thestor* individuals include 20 populations of *C. chrysaor*, 7 populations of *T. protumnus* sampled throughout their ranges in South Africa and their outgroups. Vouchers are deposited in the Museum of Comparative Zoology, Harvard University.

Taxon	Voucher	Collection Locality	Locality Coordinates
Chrysoritis chrysaor			
Chrysoritis chrysaor	AH-07-C188	Blaauwberg N.R., W. Cape	33°45.463'S: 18°26.554'E
Chrysoritis chrysaor	AH-07-C238	Blaauwberg N.R., W. Cape	33°45.463'S: 18°26.554'E
Chrysoritis chrysaor	AH-07-C239	Blaauwberg N.R., W. Cape	33°45.463'S: 18°26.554'E
Chrysoritis chrysaor	AH-07-C240	Blaauwberg N.R., W. Cape	33°45.463'S: 18°26.554'E
Chrysoritis chrysaor	AH-95-Z891	Blaauwberg N.R., W. Cape	33°45.463'S: 18°26.554'E
Chrysoritis chrysaor	AH-07-B255	Calitzdorp, W. Cape	33°30'S: 21°37'E
Chrysoritis chrysaor	AH-07-B257	Calitzdorp, W. Cape	33°30'S: 21°37'E
Chrysoritis chrysaor	AH-07-B258	Calitzdorp, W. Cape	33°30'S: 21°37'E
Chrysoritis chrysaor	AH-07-B259	Calitzdorp, W. Cape	33°30'S: 21°37'E
Chrysoritis chrysaor	AH-07-B262	Calitzdorp, W. Cape	33°30'S: 21°37'E
Chrysoritis chrysaor	AH-07-B263	Calitzdorp, W. Cape	33°30'S: 21°37'E
Chrysoritis chrysaor	AH-07-B264	Calitzdorp, W. Cape	33°30'S: 21°37'E
Chrysoritis chrysaor	AH-07-B265	Calitzdorp, W. Cape	33°30'S: 21°37'E
Chrysoritis chrysaor	AH-07-B266	Calitzdorp, W. Cape	33°30'S: 21°37'E
Chrysoritis chrysaor	AH-07-B267	Calitzdorp, W. Cape	33°30'S: 21°37'E
Chrysoritis chrysaor	AH-07-B270	Calitzdorp, W. Cape	33°30'S: 21°37'E
Chrysoritis chrysaor	AH-07-B271	Calitzdorp, W. Cape	33°30'S: 21°37'E
Chrysoritis chrysaor	AH-07-B272	Calitzdorp, W. Cape	33°30'S: 21°37'E
Chrysoritis chrysaor	AH-07-C387	Coombs, E. Cape	33 17.445 S; 26 50.922 E
Chrysoritis chrysaor	AH-12-E089	Du Toits Kloof Pass, W. Cape	35 41 52.69 S 19 04 13.27 E
Chrysoritis chrysaor	AH-12-E090	Du Toits Kloof Pass, W. Cape	36 41 52.69 S 19 04 13.27 E
Chrysoritis chrysaor	AH-12-E128	Du Toits Kloof Pass, W. Cape	33 41 52.69 S 19 04 13.27 E
Chrysoritis chrysaor	AH-12-E130	Du Toits Kloof Pass, W. Cape	34 41 52.69 S 19 04 13.27 E
Chrysoritis chrysaor	AH-12-E132	Franschhoek, W. Cape	33°53.641S 19°09.276E
Chrysoritis chrysaor	AH-95-Y661	Fraserrburg, N. Cape	
Chrysoritis chrysaor	AH-95-Y662	Fraserrburg, N. Cape	
Chrysoritis chrysaor	AP-98-W764	Gouritzmond, W. Cape	
Chrysoritis chrysaor	AP-98-W768	Gouritzmond, W. Cape	
Chrysoritis chrysaor	AH-07-C398	Great Winterberg, E. Cape	
Chrysoritis chrysaor	AH-07-C399	Great Winterberg, E. Cape	
Chrysoritis chrysaor	AH-07-C400	Great Winterberg, E. Cape	
Chrysoritis chrysaor	AH-07-C210	Karoo N.P. nr. Campsite	32°19.980'S: 22°29.494'E
Chrysoritis chrysaor	AH-07-C211	Karoo N.P. nr. Campsite	32°19.980'S: 22°29.494'E
Chrysoritis chrysaor	AH-07-C262	Karoo N.P. nr. Campsite	32°19.980'S: 22°29.494'E
Chrysoritis chrysaor	AH-07-C264	Karoo N.P. nr. Campsite	32°19.980'S: 22°29.494'E
Chrysoritis chrysaor	AH-07-C265	Karoo N.P. nr. Campsite	32°19.980'S: 22°29.494'E
Chrysoritis chrysaor	AH-07-C266	Karoo N.P. nr. Campsite	32°19.980'S: 22°29.494'E
Chrysoritis chrysaor	AH-07-C267	Karoo N.P. nr. Campsite	32°19.980'S: 22°29.494'E

Table A2.1 (Continued)

Taxon	Voucher	Collection Locality	Locality Coordinates
Chrysoritis chrysaor	AH-07-C269	Karoo N.P. nr. Campsite	32°19.980'S: 22°29.494'E
Chrysoritis chrysaor	AH-07-C270	Karoo N.P. nr. Campsite	32°19.980'S: 22°29.494'E
Chrysoritis chrysaor	AH-07-C271	Karoo N.P. nr. Campsite	32°19.980'S: 22°29.494'E
Chrysoritis chrysaor	AH-07-C272	Karoo N.P. nr. Klipspringer Pass	32°19.477'S: 22°26.712'E
Chrysoritis chrysaor	AH-07-C067	Leipoldtville, W. Cape	32°14'S: 18°28'E
Chrysoritis chrysaor	AH-07-C068	Leipoldtville, W. Cape	32°14'S: 18°28'E
Chrysoritis chrysaor	AH-07-C115	Leipoldtville, W. Cape	32°14'S: 18°28'E
Chrysoritis chrysaor	AH-07-C117	Leipoldtville, W. Cape	32°14'S: 18°28'E
Chrysoritis chrysaor	AH-07-C125	Leipoldtville, W. Cape	32°14'S: 18°28'E
Chrysoritis chrysaor	AH-07-C154	Leipoldtville, W. Cape	32°14'S: 18°28'E
Chrysoritis chrysaor	AH-06-M564	Matjiesfontein	
Chrysoritis chrysaor	AH-07-C204	Pearson Road, Nr. Jansenville, E. Cape	32°47.251'S: 24°52.116'E
Chrysoritis chrysaor	AH-07-C205	Pearson Road, Nr. Jansenville, E. Cape	32°47.251'S: 24°52.116'E
Chrysoritis chrysaor	EP-09-Z033	Port Elizabeth	
Chrysoritis chrysaor	AH-06-T729	Port Shepstone (Oslo Beach), KZN	30 46 07.5 S: 30 26 25.3 E
Chrysoritis chrysaor	AH-07-C332	Sarrisaam Farm nr. Soutfontein	30 35.57 S: 17 30.95 E
Chrysoritis chrysaor	AH-07-C334	Sarrisaam Farm nr. Soutfontein	31 35.57 S: 17 30.95 E
Chrysoritis chrysaor	AH-07-C335	Sarrisaam Farm nr. Soutfontein	32 35.57 S: 17 30.95 E
Chrysoritis chrysaor	AH-07-C336	Sarrisaam Farm nr. Soutfontein	33 35.57 S: 17 30.95 E
Chrysoritis chrysaor	AH-07-C337	Sarrisaam Farm nr. Soutfontein	34 35.57 S: 17 30.95 E
Chrysoritis chrysaor	AH-07-C338	Sarrisaam Farm nr. Soutfontein	35 35.57 S: 17 30.95 E
Chrysoritis chrysaor	AH-07-C339	Sarrisaam Farm nr. Soutfontein	36 35.57 S: 17 30.95 E
Chrysoritis chrysaor	AH-00-T287	Schonghong River Valley, Lesotho	S29˚25' / E29˚08'
Chrysoritis chrysaor	AH-07-C386	Sheldon, E. Cape	33° 02' S: 25° 56' E
Chrysoritis chrysaor	AH-07-M650	Silverstream, Bok Bay	33°35.430'S: 18°21.748'E
Chrysoritis chrysaor	AH-07-M651	Silverstream, Bok Bay	33°35.430'S: 18°21.748'E
Chrysoritis chrysaor	AH-07-M652	Silverstream, Bok Bay	33°35.430'S: 18°21.748'E
Chrysoritis chrysaor	AH-07-M653	Silverstream, Bok Bay	33°35.430'S: 18°21.748'E
Chrysoritis chrysaor	AH-07-C372	Willowmore, E. Cape	33 07 58.5 S; 23 37 35.40 E
Chrysoritis chrysaor	AH-07-C373	Willowmore, E. Cape	34 07 58.5 S; 23 37 35.40 E
Chrysoritis chrysaor	AH-07-C375	Willowmore, E. Cape	35 07 58.5 S; 23 37 35.40 E
Chrysoritis chrysaor	AAM-98-W802	Witsand	
Chrysoritis chrysaor	AH-07-C170	Yzerfontein, W. Cape	33°21.485'S: 18°09.691'E
Chrysoritis chrysaor	AH-07-C171	Yzerfontein, W. Cape	33°21.485'S: 18°09.691'E
Chrysoritis chrysaor	AH-07-C172	Yzerfontein, W. Cape	33°21.485'S: 18°09.691'E
Chrysoritis chrysaor	AH-07-C173	Yzerfontein, W. Cape	33°21.485'S: 18°09.691'E
Chrysoritis chrysaor	AH-07-C174	Yzerfontein, W. Cape	33°21.485'S: 18°09.691'E
Chrysoritis chrysaor	AH-07-M625	Yzerfontein, W. Cape	33°20.872'S: 18°09.771'E
Chrysoritis chrysaor	AH-07-M626	Yzerfontein, W. Cape	33°20.872'S: 18°09.771'E
Chrysoritis chrysaor	AH-07-M628	Yzerfontein, W. Cape	33°20.872'S: 18°09.771'E
Chrysoritis chrysaor	AH-07-M629	Yzerfontein, W. Cape	33°20.872'S: 18°09.771'E
Chrysoritis chrysaor	AH-07-M630	Yzerfontein, W. Cape	33°20.872'S: 18°09.771'E
Chrysoritis chrysaor	AH-07-M631	Yzerfontein, W. Cape	33°20.872'S: 18°09.771'E
Chrysoritis chrysaor	AH-07-M635	Yzerfontein, W. Cape	33°20.872'S: 18°09.771'E

Table A2.1 (Continued)

Taxon	Voucher	Collection Locality	Locality Coordinates
Chrysoritis chrysaor	AH-07-M636	Yzerfontein, W. Cape	33°20.872'S: 18°09.771'E
Chrysoritis chrysaor	AH-07-M637	Yzerfontein, W. Cape	33°20.872'S: 18°09.771'E
Chrysoritis chrysaor	AH-07-M638	Yzerfontein, W. Cape	33°20.872'S: 18°09.771'E
Chrysoritis chrysaor	AH-07-M639	Yzerfontein, W. Cape	33°20.872'S: 18°09.771'E
Chrysoritis chrysaor	AH-07-M641	Yzerfontein, W. Cape	33°20.872'S: 18°09.771'E
Chrysoritis chrysaor	AH-07-M642	Yzerfontein, W. Cape	33°20.872'S: 18°09.771'E
Chrysoritis chrysaor	AH-95-Z892	Yzerfontein, W. Cape	33°20.872'S: 18°09.771'E
Chrysoritis midas			
Chrysoritis midas	AH-07-C280	Karoo N.P. nr. Tower	32°16.324'S: 22°29.210'E
Chrysoritis midas	AH-07-C281	Karoo N.P. nr. Tower	32°16.324'S: 22°29.210'E
Chrysoritis midas	AH-07-C282	Karoo N.P. nr. Tower	32°16.324'S: 22°29.210'E
Chrysoritis midas	AH-07-C212	Karoo N.P. Ridge	32°15.993'S: 22°29.900'E
Chrysoritis midas	AH-12-C004	Lootsberg Pass, E. Cape	31°50.087 S; 24°51.728 E
Chrysoritis midas	AH-12-C006	Lootsberg Pass, E. Cape	31°50.087 S; 24°51.728 E
Chrysoritis midas	AH-12-C007	Lootsberg Pass, E. Cape	31°50.087 S; 24°51.728 E
Chrysoritis midas	AH-12-C008	Lootsberg Pass, E. Cape	31°50.087 S; 24°51.728 E
Chrysoritis midas	AH-12-E082	Lootsberg Pass, E. Cape	31°50.087 S; 24°51.728 E
Chrysoritis midas	AH-12-E083	Lootsberg Pass, E. Cape	31°50.087 S; 24°51.728 E
Chrysoritis midas	AH-12-E085	Lootsberg Pass, E. Cape	31°50.087 S; 24°51.728 E
Chrysoritis midas	AH-07-C112	Swaarweerberg, Sutherland, N. Cape	32°23'S: 20°35'E
Chrysoritis midas	AH-07-C113	Swaarweerberg, Sutherland, N. Cape	32°23'S: 20°35'E
Chrysoritis midas	AH-07-C118	Swaarweerberg, Sutherland, N. Cape	32°23'S: 20°35'E
Chrysoritis midas	AH-07-C119	Swaarweerberg, Sutherland, N. Cape	32°23'S: 20°35'E
Chrysoritis midas	AH-07-C120	Swaarweerberg, Sutherland, N. Cape	32°23'S: 20°35'E
Chrysoritis midas	AH-07-C121	Swaarweerberg, Sutherland, N. Cape	32°23'S: 20°35'E
Chrysoritis midas	AH-07-C122	Swaarweerberg, Sutherland, N. Cape	32°23'S: 20°35'E
Chrysoritis midas	AH-07-C133	Swaarweerberg, Sutherland, N. Cape	32°23'S: 20°35'E
Chrysoritis midas	AH-07-C134	Swaarweerberg, Sutherland, N. Cape	32°23'S: 20°35'E
Chrysoritis midas	AH-07-C135	Swaarweerberg, Sutherland, N. Cape	32°23'S: 20°35'E
Chrysoritis midas	AH-07-C141	Swaarweerberg, Sutherland, N. Cape	32°23'S: 20°35'E
Chrysoritis midas	AH-07-C142	Swaarweerberg, Sutherland, N. Cape	32°23'S: 20°35'E
Chrysoritis midas	AH-07-C143	Swaarweerberg, Sutherland, N. Cape	32°23'S: 20°35'E
Chrysoritis midas	AH-07-C144	Swaarweerberg, Sutherland, N. Cape	32°23'S: 20°35'E
Chrysoritis midas	AH-07-C150	Swaarweerberg, Sutherland, N. Cape	32°23'S: 20°35'E
Chrysoritis midas	AH-07-C151	Swaarweerberg, Sutherland, N. Cape	32°23'S: 20°35'E
Chrysoritis natalensis			
Chrysoritis natalensis	SW-09-T743	Port Shepstone (Oslo Beach), KZN	30 46 07.5 S: 30 26 25.3 E
Chrysoritis natalensis	SW-09-T744	Port Shepstone (Oslo Beach), KZN	31 46 07.5 S: 30 26 25.3 E
Chrysoritis natalensis	SW-09-T745	Port Shepstone (Oslo Beach), KZN	32 46 07.5 S: 30 26 25.3 E
Chrysoritis natalensis	SW-09-T752	Port Shepstone (Oslo Beach), KZN	33 46 07.5 S: 30 26 25.3 E

Table A2.1 (Continued)

Taxon	Voucher	Collection Locality	Locality Coordinates
Chrysoritis natalensis	SW-09-T727	Trafalgar (Umntamvuna N.Res.), KZN	31 00 24.5 S: 30 10.36.2 E
Chrysoritis natalensis	HU-08-B047		30°58.219'S: 30°16.524'E
Chrysoritis natalensis	HU-08-B048		31°04.155'S: 30°12.730'E
Chrysoritis natalensis	HU-08-B049		31°04.155'S: 30°12.730'E
Thestor dryburghi			
Thestor dryburghi	AAM-98-V085	5km West of Steinkopf, N. Cape	29°05'S: 17°35'E
Thestor dryburghi	AAM-98-Y988	North of Kamieskroon, N. Cape	30°05'S: 17°55'E
Thestor dryburghi	AAM-98-Y989	North of Kamieskroon, N. Cape	30°05'S: 17°55'E
Thestor dryburghi	AAM-98-Y990	North of Kamieskroon, N. Cape	30°05'S: 17°55'E
Thestor dryburghi	AAM-98-Y991	North of Kamieskroon, N. Cape	30°05'S: 17°55'E
Thestor dryburghi	AAM-98-Y992	North of Kamieskroon, N. Cape	30°05'S: 17°55'E
Thestor protumnus			
Thestor protumnus	AH-07-C274	Noupoort, E. Cape	31°12.323'S: 25°06.448'E
Thestor protumnus	AH-07-C275	Noupoort, E. Cape	31°12.323'S: 25°06.448'E
Thestor protumnus	AH-07-C278	Noupoort, E. Cape	31°12.323'S: 25°06.448'E
Thestor protumnus	AH-07-C406	Picketberg, W. Cape	
Thestor protumnus	AH-07-C407	Picketberg, W. Cape	
Thestor protumnus	AH-07-C409	Picketberg, W. Cape	
Thestor protumnus	AH-07-C410	Picketberg, W. Cape	
Thestor protumnus	AH-07-C411	Picketberg, W. Cape	
Thestor protumnus	AH-07-C412	Picketberg, W. Cape	
Thestor protumnus	AH-07-C176	Yzerfontein, W. Cape	33°20.811'S: 18°09.944'E
Thestor protumnus	AH-07-C178	Yzerfontein, W. Cape	33°20.811'S: 18°09.944'E
Thestor protumnus	AH-07-C179	Yzerfontein, W. Cape	33°20.811'S: 18°09.944'E
Thestor protumnus	AH-07-C180	Yzerfontein, W. Cape	33°20.811'S: 18°09.944'E
Thestor protumnus	AH-07-C181	Yzerfontein, W. Cape	33°20.811'S: 18°09.944'E
Thestor protumnus	AH-07-C182	Yzerfontein, W. Cape	33°20.811'S: 18°09.944'E
Thestor protumnus	AH-07-C183	Yzerfontein, W. Cape	33°20.811'S: 18°09.944'E
Thestor protumnus	AH-07-C184	Yzerfontein, W. Cape	33°20.811'S: 18°09.944'E
Thestor protumnus	AH-07-C185	Yzerfontein, W. Cape	33°20.811'S: 18°09.944'E
Thestor protumnus	AH-07-C186	Yzerfontein, W. Cape	33°20.811'S: 18°09.944'E
Thestor protumnus aridus	AH-06-M538	30km North of Steinkopf, N. Cape	29°01.5'S: 17°49.88'E
Thestor protumnus aridus	AH-06-M539	30km North of Steinkopf, N. Cape	29°01.5'S: 17°49.88'E
Thestor protumnus aridus	AH-07-C094	30km North of Steinkopf, N. Cape	29°01.5'S: 17°49.88'E
Thestor protumnus aridus	AH-07-C095	30km North of Steinkopf, N. Cape	29°01.5'S: 17°49.88'E
Thestor protumnus aridus	AH-07-C096	30km North of Steinkopf, N. Cape	29°01.5'S: 17°49.88'E
Thestor protumnus aridus	AH-07-T068	30km North of Steinkopf, N. Cape	29°01.5'S: 17°49.88'E
Thestor protumnus aridus	AH-07-T069	30km North of Steinkopf, N. Cape	29°01.5'S: 17°49.88'E
Thestor protumnus aridus	AH-07-T070	30km North of Steinkopf, N. Cape	29°01.5'S: 17°49.88'E
Thestor protumnus aridus	AH-07-T071	30km North of Steinkopf, N. Cape	29°01.5'S: 17°49.88'E
Thestor protumnus aridus	AH-07-T073	30km North of Steinkopf, N. Cape	29°01.5'S: 17°49.88'E

Table A2.1 (Continued)

Taxon	Voucher	Collection Locality	Locality Coordinates
Thestor protumnus aridus	AH-07-T074	30km North of Steinkopf, N. Cape	29°01.5'S: 17°49.88'E
Thestor protumnus aridus	AH-07-T075	30km North of Steinkopf, N. Cape	29°01.5'S: 17°49.88'E
Thestor protumnus aridus	AH-07-T077	30km North of Steinkopf, N. Cape	29°01.5'S: 17°49.88'E
Thestor protumnus aridus	AH-07-T078	30km North of Steinkopf, N. Cape	29°01.5'S: 17°49.88'E
Thestor protumnus aridus	AH-07-T079	30km North of Steinkopf, N. Cape	29°01.5'S: 17°49.88'E
Thestor protumnus aridus	AH-07-T080	30km North of Steinkopf, N. Cape	29°01.5'S: 17°49.88'E
Thestor protumnus aridus	AH-99-U421	Die Kruis, nr. Garies, N. Cape	
Thestor protumnus aridus	AH-99-U422	Die Kruis, nr. Garies, N. Cape	
Thestor protumnus aridus	AH-07-P813	Shaws Pass, Caledon, W. Cape	34°18.656'S: 19°25.069'E
Thestor protumnus aridus	AH-07-P814	Shaws Pass, Caledon, W. Cape	34°18.656'S: 19°25.069'E
Thestor protumnus aridus	AH-07-P815	Shaws Pass, Caledon, W. Cape	34°18.656'S: 19°25.069'E
Thestor protumnus aridus	AH-07-P817	Shaws Pass, Caledon, W. Cape	34°18.656'S: 19°25.069'E
Thestor protumnus aridus	AH-07-P818	Shaws Pass, Caledon, W. Cape	34°18.656'S: 19°25.069'E
Thestor protumnus aridus	AH-07-P819	Shaws Pass, Caledon, W. Cape	34°18.656'S: 19°25.069'E
Thestor protumnus aridus	AH-07-P820	Shaws Pass, Caledon, W. Cape	34°18.656'S: 19°25.069'E
Thestor protumnus aridus	AH-07-P821	Shaws Pass, Caledon, W. Cape	34°18.656'S: 19°25.069'E
Thestor protumnus aridus	AH-07-P822	Shaws Pass, Caledon, W. Cape	34°18.656'S: 19°25.069'E
Thestor protumnus aridus	AH-07-P823	Shaws Pass, Caledon, W. Cape	34°18.656'S: 19°25.069'E
Thestor protumnus aridus	AH-07-P824	Shaws Pass, Caledon, W. Cape	34°18.656'S: 19°25.069'E
Thestor protumnus aridus	AH-07-P825	Shaws Pass, Caledon, W. Cape	34°18.656'S: 19°25.069'E
Thestor protumnus aridus	AH-07-P826	Shaws Pass, Caledon, W. Cape	34°18.656'S: 19°25.069'E
Thestor protumnus aridus	AH-07-P827	Shaws Pass, Caledon, W. Cape	34°18.656'S: 19°25.069'E
Thestor protumnus aridus	AH-07-P829	Shaws Pass, Caledon, W. Cape	34°18.656'S: 19°25.069'E
Thestor protumnus mijburghi	AAM-98-V077	10km North of Steinkopf, N. Cape	29°05'S: 17°50'E
Thestor protumnus mijburghi	AAM-98-V078	10km North of Steinkopf, N. Cape	29°05'S: 17°50'E
Thestor protumnus mijburghi	AAM-98-V079	5km North of Aribes Riverbed, N. Cape	29°00'S: 17°50'E
Thestor protumnus mijburghi	AAM-98-V080	5km North of Aribes Riverbed, N. Cape	29°00'S: 17°50'E
Thestor protumnus mijburghi	AAM-98-V092	5km North of Aribes Riverbed, N. Cape	29°00'S: 17°50'E
Thestor protumnus mijburghi	AH-98-Y444	North West of Steinkopf, N. Cape	29°05'S: 17°35'E
Thestor protumnus protumnus	AH-07-P523	Redhill, Simonstown, W. Cape	34°11.188'S: 18°24.105'E
Thestor protumnus protumnus	AH-07-P524	Redhill, Simonstown, W. Cape	34°11.188'S: 18°24.105'E
Thestor protumnus protumnus	AH-07-P525	Redhill, Simonstown, W. Cape	34°11.188'S: 18°24.105'E
Thestor protumnus protumnus	AH-07-P526	Redhill, Simonstown, W. Cape	34°11.188'S: 18°24.105'E

Table A2.1 (Continued)

Taxon	Voucher	Collection Locality	Locality Coordinates
Thestor protumnus protumnus	AH-07-P527	Redhill, Simonstown, W. Cape	34°11.188'S: 18°24.105'E
Thestor protumnus protumnus	AH-07-P552	Redhill, Simonstown, W. Cape	34°11.188'S: 18°24.105'E
Thestor protumnus protumnus	AH-07-P553	Redhill, Simonstown, W. Cape	34°11.188'S: 18°24.105'E
Thestor protumnus protumnus	AH-07-P554	Redhill, Simonstown, W. Cape	34°11.188'S: 18°24.105'E
Thestor protumnus protumnus	AH-07-P555	Redhill, Simonstown, W. Cape	34°11.188'S: 18°24.105'E
Thestor protumnus protumnus	AH-07-P556	Redhill, Simonstown, W. Cape	34°11.188'S: 18°24.105'E
Thestor protumnus protumnus	AH-07-P557	Redhill, Simonstown, W. Cape	34°11.188'S: 18°24.105'E
Thestor protumnus protumnus	AH-07-P558	Redhill, Simonstown, W. Cape	34°11.188'S: 18°24.105'E
Thestor protumnus protumnus	AH-07-P831	Redhill, Simonstown, W. Cape	34°11.188'S: 18°24.105'E
Thestor protumnus protumnus	AH-07-P832	Redhill, Simonstown, W. Cape	34°11.188'S: 18°24.105'E
Thestor protumnus protumnus	AH-07-P835	Redhill, Simonstown, W. Cape	34°11.188'S: 18°24.105'E
Thestor protumnus protumnus	AH-07-P836	Redhill, Simonstown, W. Cape	34°11.188'S: 18°24.105'E
Thestor protumnus protumnus	AH-07-P837	Redhill, Simonstown, W. Cape	34°11.188'S: 18°24.105'E
Thestor protumnus protumnus	AH-07-P838	Redhill, Simonstown, W. Cape	34°11.188'S: 18°24.105'E
Thestor protumnus protumnus	AH-07-P839	Redhill, Simonstown, W. Cape	34°11.188'S: 18°24.105'E

Table A2.2 Primers used in this study.

Locus	Direction	Sequence	Reference
COI			
LCO1490	F	GGTCAACAAATCATAAAGATATTGG	Folmer *et al.* 1994
Nancy	R	CCCGGTAAAATTAAAATATAAACTTC	Simon *et al.* 1994
TN2126	F	TTGAYCCTGCAGGTGGWGGAG	Eastwood, unpublished
Hobbes	R	AAATGTTGNGGRAAAATGTTA	Monteiro and Pierce 2001
ITS2			
ITS3	F	GCATCGATGAAGAACGCAGC	White *et al.* 1990; Wiemers *et al.* 2010
ITS4	R	TCCTCCGCTTATTGATATGC	White *et al.* 1990; Wiemers *et al.* 2010

Table A2.3a Effective population sizes and genetic diversity indices of *C. chrysaor* for each locality calculated from COI.

	Sample Size	H	Hd	S	p	Theta(S)
Blaauwberg N.R.	5	3	0.80 +/- 0.16	3	0.00118 +/- 0.00100	1.44 +/- 1.02
Calitzdorp	13	6	0.78 +/- 0.10	14	0.00342 +/- 0.00205	4.51 +/- 2.02
Coombs	1	1	1.00 +/- 0.00	0	0.00000 +/- 0.00000	0.00 +/- 0.00
Du Toits Kloof Pass	4	3	0.83 +/- 0.22	12	0.00601 +/- 0.00427	6.55 +/- 3.86
Franschhoek	1	1	1.00 +/- 0.00	0	0.00000 +/- 0.00000	0.00 +/- 0.00
Fraserrburg	2	2	1.00 +/- 0.50	3	0.00251 +/- 0.00291	3.00 +/- 2.45
Gouritzmond	2	1	0.00 +/- 0.00	0	0.00000 +/- 0.00000	0.00 +/- 0.00
Great Winterberg	3	3	1.00 +/- 0.27	2	0.00112 +/- 0.00115	1.33 +/- 1.10
Karoo N.P.	11	8	0.89 +/- 0.09	10	0.00192 +/- 0.00129	3.41 +/- 1.66
Leipoldtville	6	4	0.80 +/- 0.17	19	0.00778 +/- 0.00482	8.32 +/- 4.25
Matjiesfontein	1	1	1.00 +/- 0.00	0	0.00000 +/- 0.00000	0.00 +/- 0.00
Pearson Road	2	2	1.00 +/- 0.50	3	0.00252 +/- 0.00291	3.00 +/- 2.45
Port Elizabeth	1	1	1.00 +/- 0.00	0	0.00000 +/- 0.00000	0.00 +/- 0.00
Sarrisaam Farm	7	4	0.81 +/- 0.13	4	0.00136 +/- 0.00104	1.63 +/- 1.03
Lesotho	1	1	1.00 +/- 0.00	0	0.00000 +/- 0.00000	0.00 +/- 0.00
Sheldon	1	1	1.00 +/- 0.00	0	0.00000 +/- 0.00000	0.00 +/- 0.00
Silverstream	4	4	1.00 +/- 0.18	7	0.00308 +/- 0.00234	3.82 +/- 2.38
Willowmore	3	3	1.00 +/- 0.27	10	0.00560 +/- 0.00453	6.67 +/- 4.33
Witsand	1	1	1.00 +/- 0.00	0	0.00000 +/- 0.00000	0.00 +/- 0.00
Yzerfontein 2	19	14	0.96 +/- 0.03	20	0.00345 +/- 000201	5.72 +/- 2.29

H=No. of haplotypes, Hd=Gene diversity, S=No. of polymorphic sites, p=Nucleotide diversity, Theta(S)=2Neu, Ne=Effective population size,
u=mutation rate

Table A2.3b Effective population sizes and genetic diversity indices of *C. chrysaor* for each locality calculated from ITS2.

	Sample Size	H	Hd	S	p	Theta(S)
Blaauwberg N.R.	5	3	0.70 +/- 0.22	5	0.00304 +/- 0.00241	1.92 +/- 1.27
Calitzdorp	13	4	0.65 +/- 0.11	5	0.00220 +/- 0.00162	0.64 +/- 0.49
Coombs	1	1	1.00 +/- 0.00	0	0.00000 +/- 0.00000	0.00 +/- 0.00
Du Toits Kloof Pass	4	3	0.83 +/- 0.22	2	0.00160 +/- 0.00159	1.09 +/- 0.88
Franschhoek	1	1	1.00 +/- 0.00	0	0.00000 +/- 0.00000	0.00 +/- 0.00
Fraserrburg	2	1	0.00 +/- 0.00	0	0.00000 +/- 0.00000	0.00 +/- 0.00
Gouritzmond	2	1	0.00 +/- 0.00	0	0.00000 +/- 0.00000	0.00 +/- 0.00
Great Winterberg	3	3	1.00 +/- 0.27	4	0.00425 +/- 0.00381	1.33 +/- 1.10
Karoo N.P.	11	4	0.67 +/- 0.12	6	0.00277 +/- 0.00196	0.68 +/- 0.52
Leipoldtville	6	3	0.60 +/- 0.22	8	0.00427 +/- 0.00304	3.07 +/- 1.76
Matjiesfontein	1	1	1.00 +/- 0.00	0	0.00000 +/- 0.00000	0.00 +/- 0.00
Pearson Road	2	2	1.00 +/- 0.50	5	0.00795 +/- 0.00871	2.00 +/- 1.73
Port Elizabeth	1	1	1.00 +/- 0.00	0	0.00000 +/- 0.00000	0.00 +/- 0.00
Sarrisaam Farm	7	2	0.57 +/- 0.12	6	0.00549 +/- 0.00365	2.04 +/- 1.22
Lesotho	1	1	1.00 +/- 0.00	0	0.00000 +/- 0.00000	0.00 +/- 0.00
Sheldon	1	1	1.00 +/- 0.00	0	0.00000 +/- 0.00000	0.00 +/- 0.00
Silverstream	4	2	0.50 +/- 0.27	4	0.00320 +/- 0.00268	1.64 +/- 1.19
Willowmore	3	1	0.00 +/- 0.00	0	0.00000 +/- 0.00000	0.00 +/- 0.00
Witsand	1	1	1.00 +/- 0.00	0	0.00000 +/- 0.00000	0.00 +/- 0.00
Yzerfontein 2	19	5	0.46 +/- 0.14	4	0.00082 +/- 0.00081	0.86 +/- 0.55

H=No. of haplotypes, Hd=Gene diversity, S=No. of polymorphic sites, p=Nucleotide diversity, Theta(S)=2Neu, Ne=Effective population size,
u=mutation rate

Table A2.4a Genetic diversity indices of *T. protumnus* for each locality calculated from COI.

	Sample Size	H	Hd	S	p	Theta(S)
Noupoort	3	1	0.00 +/- 0.00	0	0.00000 +/- 0.00000	0.00 +/- 0.00
Picketberg	6	1	0.00 +/- 0.00	0	0.00000 +/- 0.00000	0.00 +/- 0.00
Yzerfontein	10	1	0.00 +/- 0.00	0	0.00000 +/- 0.00000	0.00 +/- 0.00
30km N of Steinkopf	16	3	0.24 +/- 0.13	2	0.00021 +/- 0.00028	0.60 +/- 0.45
10km N of Steinkopf	1	1	0.00 +/- 0.00	0	0.00000 +/- 0.00000	0.00 +/- 0.00
NW of Steinkopf	1	1	0.00 +/- 0.00	0	0.00000 +/- 0.00000	0.00 +/- 0.00
5km N of Aribes	1	1	0.00 +/- 0.00	0	0.00000 +/- 0.00000	0.00 +/- 0.00
Die Kruis	2	2	1.00 +/- 0.50	1	0.00084 +/- 0.00119	1 .00+/- 1.00
Shaw's Pass	15	2	0.42+/- 0.11	1	0.00035 +/- 0.00038	0.31 +/- 0.42
Redhill	19	5	0.59 +/- 0.12	6	0.00021 +/- 0.00028	1.72 +/- 0.88

H=No. of haplotypes, Hd=Gene diversity, S=No. of polymorphic sites, p=Nucleotide diversity, Theta(S)=2Neu, Ne=Effective population size,
u=mutation rate

Table A2.4b Genetic diversity indices of *T. protumnus* for each locality calculated from ITS2.

	Sample Size	H	Hd	S	p	Theta(S)
Noupoort	3	2	0.67 +/- 0.31	6	0.00714 +/- 0.00607	2.00 +/- 1.51
Picketberg	6	2	0.33 +/- 0.22	3	0.00175 +/- 0.00156	1.31 +/- 0.91
Yzerfontein	10	3	0.69 +/- 0.10	4	0.00416 +/- 0.00279	1.41 +/- 0.86
30km N of Steinkopf	16	2	0.33 +/- 0.13	3	0.00174 +/- 0.00140	0.00 +/- 0.00
10km N of Steinkopf	1	1	0.00 +/- 0.00	0	0.00000 +/- 0.00000	0.00 +/- 0.00
NW of Steinkopf	1	1	1.00 +/- 0.00	0	0.00000 +/- 0.00000	0.00 +/- 0.00
5km N of Aribes	1	1	0.00 +/- 0.00	0	0.00000 +/- 0.00000	0.00 +/- 0.00
Die Kruis	2	1	0.00 +/- 0.00	0	0.00000 +/- 0.00000	0.00 +/- 0.00
Shaw's Pass	15	2	0.25 +/- 0.13	3	0.00131 +/- 0.00115	0.00 +/- 0.00
Redhill	19	2	0.28 +/- 0.12	1	0.00049 +/- 0.00062	0.29 +/- 0.29

H=No. of haplotypes, Hd=Gene diversity, S=No. of polymorphic sites, p=Nucleotide diversity, Ne=Effective population size

APPENDIX 3

Further life history observations in the myrmecophilous genus *Chrysoritis* Butler, plus notes on dwarfism in reared specimens[2]

Co-author: Alan Heath*
*Alan Heath is the first author on this paper.

Abstract

Associated host-ant species and larval host-plants are recorded for *Chrysoritis pelion* (Pennington, 1953), *C. irene* (Pennington, 1968) and *C. natalensis* (Van Son, 1966). Instances of undersize adults obtained when reared in captivity from eggs, without ant attendance, are recorded and discussed.

Introduction

Clark and Dickson (1971) produced the first major publication dealing with the life histories of South African lycaenids; however, they focused more heavily on the morphology of the juvenile stages than on their associated ants and natural larval host-plants. Subsequent publications on southern African butterflies, e.g. Pringle *et al.* (1994), Heath (1997) and others added to the information on these associations, in particular Kroon (1999) who compiled associated data for all Lepidoptera of Southern Africa.

Heath and Claassens (2003) reviewed the ant-associations for all southern African lycaenids and claimed that over three quarters of them are ant-associated (myrmecophilous). Of these, two thirds are considered to be obligately associated, where larvae are always tended by ants, and that without them, the mortality would rise significantly (see Pierce *et al.*, 1997; 2002). Hence these ant-associations are a vital component of a myrmecophilous lycaenid's survival needs. A summary of all known trophic and ant-associations for the genera *Chrysoritis* Butler, 1898 and *Aloeides* Hübner, 1819 was produced by Heath *et al*. (2008); the species in both these genera being regarded as obligately myrmecophilous (Heath and Claassens 2003). As part of an ongoing research into these relationships we record three new life history accounts herein for the genus *Chrysoritis.*

In rearing *Chrysoritis* butterflies from egg to adult in captivity, it has usually been convenient to do so without the presence of ants; however the resulting adults have been invariably undersize (pers. observ. A Heath). This phenomenon is discussed herein.

Material and Methods

Heath and Claassens (2003:2) described the method used to induce oviposition among captive *Chrysoritis* adults. The method adopted for rearing the *C. irene* larvae

[2] Appendix published: Heath, A. and Kaliszewska, Z. A. (2012), Further life history observations in the myrmecophilous genus *Chrysoritis* Butler, plus notes on dwarfism in reared specimens. Metamorphosis, 23: 16-23.

was as follows: The first instar larvae were each transferred to a separate potted *Tetraena retrofracta* (Thunb.) Beier and Thulin (Zygophyllaceae) plant covered in netting (see Figure A3.7) by means of a fine paintbrush. The plant's stem was loosely wrapped in dark netting to serve as a larval refuge and the pot was placed in partial shade, but otherwise it was open to the elements. Care was taken to limit the amount of water applied to the base of the pot. A fine mist-spray was applied to the upper part of the plant on most mornings to simulate morning mist. This was found to be most important during the pupal stage, as the pupae could otherwise dehydrate.

The plant used for rearing *C. irene* was not its normal larval host-plant, and was chosen as a substitute for convenience, e.g. its smaller size and availability. In nature *T. retrofracta* is commonly used by *C. thysbe osbecki* (Aurivillius) and others in the genus.

When searching for juvenile stages in the veld the method has been to search the base of potential food-plants for the presence of ants. Where potential ant-associates occur, a more detailed search is made among leaf-litter, curled leaves and other refuges close to the host-plant. On finding larva or pupae, some attending ants are collected for later identification and some for attending the larva or pupa. Further discussion on rearing larvae found in the veld is given in Heath and Pringle (2007).

Figure A3.1 Blue Mountain Pass locality for *C. pelion*.

Results

***Chrysoritis pelion* (Pennington)**

Four visits were made by the authors in January 2011 to the summit of Blue Mountain Pass in Lesotho (Figure A3.1) in an attempt to establish the ant associate and larval host-plant used by *C. pelion* in that locality. The weather was cool, generally overcast with occasional showers, and infrequent periods of sunshine, hence

only one adult female specimen was seen on the wing, despite extensive searches. On the 13th of January three pupae were discovered at the base of a *Thesium* plant growing flat against a rock face (Figure A3.2) at 29°25.98′S, 27°58.01′E, 2664 m.

Figure A3.2 *Thesium* sp. where three *C. pelion* pupae were found.

A sample of *Crematogaster* sp. ants that were tending the pupae was taken for identification. One female and two male *C. pelion* emerged from the pupae (Figure A3.3) a few days later. A further search was made in the area, and on the 14th of January a late instar larva (Figure A3.4) was found at the base of a *Thesium* sp. plant. A sample of *Crematogaster* sp. ants tending the larva was also collected for identification. The *C. pelion* larva was much darker than typical *Chrysoritis* larvae.

Figure A3.3 *Crematogaster sp.* ant tending a *C. pelion* pupa.

Figure A3.4 *C. pelion* 5th (penultimate) instar larva.

At first sight the *C. pelion* larva appeared to be plain dark grey, although a closer examination showed it to be a dull reddish-brown and underneath the dark color it had a similar pattern to other late instar larvae in the *C. thysbe* species group. Both ant samples were later identified as *Crematogaster* sp. near *peringueyi* Emery. The species of *Thesium* was common in the area but many of the plants growing amongst the grass gave the appearance of grass having been grazed, and so might easily be overlooked.

***Chrysoritis natalensis* (van Son)**

A known locality for *C. natalensis* at Umtamvuna Nature Reserve, KwaZulu Natal, 31°00.39′S, 30°10.59′E, 355 m, was visited on 8th January 2011. Four males were seen and following an examination of a bush of *Osteospermum moniliferum* L. (Asteraceae) (= *Chrysanthemoides monilifera*), a third instar larva was found in a curled dead leaf. It was attended by the ant *Crematogaster* sp. near *liengmei* Emery. Shortly after, a final instar at a pre-pupation stage was found in a cluster of dead leaves; this too was attended by the same species of ant. The latter larva was collected; it pupated the next day and later eclosed as a female *C. natalensis.* G.A. Henning is recorded similarly finding pupae and larvae of *C. natalensis* attended by *Crematogaster* ants (Pringle *et al.,* 1994).

***Chrysoritis irene* (Pennington)**

A well-known locality (Figure A3.5) for *C. irene* above the summit of Du Toit's Kloof Pass, 33°41.88′S, 19°04.22′E, 884 m, near Paarl was visited on October 19th 2010 in order to discover the butterfly's life history. A female was seen to oviposit on a dead stem resting against a plant with yellow flowers (Figure A3.6), growing close to the base of the cliff.

Figure A3.5 *C. irene* habitat; Du Toit's Kloof Pass.

Figure A3.6 *Dimorphotheca chrysanthemifolia* (Vent.) DC.

The egg and the live female were collected. Samples of the plant and of *Crematogaster* close to *C. peringueyi* Emery ants found at the base of the plant were also collected for identification purposes. The plant was later identified as the 'Chrysanthemum-leaved Cape Marigold', *Dimorphotheca chrysanthemifolia* (Vent.) DC. (Asteraceae); however, the authors do not wish to imply that this is the only foodplant of *C. irene*.

In captivity the female was induced to lay two more eggs. The resulting larvae were raised on a potted *Tetraena retrofracta* (Thunb.) Beier and Thulin (Zygophyllaceae) plant covered in netting (Figure A3.7). At no time were ants or other insects allowed access to the larvae. The three larvae rested and later pupated within the stem wrapping; two males and a female eclosed in late February 2011. These specimens were noticeably smaller than wild ones, with the two males each having a wingspan (set) of 20.5 mm. This compared with an average wingspan of 26.5 mm (n = 15) among free-flying males, hence the two males reared in captivity had a reduction in wingspan of almost 23%. The one female was similarly undersize.

Figure A3.7 Potted plant used for rearing *C. irene*.

Figure A3.8 *C. irene* 3rd instar, tended by *Crematogaster sp*. ant.

On 1st March 2011 the authors paid another visit to the same locality above Du Toit's Kloof Pass. The autumn brood was in evidence, with eight males and two females having been seen on the wing in this small locality (~8 m x 80 m) below the cliff face. Early stages were sought amongst the *Dimorphotheca* plants at the foot of the cliff face. One 3rd instar larva was discovered in a brown curled-up leaf, the edges held together by silk (Figure A3.8). The larva was tended by a single *Crematogaster* sp. ant that was highly protective and unwilling to leave its charge. In the process of collecting a few more ants at the base of one of the plants, a pooter was used. On examining the collected ants, a 3rd instar larva was discovered among them, having been aspirated accidentally from the base of the plant.

Discussion

Dwarf adults

Over the past 25 years many *Chrysoritis* species and subspecies have been reared in captivity by the first author. This was originally done to observe their morphology through different stages of development; hence they were reared from egg to adult. In all such cases, except for *C. dicksoni* (Gabriel), they were reared without ants being present, and without exception these resulted in undersize adults (Heath and Pringle 2007). In most of these cases, the appropriate plant was used to feed the larvae; however, an alternative plant species was used in some instances. The larval host-plant was usually grown in a pot as shown in Figure A3.7, outdoors but in partial shade.

In 2010 four *C. lycegenes* (Trimen) individuals were reared from egg and successfully fed on *Acacia karoo* Hayne (Fabaceae) without ants being present, but as with other *Chrysoritis* species reared, these also eclosed significantly undersize (A. Heath

and A. Morton unpubl.). In 1990 a number (>30) of *C. chrysaor* were reared in captivity under similar conditions by the first author. The adults were all undersize in varying degrees, and it was noticed that the adults eclosing later were progressively smaller than those eclosing earlier. Rearing large numbers of the palaearctic lycaenid *Polyommatus icarus* (Rottemburg) on two of its natural larval host-plants, without ant presence, resulted in dwarf adults (pers. observ. A. Heath). Similar results were obtained with *P. icarus* and *Zizeeria knysna* (Trimen) (K. Fiedler, pers. comm.).

Not all rearing of lycaenids results in dwarf specimens. In a study of the Australian lycaenid *Jalmenus evagoras* (Donovan), reared under natural conditions, it was found that larvae tended by ants developed more quickly than larvae that were not tended; however, they pupated at a significantly lower weight than their untended counterparts, and the adults that emerged from these pupae were smaller (Pierce *et al*., 1987). In contrast to this, the same species reared under artificial conditions, both with and without ants at Harvard University generally produced smaller adults than those found in the field with ants (R. Eastwood, pers. comm.). Despite being anecdotal, this last observation suggests that artificial as opposed to natural conditions may influence the size of the adult.

Figure A3.9 Final instar larva of *C. lycegenes* with *Crematogaster* sp. ants gathered around its head (Photo: Richard Kinvig).

We have considered the absence of ants as a potential cause of undersize adults, but current life history knowledge (Heath *et al*., 2008) mitigates against some of the more obvious possibilities of aphytophagy, such as carnivory or trophallaxis. Except for *C. dicksoni* (Gabriel) (see Heath 1998), trophallaxis has never been observed in this genus,

whose larvae are assumed to be wholly phytophagous but always ant-attended (Heath and Claassens 2003; Heath and Pringle 2007). There are usually only one or two ants constantly attending a larva in early instars, with later instars often having more ants attending. The ants palpate the dorsal nectary organ (DNO) at regular intervals, seeking the honeydew secretion (Clark and Dickson 1971). Attendant ants are almost always present at the DNO, but in January 2010 we observed and photographed ants clustering around the head of 4th instar larvae of *C. lycegenes* (Trimen). A fine photograph of this same phenomenon taken in December 2008 by Richard Kinvig (Figure A3.9) also shows a concentration of ants around the head of a final instar *C. lycegenes* larva. In these instances, without magnification, it is impossible to see what exactly is taking place, but it is unusual to see such activity at the head of a larva. Perhaps in this and other species of *Chrysoritis*, aphytophagous behaviour can occur and trophic interchange takes place. A further possible explanation for these observations with *C. lycegenes* is that each of them could have been in the early stages of ecdysis at the times of the photographs, and this process would doubtless attract the ants' attention.

Persistent attention by ants tends to keep the larvae active, and we suspect that this stimulates them to feed more frequently than would be the case without ants, as they need to renew their metabolic resources to provide honeydew for their host ants. Hence the absence of ants could conceivably result in less well-fed and inferior larvae, resulting in smaller adults.

In their study of *Orachrysops niobe* (Trimen) Edge and van Hamburg (2010) found that its final two larval instars (3 and 4) fed exclusively on the rootstock of its leguminous host plant under natural conditions but that larvae reared in captivity only on plant cuttings resulted in dwarf adults.

Unsuitable or poor quality of host plant is known to be one cause of dwarfism both in nature and in captivity (R. Eastwood, K. Fiedler, pers. comm.). Although supposedly healthy potted plants were used in rearing *Chrysoritis* species under artificial conditions, the protective measures might have adversely affected the plants, and/or the larva and adult. To summarize, the one common factor involved in these instances of under-size adults appears to be the artificial conditions. This could be because the larvae and host-plant are under some form of protective cover and not fully exposed to the elements; however, further research would be required if a quantitative conclusion is to be reached.

Acknowledgements

We acknowledge the Western Cape Nature Conservation Board and the KwaZulu-Natal Nature Conservation Board for permits to study and collect material under their jurisdiction. We thank the Putnam Expedition Grant Committee (USA) for funding our travels. We are grateful to Simon Joubert for drawing our attention to the *C. lycegenes* photograph by Richard Kinvig. We especially wish to thank Andrew Morton for arranging for the Compton Herbarium (SANBI), Kirstenbosch, Cape Town to identify the *C. irene* foodplant. We thank Nokuthula Mbanyana, Iziko South African Museum for identifying the ants. Finally, we thank Steve Woodhall for taking us to Umtamvuna; also for his and Jayne's hospitality during our stay with them.

APPENDIX 4

Field notes including a summary of trophic and ant-associations for the butterfly genera *Chrysoritis* Butler, *Aloeides* Butler and *Thestor* Hübner (Lepidoptera: Lycaenidae) from South Africa[3]

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Abstract

Eighteen life history observations on lycaenid taxa are reported, and discussed for the first time, and updated tables of all known ant symbionts and food-plants for *Chrysoritis* and *Aloeides* are presented. Interactions between first instar larvae of *Thestor rileyi* Pennington and their host ants are described. The occurrence of larvae of *Aloeides bamptoni* Tite and Dickson and *A. nollothi* Tite and Dickson with their ant-associates and food-plants are reported, and the oviposition behaviour of *A. barklyi* (Trimen) and the ecology of *A. pringlei* Tite and Dickson is described. An ant-associate and a new food-plant are recorded for *C. braueri* (Pennington), and ant associates are inferred for *Chrysoritis aridus* (Pennington) and for *C. natalensis* (van Son) using new evidence. Further evidence for the ant-associate and food-plant of *C. chrysantas* (Trimen) is presented. Food-plants and ant-associates are determined for *C. trimeni* (Riley) and *C. pan lysander* (Pennington), and for the first time they are recorded occurring in the same locality. A new locality for *C. zonarius coetzeri* Dickson and Wykeham is recorded. Each observation is reported and discussed in context, and in relation to previously published work.

Introduction

Of the 668 species of butterflies in South Africa, almost half (318) are in the family Lycaenidae (see Woodhall 2005). Just over half (55%) of these lycaenids are obligately associated with ants during their juvenile stages (Heath and Claassens 2003); hence they would not survive in the wild without their ant associates (Pierce *et al.* 2002). Therefore, knowledge of the ant associates, in addition to the host plants, is crucial for the conservation of these South African lycaenids. Williams (1996) calculated that no more than 25% of lycaenid life histories were fully known in this region, and of the myrmecophilous Miletinae and Aphnaeini, which are the subject of this paper, less than 19% were known. A major impediment to studying the life histories of myrmecophilous lycaenids is that many live in subterranean ant nests for at least part of their juvenile period. This makes them difficult to locate and almost impossible to observe in the wild. In some habitats, ant nests can be found under rocks. But even where suitable rocks exist, they are often disturbed or overturned by baboons searching for insects and scorpions (Heath and Claassens 2003). Studying the behaviour of lycaenids in ant nests requires

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maintaining a healthy nest of ants and lycaenids in an artificial environment. This presents additional challenges (see Claassens, 1974). One of the first steps in this process is to discover the correct species of ant. Whilst this may seem straightforward, the taxonomy of ant genera such as *Anoplolepis* and *Crematogaster* is not (H.G. Robertson, pers comm.).

From 1940 until his death in 1991, Charles Dickson published numerous papers, *inter alia* on life histories of lycaenids. Much of his early work was collated by Clark and Dickson (1971) in the first major review of juvenile stages of South African Lycaenidae. Since this seminal publication many researchers have contributed additional knowledge on the life histories of Aphnaeini and *Thestor* butterflies. These include Claassens and Dickson (1977), Dickson and Kroon, (1978), Henning, S.F. (1983a, b, 1984a, b), Cottrell, (1978, 1984, 1985), Schlosz and Brinkman (1991), Owen-Johnston (1991), Fiedler (1991), Henning, G.A. (1993), Pringle, *et al.* (1994), Heath and Brinkman (1995), Williams (1996), Williams and Joannou (1996), Heath (1995, 1997, 1998), Claassens and Heath (1997, 2003). Kroon (1999) compiled a list of food plants of all southern African Lepidoptera from published sources. More recently Heath and Claassens (2000, 2003) summarized the ant-associations and life histories of the southern African lycaenids, focusing on the genera *Thestor, Chrysoritis* and *Aloeides*. Pierce, *et al.* (2002) reviewed the ecology and evolution of myrmecophily among the Lycaenidae. Edge (2005b), discussed the ecology of a subspecies of *Aloeides pallida* Riley. Williams (2006) recorded new oviposition behaviours in three lycaenids for the first time. Edge and Pringle (2006) published observations on the life history of *Chrysoritis braueri* (Pennington), and Heath and Pringle (2007) discussed and selectively illustrated some life history characteristics of *Chrysoritis* species. Here we describe 18 new observations of 17 lycaenid taxa and list known ant-associates and food-plants of *Aloeides* species (Table A4.1) and all 72 *Chrysoritis* taxa (Table A4.2). Each of the current authors has at one time or another assisted in uncovering some of the life history data presented here.

Materials and Methods

Localities in Namaqualand were visited and were mostly those already well known to lepidopterists, as it was life history data of lycaenid butterflies that was sought. *Thestor* eggs were obtained in the same manner as described in Claassens and Heath (1997) and Heath and Claassens (2000). A female *Thestor riley* Pennington was collected on 1st January 2007 from high up on the Helderberg Mountain, Somerset West. A partial nest of *Anoplolepis custodiens* (F. Smith) (Formicinae) ants was also collected from the same locality, and housed in a formicarium (see Claassens 1974, Claassens and Heath 1997 and Heath and Claassens 2000). Searching for juvenile stages of *Chrysoritis* and *Aloeides* species consisted of examining the bases of potential food-plants at localities where the butterflies are known to fly. A much closer inspection was made if ants were present on the plant. Late instar larvae were sometimes collected and reared to adults to confirm their identity. These larvae were each placed in a small plastic container (3cm diameter x 12cm deep) together with two attending ants and sprigs of the food-plant. Usually, only the final instar larvae were collected, as the sprigs of food-plant seldom stay fresh for more than a few days, and earlier instars would be unlikely to survive. For each butterfly larva collected, a further dozen ants were placed in a vial containing ethanol for subsequent morphological and molecular study. Wherever possible, digital

photographs were taken of larvae *in situ*, whilst food-plant and all relevant data were recorded in a personal database (AH). Some of these photographs are reproduced here; all taken by the authors, except for the adult *Chrysoritis natalensis*, taken by R. Dobson. Note that many earlier publications list *Zygophyllum* as a food-plant of *Chrysoritis* and *Aloeides* species. All of these *Zygophyllum* species are currently placed in the genera *Roepera* or *Tetraena* (Zygophyllaceae); hence *Zygophyllum flexuosum* is now known as *Roepera flexuosa,* and *Z. retrofractum* is now *Tetraena retrofracta*. These changes, and others, have been incorporated in the tables of food-plants and ant-associations for *Chrysoritis* and *Aloeides* given below. Several new ant-association and food-plant records are included and are based on unpublished observations (AH) during the past four years. We have attempted to list the earliest published record for each food-plant entry in the tables. Currently, the principal way to separate *Crematogaster liengmei* For. from *C. peringueyi* Emery is by the number of antennal segments; the former having 10 and the latter 11. It is possible that some ant taxa contain cryptic species (see Heath, 1997; Eastwood *et al.*, 2006); however, ants are treated herein according to current taxonomy. Identification (AH) of ants was based on earlier identifications by, and subsequent consultations with, Dr. H.D. Robertson.

Results and Discussion

1. *Thestor rileyi* Pennington, 1956

A female *T. rileyi* was allowed to oviposit on the sides of a cardboard box without ants being present. A week later the eggs were placed in the atrium of a formicarium. The ants (*Anoplolepis custodiens* Smith (Formicinae)) showed some interest in the eggs, with up to four ants at times attending a single egg. The eggs started hatching eleven days after being laid and the ants showed considerable interest when the larvae emerged from the shells (Figures A4.1–4). The eggshells were not eaten by the larvae. The larvae would occasionally rear up in front of an ant as if begging to be fed but trophallaxis was not observed. Ants would wave their antennae over the larvae and eventually pick one up and take it into the nest. Inside the plaster nest, the larvae were placed near the perimeter; some of these had an ant in attendance. A few days later, all the larvae were dead, but there was no evidence that the ants had deliberately killed the larvae (see also Heath and Claassens 2003).

Figure A4.1 *Anoplolepis custodiens* ants tending eggs and first instar larva of *Thestor rileyi.*

Figure A4.2 *Anoplolepis custodiens* ants tending first instar larvae of *Thestor rileyi.*

Figure A4.3 *Anoplolepis custodiens* ants tending first instar larva of *Thestor rileyi.*

Figure A4.4 *Anoplolepis custodiens* ants tending first instar larva of *Thestor rileyi.*

The behaviour of these newly-hatched *Thestor rileyi* larvae in captivity was similar to that observed on earlier occasions in *T. yildizae* Koçak and *T. rileyi* by Heath and Claassens (2000, 2003). The rearing up behaviour of the larvae may suggest that they 'expected' an interaction, and their actions may have induced the ants to grasp them and take them into the nest. The puzzle arising from the subsequent death of the larvae (on this and earlier occasions) is to identify their natural food during the early instars, since they are all assumed to have starved to death, as opposed to having been killed. Williams and Joannou (1996) raised the first three instars of *T. basutus capeneri* Dickson on the grass-infesting coccid *Pulvinaria iceryi* (Signoret) (Hemiptera: Coccidae) infesting grass, but the montane fynbos habitat of *T. yildizae* and *T. rileyi* does not normally support grass. Several attempts have been made to locate Hemiptera within the colonies of *T. yildizae* and *T. rileyi* but without success. However, in the final two instars both species are known to feed (trophallaxis) on the regurgitations of *Anoplolepis custodiens* ants (Claassens and Heath 1997; Heath and Claassens 2000). The observation, in captivity, of first instar larvae of *T. yildizae* and *T. rileyi* being carried into the nest suggests that this would also happen under natural conditions and form part of their normal behaviour. There was no sign of the larvae being eaten by the ants. One possibility is that in nature the larvae feed on organic detritus within a natural ant nest. Since an accumulation of detritus is usually absent in newly created artificial nests, this would explain the mortality of larvae reared in artificial conditions. Organic detritus is considered to be a probable supplementary food source for late instar *T. basutus* (Heath and Claassens 2003: 9) and it is also implied ("droppings") by Clark and Dickson (1971: 253) for *T. protumnus aridus* van Son. A less likely possibility is that a very small percentage of larvae manage to infiltrate the area where ant larvae are tended, and are thereafter sustained in cuckoo fashion by trophallaxis, or by feeding on the brood. A more complete understanding of the early behaviour of *Thestor* larvae is critical to developing conservation strategies for these species.

2. *Aloeides barklyi* (Trimen, 1874)

On 3rd September 2006 several *A. barklyi* (Figure A4.5) adults of both sexes, were observed flying on a gentle north-west slope at Grootvlei Pass (30°12.945'S: 17°45.032'E) south-west of Kamieskroon. Four females were separately observed and each one oviposited directly on the hard-packed sandy-gravel substrate, never on or close to a plant. Oviposition occurred on the ground, both in direct sunlight between various aridaceous plants, and in shade below small rock overhangs. Although a variety of plants were in the vicinity, none appeared to be likely as food-plants, and the observed females favoured none. A few ants were observed in the vicinity of the oviposition sites and these were later identified as *Lepisiota capensis* Mayr (Formicinae). One egg was collected and placed in a vial of ethanol. The ovipositing behaviour of *A. barklyi* females strongly suggests an aphytophagous life history. There are records of phytophagous *Aloeides* (and the closely related genus *Erikssonia* Trimen) ovipositing on or in soil (Heath and Claassens 2003: 11) but in these cases it took place beneath or beside a food-plant. In the case of *A. barklyi* oviposition was not associated with any plant, but appeared to be associated instead, with ant trails. *Lepisiota* ants have repeatedly been recorded associating with *Aloeides* and *Erikssonia* larvae (see Heath and Claassens 2003; Henning, 1984a; Edge 2005b; Williams 2006, and this paper). Grootvlei Pass (Figure A4.6) is easily accessible and on a gentle slope with few rocks, unlike most other *A. barklyi* localities, and seems ideally suited to further studies.

Figure A4.5 Underside of gravid female of *Aloeides barklyi*. Grootvlei Pass, south-west of Kamieskroon.

Figure A4.6 Habitat of *Aloeides barklyi* and *A. damarensis*, Groovlei Pass, south-west of Kamieskroon.

3. *Aloeides damarensis* (Trimen, 1891)

Clark and Dickson (1971) recorded an attempt to rear a specimen of *A. d. damarensis* from an ovum on a species of *Aspalathus* (Fabaceae). Unfortunately, the larva died before completing its second instar, and the reason for its death was not given. *A. d. damarensis* adults occur together with *A. barklyi* at Grootvlei Pass, neither of whose food-plant could be determined, and so they could also possibly be aphytophagous.

4. *Aloeides nollothi* Tite and Dickson, 1977

Several larvae and pupae of *A. nollothi* have been collected at Groenriviermond over the past few years (AH). The larvae were found feeding on a species of *Roepera*

(Zygophyllaceae) and on *Hermannia* (Sterculiaceae), and were always attended by *Lepisiota capensis* Mayr ants (Figure A4.7). On one occasion at Hondeklip Bay, as many as eight 3rd instar larvae were found together beneath *Roepera flexuosa* (Eckl. and Zeyh.) Beier and Thulin (Figure A4.8). The larvae normally rest in crevices in the plant stem, often below the surrounding substrate. The pupae are sometimes found where the larvae had previously rested, or in the surface leaf litter surrounding the food-plant.

Figure A4.7 Late instar larvae of *Aloeides nollothi* with a *Lepisiota capensis* ant.

Figure A4.8 Eight third instar larvae of and *Aloeides nollothi* and a *Lepisiota capensis* ant.

5. *Aloeides bamptoni* Tite and Dickson, 1977

On 24th August 2005 a search was made for juvenile stages of *A. bamptoni* Tite and Dickson at a locality 10 km north of Steinkopf (29°11.821'S: 17°48.615'E). The adults were flying in a dry gulley on the east side of the N7 road. In the middle of this population a 4th instar larva was found feeding on a species of *Hermannia* and tended by *Lepisiota capensis* ants (Figure A4.9). The larva was retained in a vial of ethanol for subsequent DNA analysis.

Figure A4.9 Fourth instar larva of *Aloeides bamptoni* feeding on a *Hermannia* species.

Figure A4.10 Habitat of *Aloeides pringlei* on the slopes of The Groote Winterberg, Eastern Cape.

6. *Aloeides pringlei* Tite and Dickson, 1976

A search was made (AH) on 15th November 2004 for early stages of *A. pringlei* at its type locality on the slopes of the Groote Winterberg (Figure A4.10) in the Eastern Cape Province. The adults of both sexes were flying in good numbers, so most of the pupae would have eclosed. However, a search in an ant nest beneath a rock revealed a final instar larva, which was quickly herded out of sight by host ants before it could be photographed. Later, a pupa was found in a nest beneath another rock (Figure A4.11); a female emerged a few days later. The larva and pupa were found in separate nests of *Lepisiota capensis* ants hidden beneath moderate-sized flat slabs of rock. Neither of these nests was within two metres of any plant other than grass, although a species of *Felicia* (Asteraceae) was common in the general area. In view of the close relationship with *A. pallida* Riley, it seems probable that the larvae are aphytophagous or partly so. This suggestion is based on the observations of Heath and Claassens (2003) who recorded *A. p. grandis* Tite and Dickson in captivity, feeding solely on ant eggs during four months of its final instar (Figure A4.12). An undescribed subspecies of *A. pallida* Riley was observed ovipositing on the substrate close to an entrance to an ant nest (Edge 2005b), suggesting the first instar would probably enter, or be taken into the nest, on hatching. These observations almost certainly imply an aphytophagous life history.

Figure A4.11 A pupa of *Aloeides pringlei* in a *Lepisiota capensis* ant nest beneath a slab of rock.

Figure A4.12 Final instar larva of *Aloeides pallida grandis* in captivity, being tended and fed by *Lepisiota capensis* ants.

7. *Aloeides apicalis* Tite and Dickson, 1968
In 1999, larvae were found under a flat stone in a corral beneath *Aspalathus spinosa* L. (Fabaceae) at Koringsberg, Moorreesburg, attended by *Monomorium fridae* Forel (Myrmecinae) ants. The ant nest was located a few metres away (Heath and Claassens 2000). On 20th September 2007, two pupae were found in the sand beneath two plants of *Roepera teretifolia* (Schltr.) Beier and Thulin (Zygophyllaceae) 7 km south of Lambert's Bay. These pupae were also tended by *M. fridae* Forel ants from a nest several metres away. The pupae eclosed as *A. apicalis* several days later. The dorsal nectary organ (DNO) was absent in all the final instar larvae, although examination of a frozen section revealed an underlying DNO structure beneath the cuticle (AH unpubl.). In these and other cases where early stages have been found (AH), they were closely associated with a food-plant and are regarded here as phytophagous.

8. *Chrysoritis braueri* (Pennington, 1967)
On the 27th November 2007, AH and ZAK were shown a locality (32°25.831'S: 26°10.775'E) for *C. braueri* by Ernest Pringle, as well as a known food-plant *Lotononis carnosa carnosa* (Eckl. and Zeyh.) Benth. (Fabaceae) of this butterfly (see Edge and Pringle 2006; also addendum in *Metamorphosis* 18: 45 (2007). The site (Figure A4.13) was revisited the following day and several clumps of a species of *Thesium* Linnaeus (Santalaceae) in the vicinity of the *C. braueri* population were observed and some of these were carefully examined. A total of twelve larvae were discovered at the base of various clumps of *Thesium.* One larva was found inside a fibrous shelter made by the ants and attached to the side of a rock (Figure A4.14). Three of the larvae were in their final instars and hence were collected together with food-plant and ants (Figure A4.15). The younger larvae (Figure A4.16) were left *in situ*, except for two that were accidentally damaged; these were placed in a vial of ethanol for subsequent molecular study. The three final instar larvae continued to feed on the *Thesium* and were attended by ants in captivity. They subsequently pupated and emerged (16th–18th Dec. 2007) as one male and two female *C. braueri*. The ants infested a number of *Thesium* clumps, but a carton nest (partly hidden between rocks) was clearly the epicentre of the ant colony. Subsequent examination of the ants showed they were referable to a *Crematogaster* sp. near *peringueyi* Emery.

Figure A4.13 Habitat (foreground) of *Chrysoritis braueri* on the farm Huntly Glen near Bedford, Eastern Cape.

Figure A4.14 Penultimate instar larva of *Chrysoritis braueri* in a partly opened fibre shelter constructed by *Crematogaster peringueyi* ants.

Figure A4.15 Final instar larva of *Chrysoritis braueri* tended by a *Crematogater peringueyi* ant.

Figure A4.16 Fourth instar larva of *Chrysoritis braueri* on a species of *Thesium*.

9. *Chrysoritis pan lysander* (Pennington, 1962) (=*C. williami* Heath)

Four of the authors visited the Kleinsee area in September 2006, and some early instar larvae believed to be *C. pan lysander* were found on *Roepera flexuosa* (Eckl. and Zeyh.) Beier and Thulin (Zygophyllaceae), where they were tended by *Crematogaster liengmei* For. (Myrmicinae) ants. Unfortunately, they could not be reared because the food-plant, once cut, is short-lived. Two further visits were made to the Kleinsee area during August and September 2007. On 20th August 2007, 10 km south of Kleinsee (29°43.968'S: 17°05.467'E) final instar larvae of *C. pan lysander* were found feeding on *Roepera flexuosa* and tended by *C. liengmei* ants. These larvae pupated and emerged as *C. p. lysander* some weeks later.

Figure A4.17 Female *Chrysoritis pan lysander* alighting on a larval food-plant, *Atriplex bolusii*.

Figure A4.18 Penultimate instar larva of *Chrysoritis pan lysander* being tended by *Crematogaster* peringueyi ants at Leipoldtville.

10. *Chrysoritis pan lysander* (Pennington, 1962) (=*Poecilmitis atlantica* Dickson)

On the 10th August, 2005, beside the T-junction north-west of Leipoldtville (32°14'S: 18°28'E), several females of *C. pan lysander* (Pennington) were observed ovipositing on different *Atriplex bolusii* C.H. Wright (Amaranthaceae) plants (Figure A4.17) infested with *Crematogaster peringueyi* Emery (Myrmicinae) ants. A pupa was found at the base of one of these plants that later eclosed as a female *C. pan lysander*. On 11th December 2007, at precisely the same locality, a penultimate instar larva was found (Figure A4.18). Adult females have also been seen in close proximity to *Chrysanthemoides incana* (Burm. f.) Norl. (Asteraceae) at Leipoldtville and at nearby Lambert's Bay, and it is probable that both food-plants are used in this general area (see also Dickson and Kroon 1978).

11. Dual ant-associates for *Chrysoritis perseus* (Henning 1977) and *C. pan* (Pennington 1962)

At Leipoldtville, *C. pan lysander* has been observed associating with the ant *Crematogaster peringueyi* (11 antennal segments), whilst nearer Cape Town, the nominate subspecies associates with *C. liengmei* (10 antennal segments). The populations of *C. pan lysander* further north in the Namaqualand west coast region (Groenriviermond to Kleinsee) also associate with *C. liengmei* ants. A similar dichotomy of ant-association occurs with *Chrysoritis perseus* (W.H. Henning). At Hondeklip Bay, this species associates with the ant *C. melanogaster,* but at Lambert's Bay it associates with *C. peringueyi*. Between these two localities, at Groenriviermond, they associate with one of either ant species (Heath and Pringle 2007). Until recently, it was thought that each *Chrysoritis* species associated with only one species of ant, but these myrmecophilous associations appear to vary geographically; a situation that could lead to genetic divergence between butterfly populations of the same species.

12. *Chrysoritis trimeni* (Riley, 1938)

On 24th August 2004 at McDougalls Bay, Port Nolloth, a final instar larva was found on *Roepera morgsana* (L.) Beier and Thulin (Zygophyllacae) tended by

Crematogaster peringueyi ants. The following day, further inland at 13 km east of Port Nolloth (29°17.403'S: 16°59.629'E), two final instar larvae were found under a species of *Thesium* (Santalaceae), also tended by *C. peringueyi* ants. All of these emerged as *C. trimeni* (Heath and Pringle 2007). On 19th August, 2007 a second instar and two final instar larvae of *C. trimeni* were found just behind the coastal dunes (29°43.110'S: 17°03.706'E) south of Kleinsee, feeding on *Roepera flexuosa* (Eckl. and Zeyh.) Beier and Thulin (Zygophyllacae) (Figure A4.19). Also, two pupae were found under *Osteospermum oppositifolium* (Aiton) B.Nord. (Compositae). Both pupae and larvae were tended by *C. peringueyi* ants. The larvae eventually pupated, and all eclosed some weeks later as *C. trimeni*.

13. Multiple food-plants

The food-plants of *Chrysoritis trimeni* are now known to include four species in the families Zygophyllaceae, Santalaceae and Asteraceae (see above), which represents an unusually broad diet for a fairly local species. Two food-plants are now recorded for *C. braueri* (Santalaceae and Fabaceae), where previously it was thought to utilize only one (Edge and Pringle 2006 and this study). Five food-plants, belonging to three families, are recorded for *C. pan*. Seven food-plant species are recorded for *C. palmus* and nine recorded so far for *C. thysbe*. All these observations demonstrate that, given the presence of suitable ants, these species are able to exploit a wide variety of plants (Heath and Pringle 2007 and this study).

Figure A4.19 Final instar larva of *Chrysoritis trimeni* being tended by a *Crematogaster* peringueyi ant at Kleinsee.

Figure A4.20 Male of *Chrysoritis natalensis*.

14. *Chrysoritis trimeni* and *Chrysoritis pan lysander*

In the general area 5–15 km south of Kleinsee, *C. pan lysander* and *C. trimeni* were found flying parapatrically. In general, males of *C. pan lysander* in this region prefer the smaller prominences, whilst those of *C. trimeni* prefer flatter ground or shallow depressions closer to the sea-shore. Individuals of *C. trimeni* in this area varied considerably in size. The smaller specimens were indistinguishable from those of *C. pan lysander* (=*williami*), except the latter usually had lighter undersides to the hind wings. This distinction is not consistent however, as winter specimens of *C. pan lysander* also have dark undersides (AH pers obs.). Note that the black upper side wing margins of

many (but not all) *C. trimeni* from Kleinsee are proportionally broader, and the black spots are larger, than in typical specimens further north at McDougall's Bay. Hence the two taxa, *lysander* and the smaller specimens of *trimeni*, are hard to differentiate. Over the years, several of these smaller specimens have been collected from 10–13 km east of Port Nolloth, these had proportionally larger spots and broader margins than those from the nearby type locality at McDougall's Bay. As a result, they also resemble *C. pan lysander*, but their juvenile stages associate with *Crematogaster peringueyi* ants, as do those of *C. trimeni*. However, in the west coast region north of Lambert's Bay, including Kleinsee, *C. pan lysander* associates with *Crematogaster liengmei* ants (see Heath and Pringle 2007: 24). In his description of *Poecilmitis dicksoni* (here treated as *C. pan lysander*), W.H. Henning (1977) noted that it had often been confused with *C. trimeni,* and went on to state that it differed by being smaller, having a broader black border, darkened veins and paler 'flatter' underside. It has become apparent that none of these characters is consistent; hence the two taxa may still be confused. Until now, *C. pan lysander* and *C. trimeni* have not been recorded flying in the same area. One wonders how closely related they are, and if *C. trimeni* could perhaps have split (speciated) from the common ancestor of *C. pan lysander* and *C. trimeni* in this Kleinsee area, by means of a shift in ant-associate. Hopefully the former question at least, will be answered using molecular data.

15. *Chrysoritis aridus* (Pennington, 1953)

A known collecting locality for *C. aridus* (31°00.958'S: 17°47.133'E) near Kotzesrus was visited on the 2nd and again on the 9th of September 2007. No juvenile stages were found, but the predominant species of ant in the area was found to be *Crematogaster melanogaster* Emery, which was present on some plants of both a *Roepera* sp. and *Thesium*. We strongly suspect that *C. melanogaster* may be the ant associate of *C. aridus* in this locality.

16. *Chrysoritis natalensis* (van Son, 1966)

A visit to Oslo Beach, KwaZulu-Natal was made on 2nd December 2007 to search for the ant associate of *C. natalensis* (Figure A4.20). No adults were seen at the known locality but *Crematogaster* ants were present among the *Chrysanthemoides monilifera* (L.) Norl. (Asteraceae) at the precise spot where females have been captured in the past (S.F. Woodhall, pers. comm.). Samples were taken of these ants, which were subsequently identified as *Crematogaster liengmei*. *C. monilifera* has been recorded as one of the two food-plants of *C. natalensis* (Pringle, *et al.* 1994). Oviposition has also been recorded on *C. monilifera* in the presence of *C. liengmei* ants (Richard Dobson, pers. comm.).

17. *Chrysoritis chrysantas* (Trimen, 1868)

Heath and Pringle (2007: p.8) noted that a larva believed to be that of *C. chrysantas* had earlier been found on *Salsola tuberculata* (Moq.) Fenzl. (Chenopodiaceae) attended by *Crematogaster melanogaster* Emery (Myrmicinae) ants north of Wallekraal. On 14th October, 2007, at precisely the same arid locality, a female *C. chrysantas* (Figure A4.21) was observed ovipositing on the same species of plant, on

which *C. melanogaster* ants were also present (Figure A4.22). This lends further strong support for these being respectively, a food-plant and ant-associate for this butterfly.

Figure A4.21 Female of *Chrysoritis chrysantas* basking.

Figure A4.22 Female of *Chrysoritis chrysantas* resting on *Salsola tuberculata.*

18. *Chrysoritis zonarius coetzeri* Dickson and Wykeham, 1994

Four specimens of *C. zonarius coetzeri* were collected from 8 km east of Hondeklip Bay (30°20.268'S: 17°21.659'E) on 10th September 2007. They were flying around the silvery-blue variety of *Chrysanthemoides incana* (Burm. f.) Norl. (Asteraceae) bushes. Two of these specimens were placed in ethanol vials for subsequent DNA analysis. The discovery of *C. zonarius coetzeri* close to Hondeklip Bay is remarkable, as this is the first published record of this insect so far north, being over 200 km north-west of its previously only known (type) locality at Nieuwoudtville, and confirms a sight record by Harald Selb (pers. comm.) a few days earlier. This new locality record opens up a possibility that other local populations of this tiny butterfly may occur in Namaqualand.

Conclusion

Only four species of ants, belonging to two myrmicine genera, associate with the 42 currently acknowledged species of *Chrysoritis* (listed in Table A4.2); while 19 plant genera from 13 families have so far been recorded as food-plants. As many as nine species of food-plant may be used by a single *Chrysoritis* species; and some plants are used by many species, e.g. *Thesium* is a known food-plant for 23 taxa and *Roepera* for 26 taxa. In this context it appears unlikely that speciation has occurred as a result of a food-plant switch; however, an ant switch seems far more likely (see Pierce, 1984, 1987). *Chrysoritis* species that associate with two different ant species, e.g. *C. perseus* and *C. pan* could each be destined to split in accordance with their ant associates, especially if their distributions have geographic affinities. We have postulated above, that *C. trimeni* might have originated in this way. It remains for molecular studies to throw further light on these hypotheses. Based on small structural variations that H.G. Robertson found (Heath, 1997: 39) between populations of *Crematogaster* ants, particularly *C. liengmei,* it is possible that cryptic species may exist among these ants. This would further support the concept of diversification in *Chrysoritis* being associated with shifts in ant associates.

Determining accurate ant-associations can occasionally be difficult. For example, a *Camponotus* species of ant has, at times, been found together with a *Myrmicaria* or *Crematogaster* species of ant and close to *Chrysoritis* juveniles (Heath and Pringle 2007: 8). Such instances have led to the mistaken conclusion that the former ants were the ant associates in those cases. Similar confusion occurred in a situation where a pupa of *Aloeides d. dentatis* was found in close proximity to a *Camponotus* ant, which happened to share space beneath a stone with its natural ant-associate *L. capensis* (see Pringle *et al*., 1994).

Aphytophagy is known in only one species of *Chrysoritis*, namely *C. dicksoni* (Gabriel). In this instance, reliance on trophallaxis was observed in the first, second, and final larval instar; other instars not having been studied (Heath 1998). Coupled with observations that oviposition occurs on a wide variety of plants that larvae refused to eat (Clark and Dickson 1971), parsimony would suggest that aphytophagy is probably the behaviour in all larval instars. In *Aloeides*, some species appear to be aphytophagous. Unfortunately, none of these have been studied throughout their juvenile stages. The final instar larva of *A. pallida grandis* was observed, in captivity, to feed solely on ant eggs, and did not forage outside the ant nest during its four months as a final instar larva (Heath and Claassens 2000, 2003). In captivity, the larvae of some *Aloeides* species are recorded to have survived feeding on vegetation into their second instar and then died, e.g. *A. p. pallida* and *A. d. damarensis* (Clark and Dickson 1971). Reasons for these deaths were not given, but the possibility exists that these larvae are aphytophagous in nature as we have suggested above, in which case their ability to feed on vegetation throughout their first instar may simply be a relic of ancestral phytophagy.

The dorsal nectary organ (DNO) first appears in the 3rd larval instar of *Aloeides* species (Clark and Dickson 1971; Heath and Claassens 2000) and is present in subsequent instars; however, in some species the DNO is lost in the final instar. This loss is known to occur in *A. depicta* Tite and Dickson, *A. pallida* Tite and Dickson, *A. thyra* (Linn.)*, A. apicalis* Tite and Dickson, and *A. dentatis* (Swierstra) (see Heath and Claassens 2000, 2003; S.F. Henning 1983a). As the DNO can play a vital role in the maintenance of ant-lycaenid association (Pierce, *et al.* 2002), so its loss implies that a change in the relationship between ant and lycaenid may have taken place. We can only speculate as to why these changes occur, along with so many other intriguing and, as yet, unanswered questions concerning the juvenile stages of these myrmecophilous lycaenids.

Table A4.1. Trophic and ant-associates for the genus *Aloeides*
Three ant taxa are recorded, namely *Lepisiota capensis* (Mayr) (Formicinae), *Monomorium fridae* Forel (Myrmecinae) and *Pheidole capensis* Mayr (Myrmecinae)

Aloeides taxon	Ant species	Food-plant
A. apicalis	[27]*M. fridae*	[6]*Aspalathus spinosa* L. (Fabaceae); [8]*Roepera teretifolia* (Schltr.) Beier and Thulin; (Zygophyllaceae)
A. aranda	[27]*P. capensis*	[11]Oviposited in sand beneath *Aspalathus* sp. (Fabaceae)
A. bamptoni	[8]*L. capensis*	[8]*Hermannia* sp. (Sterculiaceae)
A. barklyi	*[8]*L. capensis*	*[8]Possibly aphytophagous (by inference)
A. caffrariae	Unrecorded	*[34]*Aspalathus* sp. (Fabaceae)
A. carolynnae carolynnae	Unrecorded	*[13]*Aspalathus* sp. (Fabaceae)
A. clarki [see note 1 below]	[30]*Monomorium* sp.	*[1]Reared to 4th instar on *Aspalathus* sp.; [34]Oviposited in sand below *Aspalathus* sp. (Fabaceae)
A. damarensis damarensis	Unrecorded	*[8]Possibly aphytophagous. [1]Partly reared on *Aspalathus* sp. but died during 2nd instar
A. damarensis mashona	Unrecorded	*[13]*Aspalathus* sp. (Fabaceae)
A. dentatis dentatis	[28]*L. capensis*	[28]*Hermannia depressa* N.E. Br. (Sterculiaceae); [29]*Lotonotis eriantha* Benth. (Fabaceae)
A. dentatis maseruna	[1]*L. capensis*	[29]*Hermannia jacobeifolia* (Turcz.) R.A.Dyer (Sterculiaceae)
A. depicta	[31]*L. capensis*	*[1]Reared to 4th instar on *Aspalathus* sp. (Fabaceae)
A. gowani	Unrecorded	*[1]Reared through on *Aspalathus* sp. (Fabaceae)
A. henningi	Unrecorded	*[1]Reared through on *Aspalathus* sp. (Fabaceae); [13]*Hermannia depressa* N.E. Br.
A. lutescens	Unrecorded	*[13]*Aspalathus* sp. (Fabaceae)
A. molomo krooni	Unrecorded	[29]*Sida ovata* Forssk. (Malvaceae)
A. molomo coalescens	Unrecorded	*[11]Oviposited in sand beneath *Gnidia* sp. (Thymelaeaceae)
A. nollothi	[6]*L. capensis*	[6]*Hermannia* sp. (Sterculiaceae); [6]*Roepera flexuosa* (Eckl. and Zeyh.) Beier and Thulin (Zygophyllaceae)
A. pallida pallida	Unrecorded	[1]Reared only to 2nd instar on *Aspalathus* sp.
A. pallida grandis	[27]*L. capensis*	*[8]Possibly aphytophagous ([27]carnivorous final instar)
A. pallida ssp (undescribed)	[33]*L. capensis*	*[33]Aphytophagous (by inference)
A. pierus	[9]*L. capensis*	[1]*Aspalathus* sp. (Fabaceae)

A. pringlei	[8]*L. capensis*	*[8]Aphytophagous (by inference)
A. rossouwi	[28]*Lepisiota* sp.	Unknown
A. susanae	Unrecorded	[34]Oviposited on small prostrate legume
A. thyra thyra	[26]*L. capensis*	[26]*Aspalathus laricifolius* Berg.; [35]*A. acuminatus* Lam.; [35]*A. cymbiformis* DC; *A. acuminatus* Lam. subspecies *pungens* (Thunb.) R. Dahlgr (Fabaceae)
A. t. trimeni	Unrecorded	*[1]Reared through on *Aspalathus* sp. (Fabaceae); [13]*Hermannia depressa* N.E. Br. (Sterculiaceae)

Note 1: *Aspalathus spinosa* var. *spinosa* is common at the two Coega colonies of *Aloeides clarki*, but absent from the colony at Sundays River mouth. The plant under which the stone was situated and where a 4th instar larva was found (attended by a *Monomorium* sp. of ant) was *Melolobium exudans* Harv. (Fabaceae), but another nearby plant was *Nylandtia spinosa*. (L.) Dumort. (Polygalaceae) (Pringle, pers. comm.). Neither of these have been listed in the table above due to the degree of uncertainty expressed by Pringle.

References for Tables A4.1 and A4.2

[1]Clark and Dickson (1971); [2]Heath (1997a); [3]Dickson (1943); [4]Heath (2001); [5]Henning S.F.(1983a); [6]Heath (unpubl.); [7]Dickson (1948); [8]Heath *et al*. (this study, see text); [9]Clark and Dickson (1956); [10]Heath and Pringle (2007); [11]Heath and Claassens (2003); [12]Dickson (1959); [13]Pringle, *et al.* (1994); [14]Owen-Johnston (1991); [15]Heath (1998); [16]Dickson and Kroon (1978); [17]Terblanche and Hamburg (2004); [18]Dickson (1952); [19]Dickson (1975); [20]Dickson (1940); [21]Dickson (1965); [22]Dickson (1953); [23]Dickson (1947); [24]Dickson (1945); [25]Edge and Pringle (2006) + addendum in *Metamorphosis* **18**: 45 (2007); [26]Claassens and Dickson (1974); [27] Heath and Claassens (2000); [28]Henning, G.A. and Henning, S.F. (1989); [29]Henning, G.A. (1993); [30]Pringle (pers. comm.); [31]Pringle (1998); [32]Edge, (2005a); [33]Edge, (2005b); [34]Williams, (2006); [35]Claassens and Dickson (1977); *Unconfirmed

Table A4.2. Trophic and ant associates for the genus *Chrysoritis*
Four ant taxa are recorded, namely *Crematogaster liengmei* For., *C. liengmei* Emery, *C. melanogaster* Emery and *Myrmicaria nigra* (Mayr).

Chrysoritis taxa	Ant species	Larval food-plants
C. oreas	[2]*M. nigra*	[2]*Thesium* sp. (Santalaceae)
C. dicksoni	[1]*C. peringueyi*	[15]Aphytophagous (trophallaxis)
C. phosphor phosphor	Unrecorded	Unknown
C. phosphor borealis	Unrecorded	Unknown
C. chrysaor	[3]*C. liengmei*	[3]*Cotyledon orbiculata* L. (Crassulaceae); [16]*Rhus* sp. (Anacardiaceae); *[2]*Tetraena retrofracta* (Thunb.) Beier and Thulin. (Zygophyllaceae); [6]*Chrysanthemoides incana* (Burm. f.) Norl. (Asteraceae); *[2]*Acacia karoo* Hayne (Fabaceae)
C. chrysaor f. lycia	[2]*C. liengmei*	[13]*Tylecodon paniculatus* (L.f.) Toelken (Crassulaceae)
C. midas	[2]*C. peringueyi*	[2]*Diospyros austro-africana* De Winter var. *microphylla* (Ebenaceae)
C. natalensis	[2]*C. liengmei*	[13]*Chrysanthemoides monilifera* L. (Norl.) (Asteraceae); [13]*Cotyledon orbiculata* L. (Crassulaceae)
C. aethon	[4]*C. liengmei*	[14]*Rhus zeyheri* Sond. (Anacardiaceae); [4]*Crassula* sp. (Crassulaceae)
C. aureus	[4]*C. liengmei*	[5]*Clutia pulchella* L. (Euphorbiaceae); [17]*Diospyros lycioides* Desf. (Ebenaceae)
C. lyncurium	[2]*C. liengmei*	*[14]*Diospyros lycioides* Desf. (Ebenaceae)
C. lycegenes	[5]*C. liengmei*	[14]*Diospyros lycioides* Desf.; [16]*D. austro-africana* De Winter (Ebenaceae); [16]*Myrsine africana* L. (Myrsinaceae); [14]*Rhus* sp. (Anacardiaceae); [13]*Chrysanthemoides monilifera* L. (Norl.) (Asteraceae)
C. zeuxo zeuxo	[2]*C. liengmei*	[18]*Chrysanthemoides monilifera* L. (Norl.) (Asteraceae)
C. zeuxo cottrelli	[2]*C. liengmei*	[19]*Chrysanthemoides monilifera* L. (Norl.) (Asteraceae)
C. zonarius zonarius	[2]*C. peringueyi*	[16]*Chrysanthemoides incana* (Burm. f.) Norl. (Asteraceae)
C. zonarius coetzeri	[2]*C. peringueyi*	[13]*Chrysanthemoides incana* (Burm. f.) Norl. (Asteraceae)
C. felthami felthami	[2]*C. peringueyi*	[16]*Roepera flexuosa* (Eckl. and Zeyh.) Beier and Thulin; [16]*R. sessilifolia* (L.) Beier and Thulin; [20]*R. morgsana* (L.) Beier and Thulin (Zygophyllaceae)
C. felthami dukei	[6]*C. peringueyi*	[13]*Roepera flexuosa* (Eckl. and Zeyh.) Beier and Thulin; [13]*R. sessilifolia* (L.) Beier and Thulin (Zygophyllaceae)

C. pyroeis pyroeis	[7]*M. nigra*	[7]*Roepera flexuosa* (Eckl. and Zeyh.) Beier and Thulin; [16]*R. sessilifolia* (L.) Beier and Thulin; [20]*R. morgsana* (L.) Beier and Thulin (Zygophyllaceae)
C. pyroeis hersaleki	Unrecorded	[13]*Roepera flexuosa* (Eckl. and Zeyh.) Beier and Thulin; [13]*R. sessilifolia* (L.) Beier and Thulin (Zygophyllaceae)
C. chrysantas	[8]*C. melanogaster*	[8]*Salsola tuberculata* (Moq.) Fenzl. (Chenopodiaceae)
C. thysbe thysbe	[9]*C. peringueyi*	[1]*Aspalathus* spp.; [1]*Lebeckia plukenetiana* E. Mey.; (Fabaceae); [1]*Roepera flexuosa* (Eckl. and Zeyh.) Beier and Thulin; [1]*R. sessilifolia* (L.) Beier and Thulin (Zygophyllaceae); [13]*Chrysanthemoides monilifera* L. (Norl.) (Asteraceae)
C. thysbe osbecki	[6]*C. peringueyi*	[6]*Aspalathus* spp.; [1]*Lebeckia plukenetiana* E. Mey. (Fabaceae); [16]*Roepera flexuosa*.; [16]*R. morgsana* (L.) Beier and Thulin (Zygophyllaceae); [6]*Thesium* spp. (Santalaceae); [16]*Chrysanthemoides incana* (Burm. f.) Norl. (Asteraceae)
C. thysbe psyche	[2]*C. peringueyi*	[2]*Roepera* sp. (Zygophyllaceae); [6]*Thesium* spp. (Santalaceae)
C. thysbe bamptoni	[2]*C. peringueyi*	[2]*Roepera flexuosa* E. and Z. ; [6]*R. teretifolia* (Schltr.) Beier and Thulin (Zygophyllaceae); [6]*Thesium* spp. (Santalaceae); [13]*Lebeckia plukenetiana* E. Mey. (Fabaceae)
C. thysbe schloszae	[6]*C. peringueyi*	[10]*Roepera* sp. (Zygophyllaceae)
C. thysbe mithras	Unrecorded	*[32]*Chrysanthemoides monilifera* L. (Norl.) (Asteraceae)
C. thysbe whitei	[2]*C. peringueyi*	[2]*Chrysanthemoides monilifera* L. (Norl.) (Asteraceae); [13]*Roepera* sp. (Zygophyllaceae)
C. trimeni	[10]*C. peringueyi*	[8]*Roepera flexuosa* (Eckl. and Zeyh.) Beier and Thulin; [6] *R. morgsana* (L.) Beier and Thulin (Zygophyllaceae); [10]*Thesium* spp. (Santalaceae); [8]*Osteospermum oppositifolium* (Aiton) B.Nord. (Compositae)
C. pan pan	[2]*C. liengmei*	[16]*Chrysanthemoides incana* (Burm. f.) Norl. (Asteraceae)
C. pan lysander (W. Coast)	[2]*C. liengmei*	[6]*Roepera flexuosa* (Eckl. and Zeyh.) Beier and Thulin; (Zygophyllaceae); [6]*Osteospermum*; *C. oppositifolium* (Aiton) B. Nord. (Asteraceae)
C. pan lysander (Leipoldtv'll)	[8]*C peringueyi*	[10]*Atriplex bolusii* C.H. Wright (Amaranthaceae); *[16]*Chrysanthemoides incana* (Burm. F.) Norl. (Asteraceae)

C. pan henningi	[2]*C. liengmei*	[2]*Tetraena retrofracta* (Thunb.) Beier and Thulin (Zygophyllaceae)
C. azurius	[11]*C. peringueyi*	[10]*Roepera* sp. (Zygophyllaceae)
C. aridus	*[8]*C. melanogaster*	[21]*Chrysanthemoides incana* (Burm. F.) Norl. (Asteraceae); *[8]*Roepera* sp. (Zygophyllaceae); *[8]*Thesium* sp. (Santalaceae)
C. turneri turneri	Unrecorded	[22]*Roepera* sp. (Zygophyllaceae)
C. turneri wykehami	[2]*C. liengmei*	[2]*Dimorphotheca cuneata* (Thunb.) Less. (Asteraceae)
C. turneri amatola	Unrecorded	Unknown
C. uranus uranus	[2]*C. liengmei*	[2]*Centella* sp. (Apiaceae); [16]*Roepera* sp. (Zygophyllaceae); [16]*Aspalathus spinosa* L. (Fabaceae)
C. uranus schoemani	Unrecorded	[2]*Centella* sp. (Apiaceae)
C. perseus (West Coast)		[2]*C. melanogaster* [6]*Roepera flexuosa* (Eckl. and Zeyh.) Beier and Thulin (Zygophyllaceae); [2]*Thesium* sp. (Santalaceae); [6]*Osteospermum oppositifolium* (Aiton) B. Nord. (Asteraceae)
C. perseus (Lambert's Bay)		[10]*C. peringueyi* [6]*Roepera teretifolia* (Schltr.) Beier and Thulin (Zygophyllaceae); [2]*Thesium* sp. (Santalaceae)
C. adonis adonis	[2]*C. liengmei*	[16]*Roepera* sp. (Zygophyllaceae); [2]*Thesium* sp. (Santalaceae)
C. adonis aridimontis		Unrecorded Unknown
C. swanepoeli swanepoeli		[2]*C. liengmei* [13]*Thesium* sp. (Santalaceae); *[13]*Tylecodon paniculatus* (L.f.) Toelken (Crassulaceae)
C. swanepoeli hyperion		[6]*C. liengmei* [4]*Thesium* sp. (Santalaceae)
C. irene	Unknown	Unknown
C. nigricans nigricans		[2]*C. liengmei* [13]*Thesium* sp. (Santalaceae); [23]*Osteospermum polygaloides* L. (Asteraceae); *[16]*Roepera fulva* (L.) Beier and Thulin (Zygophyllaceae)
C. nigricans zwartbergae		[6]*C. liengmei* [6]*Thesium* spp. (Santalaceae); [6]*Roepera* sp. (Zygophyllaceae)
C. nigricans rubrescens		[6]*C. liengmei* [6]*Thesium* sp. (Santalaceae)
C. palmus palmus	[1]*C. peringueyi*	[24]*Berzelia intermedia* (D. Dietr.) Schltdl.; [24]*B. lanuginosa* Brongn.; [24]*B. abrotanoides* (L.) Brongn. (Bruniaceae); [21]*Chrysanthemoides monilifera* L. (Norl.); [16]*C. incana* (Burm. f.) Norl. (Asteraceae); [22]*Aspalathus sarcantha* Vog.; [16]*A. carnosa* Berg. (Fabaceae)
C. palmus margueritae		Unrecorded [Probably as for nominate subspecies]

C. brooksi	[12]*C. peringueyi*	[13]*Thesium* sp. (Santalaceae); [13]*Roepera* sp. (Zygophyllaceae); [16]*Aspalathus spinosa* L. (Fabaceae)
C. brooksi tearei	[2]*C. peringueyi*	[6]*Roepera* sp. (Zygophyllaceae)
C. daphne	[2]*C. liengmei*	[2]*Thesium* sp. (Santalaceae)
C. plutus	[2]*C. peringueyi*	[2]*Thesium* sp. (Santalaceae); [13]*Roepera* sp. (Zygophyllaceae)
C. blencathrae	[2]*C. liengmei*	[2]*Dimorphotheca venusta* (Norl.) Norl. (Asteraceae)
C. endymion	[2]*C. peringueyi*	[13]*Thesium* sp.; *[2]*Thesidium* sp. (Santalaceae)
C. rileyi	[2]*C. peringueyi*	[13]*Thesium* sp. (Santalaceae); [16]*Aspalathus* sp. (Fabaceae)
C. pyramus pyramus	[2]*C. peringueyi*	[2]*Osteospermum asperulum* (DC) Norl. (Asteraceae); [2]*Thesium* sp. (Santalaceae)
C. pyramus balli	[2]*C. peringueyi*	[2]*Dimorphotheca montana* Norl. (Asteraceae); [2]*Thesium* sp. (Santalaceae)
C. violescens	[2]*C. peringueyi*	[2]*Dimorphotheca cuneata* (Thunb.) Less. (Asteraceae)
C. beaufortius beaufortius	Unrecorded	[13]*Dimorphotheca cuneata* (Thunb.) Less. (Asteraceae); [13]*Chrysanthemoides monilifera* L. (Norl.) (Asteraceae)
C. beaufortius charlesi		[2]*C. peringueyi* [13]*Dimorphotheca cuneata* (Thunb.) Less. (Asteraceae)
C. beauf. stepheni (Calvinia)	[2]*C. peringueyi*	[2]*Dimorphotheca cuneata* (Thunb.) Less. (Asteraceae)
C. beauf. stepheni (Garies)		[2]*C. peringueyi* [2]*Osteospermum amplectans* (Harv.) Norl. (Asteraceae)
C. beauf. sutherlandensis		[6]*C. peringueyi* [6]*Dimorphotheca cuneata* (Thunb.) Less. (Asteraceae)
C. beulah	Unrecorded	Unknown
C. braueri	[8]*C. peringueyi*	[8]*Thesium* sp. (Santalaceae); [25]*Lotononis carnosa* (Eckl. and Zeyh.) Benth. ssp. *carnosa* (Fabaceae); [16]*Roepera* sp. (Zygophyllaceae)
C. penningtoni	Unrecorded	Unknown
C. orientalis	[2]*C. liengmei*	[2]*Thesium* sp. (Santalaceae)
C. pelion	Unrecorded	Unknown

Note 1: Pennington (1962) gives the food-plant of *C. pan* as *Chrysanthemoides monilifera*. This is clearly a misidentification of *C. incana*, as the former does not grow in the localities indicated, but the latter does.

Note 2: Henning, S.F. (1979) gives the food-plant of *Poecilmitis kaplani* (provisionally treated here as a polytopic population of *C. beaufortius stepheni* near Garies) as *Dimorphotheca cuneata*. This was corrected to *Osteospermum amplectans* in Heath (1997). *D. cuneata* grows in the Sutherland district, although the two plants are very similar and mature larvae transfer readily between the two (Heath and Pringle 2007).

Note 3: Dickson and Kroon (1978) gave the food-plant of *Chrysoritis aureus* as *Clutia galpinii* Pax. (=*C. pulchella*); this was repeated by Owen-Johnston (1991) and Heath (1997). *Clutia galpinii* Pax. is a synonym of *Clutia pulchella* L. (Henning, S.F. 1983a).

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