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Chrysoritis Butler (Papilionoidea: Lycaenidae: Aphnaeinae) – Part I: Molecular phylogenetic analyses of a South African genus of myrmecophilous butterflies

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Swee-Peck Quek [1] * Email: queksp@gmail.com; Ernest L. Pringle [2] Email: epringle@eastcape.net; Alan Heath [3] Email: alan.heath333@icloud.com
[1] Integrative Biology Department, University of California, Berkeley, CA, USA.
[2] Huntly Glen, Bedford, Eastern Cape 5780, South Africa.
[3] Iziko South African Museum, Cape Town, South Africa.

Abstract: The molecular data of Talavera *et al.* (2020) is re-analysed to provide a foundation for a taxonomic revision of *Chrysoritis*. A COI phylogeny recovers most of the *Chrysoritis* species as monophyletic, and a few as polyphyletic but supported by the CAD gene. In the *thysbe* clade, most species, despite occurring in sympatry with at least one other species in the clade, maintain distinctive wing facies and ecological identity without intermediate forms. Within the *thysbe* clade, which contains the majority of species, sister taxon comparisons based on the COI phylogeny suggest speciation has been predominantly allopatric and accompanied mostly by minor morphological change and sometimes also a change in male patrolling terrain and/or host ant species. The diversification of the *thysbe* clade and the taxonomic implications of our results are discussed.

Key words: Speciation, sympatry, GCFR, Cytochrome C Oxidase I, introgression, CAD, iridescence, aphytophagy, trophallaxis, hilltopping, male patrolling terrain, *Crematogaster*.

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INTRODUCTION

In this first of three papers on *Chrysoritis*, molecular phylogenetic analyses of the genus are presented, based on data originally published by Talavera *et al.* (2020) comprising COI for all specimens and three nuclear loci (CAD, EF1-alpha and Histone 3) for a quarter of them. Two papers on natural history (Heath *et al*. in press 2023a) and taxonomy (Heath *et al*. in press 2023b) follow in the next volume; they are abbreviated herein to HEA23a and HEA23b, respectively. When possible, the three papers are best read as a single publication due to extensive cross-referencing of each to the others. To maintain consistency in names across the three publications, all *Chrysoritis* names herein follow the taxonomic revision of HEA23b, thus some names used here are technically not yet valid until the publication of HEA23b shortly. They are: *C.* aridimontis (currently *C. adonis aridimontis*), *C.* zwartbergae (currently *C. nigricans zwartbergae*), *C.* amatola (currently *C. turneri amatola*), *C.* wykehami (currently *C. turneri wykehami*), *C.* stepheni (currently *C. beaufortia stepheni*), *C.* mithras (currently *C. thysbe mithras*), *C.* whitei (currently *C. thysbe whitei*), *C.* pan atlantica (currently part of *C. pan lysander*), *C.* lysander (currently part of *C. pan lysander*) and *C.* williami (currently part of *C. pan lysander*). The species epithets are not italicised in the text of this publication to recognise their provisional status. Note that HEA23b proposes other taxonomic changes, which do not appear in this article.

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Chrysoritis Butler (1897) is a southern African genus of lycaenid butterflies within the myrmecophilous subfamily Aphnaeinae (Heath, 1997; Boyle *et al*., 2014). The genus includes 43 species prior to the publication of HEA23b and diverged about 22–25 million years ago (MYA) from its sister lineage comprising the genera *Cigaritis*, *Crudaria* + *Cesa*, *Liphaphnaeus*, and *Chloroselas* + *Vansomerenia* (Kawahara *et al*., in revision). Most of *Chrysoritis'* diversity is concentrated in the Greater Cape Floristic Region (GCFR) of South Africa.

The larvae of all *Chrysoritis* species are obligately attended by ants (Heath, 1997; Heath & Claassens, 2003). The ants provide protection to the larva and in return they harvest secretions from the larva's dorsal nectary organ (Heath & Claassens, 2003: 10). With the exception of *C. dicksoni* (Gabriel, 1947), all species have phytophagous larvae. *C. dicksoni* larvae are aphytophagous and are fed by ants through trophallaxis; they are likely also fed trophic eggs by their host ants (Heath, 1998; 2014).

The taxonomy of *Chrysoritis* presents a considerable challenge in large part because of morphological uniformity across the genus. Wing venation and the ground plan of wing markings are invariant or show limited variability in *Chrysoritis*, while the degree of melanistic scaling (from highly melanistic to pallid) within species can vary from fixed to highly labile, thus these features are not taxonomically helpful. Genitalic structure, often relied upon by entomologists to discriminate among closely related species, is highly conserved in the genus and uniform in the young *thysbe* clade. Thus for most of the *thysbe* clade, adult morphology-based taxonomy ranges from challenging to intractable since the only morphological traits available are wing marking and

colouration which often do not vary in taxonomically meaningful ways.

The first attempt at inferring a molecular phylogeny of *Chrysoritis* was undertaken by Rand *et al*. (2000) with a study based on the mitochondrial gene cytochrome c oxidase I (COI). Nineteen of the 59 species recognized at that time were selected to represent the genus. Based on that study, two sets of changes were subsequently made to the taxonomy at the species level by Heath (2001 & 2011) and several other publications on *Chrysoritis* (Heath, 1997, 2001; Rand *et al*., 2000; Heath & Pringle, 2007: 35) recognized the need for a taxonomic review supported by molecular data.

The recent molecular phylogeny of *Chrysoritis* by Talavera *et al*. (2020; hereafter referred to as TEA20) showed that 28 of the then 43 species (65%) clustered into a single large clade, the *thysbe* clade, which radiated in the last ~2 MY (however, in light of the results of Kawahara *et al*. [in revision], this age may be an overestimate, given that the age estimated for *Chrysoritis* by Kawahara *et al*. is considerably younger than that of TEA20). While this clade itself was well supported, it lacked a stable and robust internal topology (Fig. S1 in TEA20), and several species were not recovered as monophyletic. TEA20 postulated that the *thysbe* clade could be viewed as consisting of a single polymorphic species comprising many subspecies.

Here we re-analyse the molecular data from TEA20 to provide a better foundation for a taxonomic revision of *Chrysoritis*, and address the question of whether the *thysbe* clade could be regarded as a single species. In HEA23a we discuss the natural history of the genus, including adult and juvenile morphological, ecological and behavioural traits, as well as an account of the aphytophagous *C. dicksoni*. A taxonomic revision of *Chrysoritis* based on the analyses presented here in combination with morphological, ecological and distributional traits, is presented in HEA23b.

MATERIALS AND METHODS

Abbreviations

TEA20: Talavera *et al.* (2020)
HEA23a: Heath *et al*. (2023a)
HEA23b: Heath *et al*. (2023b)
COI: Cytochrome c oxidase subunit I (partial gene fragment)
CAD: Carbamoyl-phosphate synthetase 2/aspartate transcarbamylase/dihydroorotase (partial gene fragment)
EF: Elongation factor 1-alpha (partial gene fragment)
H3: Histone 3 (partial gene fragment)
mtDNA: mitochondrial DNA
ML: Maximum Likelihood
MY(A): Million years (ago)
MPT: Male patrolling terrain
AMOVA: Analysis of Molecular Variance
GCFR: Greater Cape Floristic region

Sampling and natural history observations

Opportunistic field observations of adults and juvenile stages of *Chrysoritis* were made across >90 person years by E. Pringle and A. Heath combined. These observations were coupled with the rearing of juvenile stages in captivity, dissections and studies of genitalia, and the preparation of wing venation slides. The resulting reference collection of set adult specimens (A. Heath) has been accessioned and integrated into the Iziko South African Museum. A collection of host ants was also made, which has now been accessioned into the Museum ant collection. A total of 399 *Chrysoritis* samples plus four outgroup specimens from three lycaenid genera (*Pseudaletis*, *Cigaritis* and *Crudaria* in the Aphnaeinae) are included in the phylogenetic analyses; all hitherto described species of *Chrysoritis* are included. The list of samples can be found in Table S1 of TEA20, noting that some taxon names have changed as a result of the taxonomic revision presented in HEA23b.

Illustrations

All photographs and line drawings are by A. Heath except where otherwise stated. The equipment used includes an Olympus E-M5 micro 4/3 camera, 60mm Macro lens, 20mm Macro lens and Olympus Bellows. Editing, including focus-merging, was done using Affinity Photo. Images of wing scales taken at the Iziko Museum Entomology Department by S. van Noort were taken with a Leica LAS 4.9 imaging system, comprising a Leica® Z16 microscope (using either a 2X or 5X objective) with a Leica DFC450 Camera and 0.63x video objective attached. The imaging process, using an automated Z-stepper, was managed using the Leica Application Suite V 4.9 software installed on a desktop computer. Diffused lighting was achieved using a Leica LED5000 HDI dome.

Chrysoritis identifications

Identification of the specimens were established over many years and were made in reference to the type specimens and their authors' descriptions. In two cases of ambiguity, namely *C. lyndseyae vs C. thysbe bamptoni* and *C. thysbe mithras* vs *C. t. thysbe*, COI neighbour joining trees were constructed (by Z.A. Kaliszewska) during the early stages of the TEA20 study, which helped to confirm separate COI phylogenetic placements for each member in each pair.

Morphological traits

Morphological traits used for species and subspecies circumscriptions relied primarily on wing morphology, particularly the:

1) extent (including presence/absence) of silvery-blue wing scaling (which creates iridescence structurally)
2) extent of "solid blue" of the hindwing upper side
3) density of silvery-blue on both wings (thereby allowing or preventing visibility of underside markings through to the upper side)
4) clarity of hindwing underside markings (plain vs. well marked)
5) pallidity of wings (hindwing underside, or in general)
6) hindwing and forewing shape
7) length of the hindwing "tail"

Some of these traits are useful only to a limited degree (either for a single taxon or a small group of taxa, e.g., hindwing shape for *C. chrysaor natalensis*, forewing shape for *C. brooksi* and *C. palmus*, and short hindwing "tail" for *C. chrysaor, C.chrysaor natalensis,* and *C. phosphor*).

Preliminary investigations suggest that the length of the upper row of lateral setae in early first instar larvae appears to be fairly constant within a given species, and the length and shape of the tubercles housing the tentacular organs appear to differ significantly among *Chrysoritis* species (details in HEA23a). Thorough investigation of these traits is beyond the scope of this paper but should be considered in future taxonomic efforts.

Ecological traits

Heath & Pringle (2007: 38) suggested that any species within the *thysbe* clade could utilise the host plant of any other species within the clade, and so all species in the *thysbe* clade have been treated as polyphagous. Thus, host plant data are considered to be of little use in *Chrysoritis* taxonomy (see also Cottrell, 1984: 41).

TEA20 showed that *Chrysoritis* species show remarkable overlap in climatic niche, thus that trait is also not useful for taxonomy. The following ecological traits were examined for their potential in delimiting species: 1) host ant species, 2) male patrolling terrain, and 3) range overlap (sympatry vs. allopatry; see Note S1) with other *Chrysoritis* taxa. These traits are discussed below.

Host ants and plants

Host plants and ants are listed together with original sources in Heath *et al.* (2008), and TEA20 provided a broad summary of host plant genera in their Table S5. Following revised IDs of the host ants by B. Blaimer (see below) a revised list of plant and ant hosts is given in Table S1 in HEA23b. The two ant genera recorded as associated with *Chrysoritis* species are the 'Droptail' ants *Myrmicaria* Saunders, 1842 and the 'Cocktail' ants *Crematogaster* Lund, 1831, both in Myrmicinae. The majority of *Chrysoritis* species are associated with *Crematogaster*; only two species are recorded with *Myrmicaria* – *C. oreas* and *C. pyroeis*.

Host ant identification

The absence of the "correct" species of host ant, assumed to be chemically detected by the gravid female, may deter oviposition. Dr Hamish Robertson provisionally identified the ants mentioned herein from samples collected in the field that were found in association with *Chrysoritis* larvae and adults. Some of these identifications were subsequently revised by B. Blaimer (Smithsonian National Museum of Natural History) and endorsed by H.G. Robertson. The authors also referred to Peter Slingsby's recent guidebook (2017) to confirm ant identifications. The *Crematogaster* species currently known to associate with *Chrysoritis* are:

Crem. liengmei Forel, 1894
Crem. gallicola Forel, 1894
Crem. amabilis Santschi, 1911 (*cf.* Blaimer)
Crem. alulai Emery, 1901 (*cf.* Blaimer)
Crem. castanea Smith, F., 1858
Crem. melanogaster Emery, 1895
Crem. peringueyi Emery, 1895

Crematogaster peringueyi is understood to be a subjective synonym of *Crem. capensis* Mayr, 1862 (B. Blaimer & H.G. Robertson, pers. comm.); this view is as yet unpublished and so both names are currently valid and available. To avoid confusion, only the former name is used herein.

Unresolved cryptic species diversity is prevalent among ants. Even putatively well-known ant species like *Lasius niger* and *Lasius alienus* in Europe have turned out to be species complexes. Few of the ant species-level identifications listed above have been scrutinised by myrmecologists, and thus may be suspect (Fiedler, 2021). For example, *Crem. peringueyi* and *Crem. liengmei* are widespread common species that could potentially contain cryptic species (see also Bickford *et al.*, 2007) but they have yet to be studied in detail to determine if this could be the case here (B. Blaimer, pers. comm.).

Male patrolling terrain (MPT)

MPT is an informative trait in circumscribing *Chrysoritis* species. In some taxa, males congregate at specific topographic features (*e.g.*, hilltop, gulley etc.), awaiting the arrival of newly eclosed virgin females. Likewise, virgin females will seek out particular terrain features to find their mate soon after eclosing. This phenomenon, possibly a form of lekking, is sometimes known as "hilltopping." Specificity in MPT maximises the chances of finding a mate of the same species and could serve as a prezygotic barrier to gene flow between species in sympatry. MPT specificity appears to be characteristic of *thysbe* clade species and has so far not been observed in species outside the *thysbe* clade. Males of *C. dicksoni* (outside the *thysbe* clade) show aggregating behaviour but it seems not to be associated with a consistent topographical feature (see HEA23a). In non-*thysbe* clade species (noting the exception of *C. dicksoni*), most males and females congregate and mate near their host plants, and some species (e.g. *C. zonarius*) prefer to stay very close to their host plant.

Analyses of molecular data

Phylogenetic analyses

Molecular data for population and phylogenetic analyses are from TEA20. The 406 *Chrysoritis* samples reported therein were found to contain a few samples that were sequenced twice for COI. After removing the duplicates, the total number of *Chrysoritis* samples was 399 (excluding 4 outgroup samples), of which all have sequences of COI (1220 base pairs [bp]), 97 have EF (1039 bp) and CAD (745 bp) sequences, and 98 have H3 (328 bp) sequences. We also discovered that the COI sequence for sample AH06M581 (identified as *C. pan* but appearing as part of *C.* stepheni) was likely the result of contamination and we deduced the correct sequence (see Note S1). The trees presented by TEA20 based on all four genes combined lacked well supported resolution in the *thysbe* clade. Examination of the nuclear gene trees (CAD, EF and H3, unpublished, courtesy of G. Talavera) showed scant resolution within the *thysbe* clade. Thus we performed further analyses on the COI data alone (1220 nucleotides). Identical sequences were identified using Arlequin 3.5. (Excoffier *et al.*, 1992; Excoffier & Lischer, 2010) and removed prior to phylogenetic analyses, resulting in a sample set of 270. Maximum likelihood (ML) analyses were performed on the unpartitioned COI dataset using

IQTree (Nguyen *et al*., 2015; Minh *et al*., 2020) run on IQTree's web server (Trifinopoulos *et al.*, 2016) at https://www.hiv.lanl.gov/content/sequence/IQTREE/iqtree.html using the "find best and apply" substitution model setting (ModelFinder, Kalyaanamoorthy *et al.*, 2017, resulting in the TVM + F + R3 model selected using AIC, BIC and AICc), and branch support was obtained from 1000 repetitions of ultrafast bootstrap (Hoang *et al*., 2018). ML analyses were also performed on the three nuclear genes separately using the same software and settings as for the COI data.

The aligned and combined nuclear gene dataset matrix is available for download at https://doi.org/10.6084/m9.figshare.19225203 and the COI dataset matrix at: https://doi.org/10.6084/m9.figshare.19225101.

Haplotype network construction

A statistical parsimony network (Templeton *et al*., 1992) of COI haplotypes (with duplicates removed as described above) from *thysbe* clade (*sensu* TEA20) was constructed using TCS 1.21 (Clement *et al*., 2000), and the network visualised using the program tcsBU (Murias dos Santos *et al.*, 2016). The 334 samples within the *thysbe* clade collapsed into 200 unique haplotypes; however, some haplotypes differed only by missing data. A list of samples sharing identical haplotypes is provided in Table S1, available at: https://doi.org/10.6084/m9.figshare.19225038.

AMOVAs

To ascertain the degree to which genetic structure in the COI data of the *thysbe* clade can be explained by taxonomic designation versus geographic distribution, we performed analyses of molecular variance (AMOVAs) using Arlequin 3.5. (Excoffier *et al.*, 1992, Excoffier & Lischer, 2010). AMOVAs were run using four grouping schemes: 1) by species, 2) by subspecies, 3) by region, and 4) by locality group (comprising a cluster of localities in the same vicinity – see Table S2). In addition to the overall fixation index (Φ_{ST}), fixation indices for all pairwise comparisons were calculated to determine how differentiated a species or subspecies was from its sister taxon. Two samples were excluded from the AMOVAs: AH06M581 (*C. pan* which had a contaminated COI sequence, see above) and AH12C011 (a *brooksi* x *rileyi* hybrid).

Genetic distances

In this study, the authors used COI data from TEA20 to generate a pairwise COI distance matrix of *Chrysoritis* samples. Genetic distance is not used to determine rank designations or species-level splits. This table is available as a .xlsx file at: https://doi.org/10.6084/m9.figshare.17241566.v1.

RESULTS

All supplementary figures (Fig. S_) are available at https://doi.org/10.6084/m9.figshare.21341559 and all supplementary tables (Table S_) and notes (Note S_) are at https://doi.org/10.6084/m9.figshare.21588234.

Phylogenetic analyses: COI data compared with total dataset

Within *Chrysoritis* (outgroups excluded), the COI dataset (n = 399) contributed 292/1220 parsimony-informative sites, EF (n = 93) contributed 97/1039, CAD (n = 93) contributed 97/745, and H3 (n = 94) contributed 41/328 (Table S3). The COI phylogeny inferred from ML is shown in Fig. 1. Type localities are included in our sampling for 32 out of the 49 species of *Chrysoritis* (indicated in Fig. 1). As in TEA20, the COI tree shows that *Chrysoritis* is split into two major lineages – the Eastern Lineage (7 species; also known as the *chrysaor* clade in TEA20) and the Western Lineage containing the *thysbe* clade (37 species as of the taxonomic revision of HEA23b) and a grade of lineages sister to the *thysbe* clade (5 species). For comparison, the total dataset tree inferred by TEA20 (Fig. S1 in TEA20) is reproduced here in a viewer-friendly version in Fig. S2; it comprises 403 specimens (after removing doubly sequenced samples) with COI data and about quarter of that with data from nuclear gene regions. The *thysbe* clade is found in 100% of bootstrapped COI trees (Fig. 1A). Species in the *thysbe* clade are divided into six main COI lineages (Figs 1A, 1B). Their main features are:

mitochondrial (mt) clade 1 (95% bootstrap support): 11 species, plus one species shared with mt clade 3;
mt clade 2 (100% support): 1 species (*C. thysbe*);
mt clade 3 (95% bootstrap support): 15 species, plus one species shared with mt clade 1 and one species shared with mt clade 4;
mt clade 4 (100% bootstrap support): five species, plus one species shared with mt clade 3.
C. amatola + *C. violescens*: two species, 100% bootstrap support,
C. pelion: one species, 100% bootstrap support (the two specimens are identical, see Fig. S2).

Relationships among species differed slightly in the total dataset tree (Fig. S2) compared with the COI tree (Fig. 1A). The six main COI lineages listed above were present in the total dataset tree, but bootstrap values are much lower in the total dataset tree (the Ultrafast bootstrap option in IQTree, used for the COI analysis, tends to yield higher bootstrap support than standard bootstrap analysis which was used for the total dataset tree by TEA20; see Hoang *et al*. (2018)). Bootstrap values for species were also lower in the total dataset tree than in the COI tree for the reason noted above. The total dataset tree yielded polyphyletic groupings for three species (*C. pan*, *C.* williami, and *C. azurius*) that were monophyletic in the COI tree. *Chrysoritis lyndseyae* was polyphyletic in the total dataset tree, but paraphyletic in the COI tree. Thus, the COI tree aligns with classical taxonomy better than does the total dataset tree.

In the COI tree, all species that were monophyletic and represented by multiple samples were supported by bootstrap values of 95% or more. Six species were recovered as polyphyletic in the COI tree (*C. trimeni*, *C. perseus*, *C. beulah*, *C.* wykehami, *C.* aridimontis, and *C.* zwartbergae), and four as paraphyletic (*C. lyndseyae* with respect to a lineage of *C. trimeni*, *C. pan* with respect to *C. aridus*, *C.* amatola with respect to *C. violescens* and *C. zonarius* with respect to *C. zeuxo*). Further details on

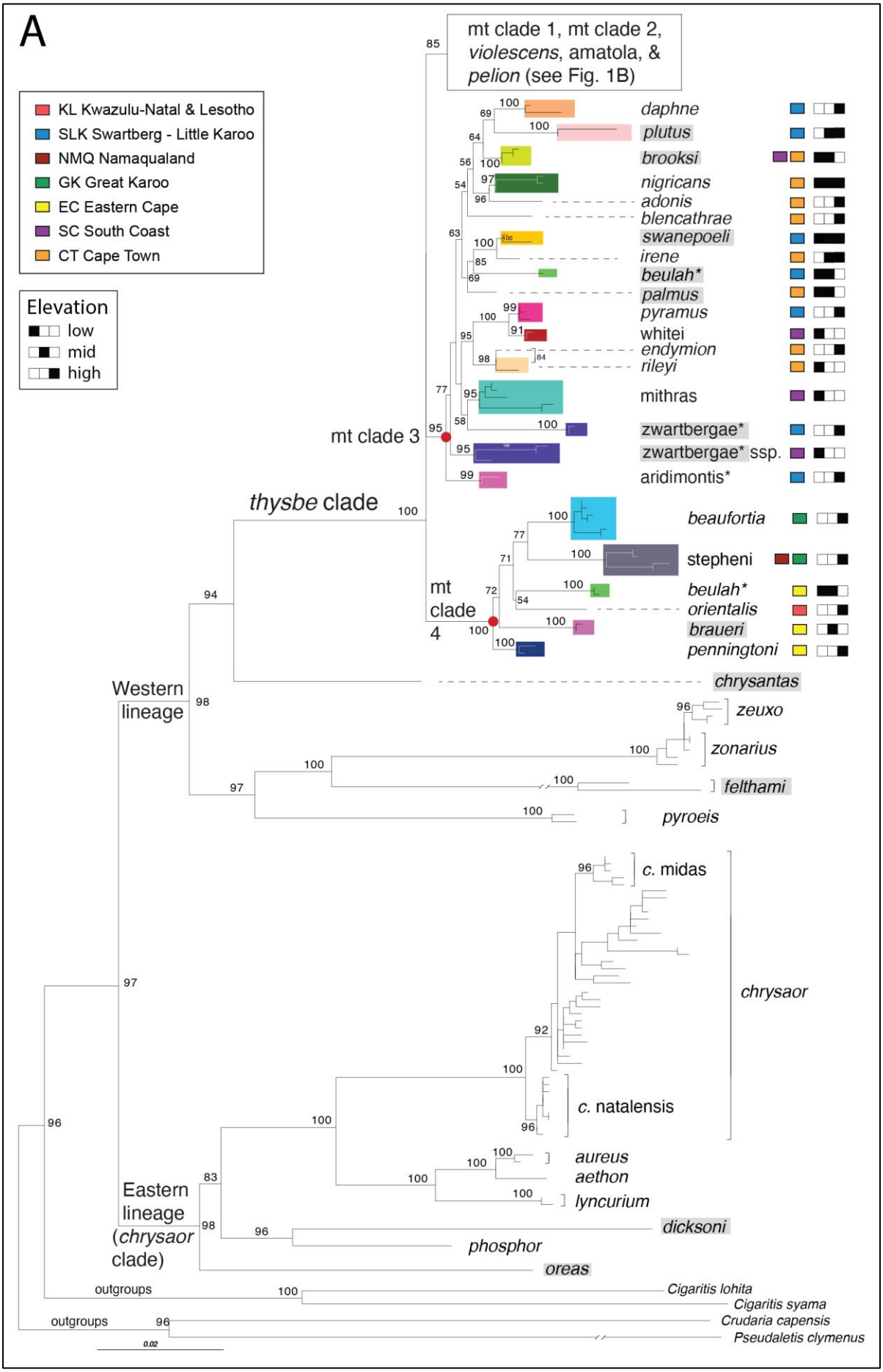

Figure 1A – COI phylogeny of *Chrysoritis* derived from ML analyses, with bootstrap support from 1000 replicates shown at nodes, and duplicated haplotypes removed. Species marked with an asterisk (*) are polyphyletic. Type localities are represented for all taxa except where species names are grey-shaded. This part shows the main tree, with a portion continued in Fig. 1B. The tree shown here is expanded in Fig. S1, where specimen labels are legible.

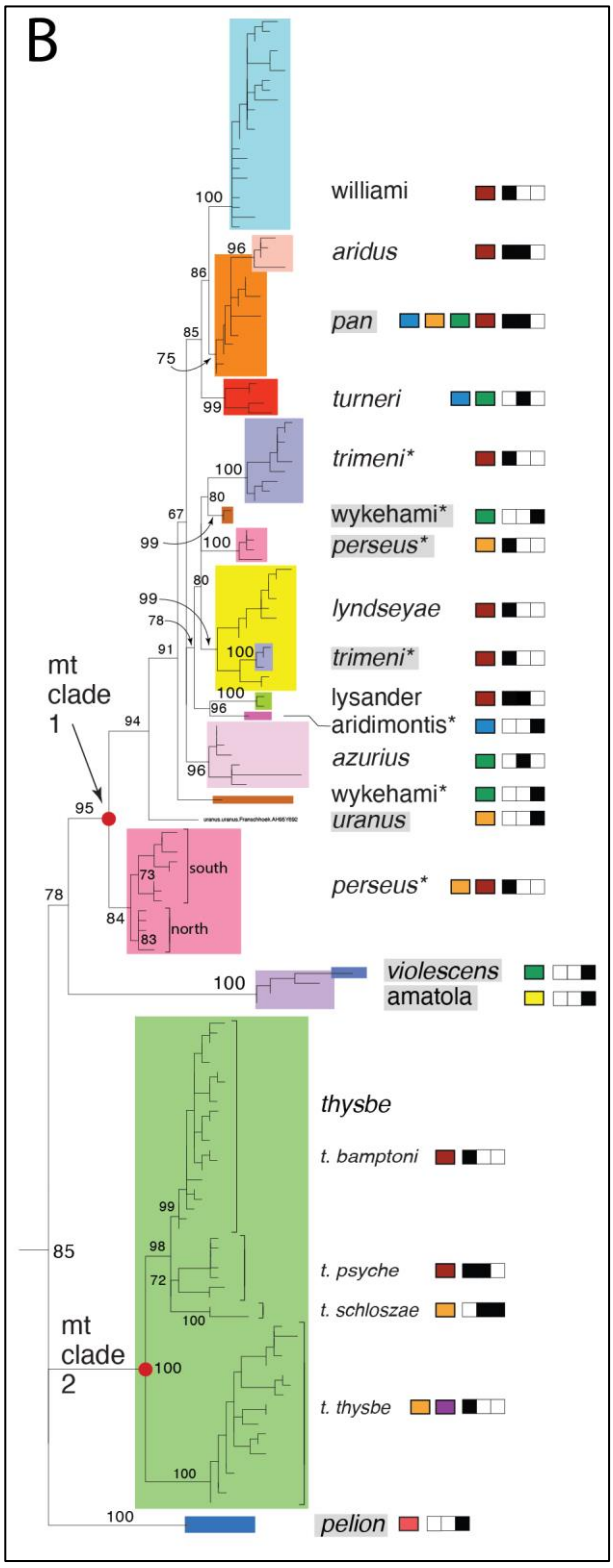

Figure 1B – Expanded portion of Fig. 1A

these COI-polyphyletic species are provided in the next section, as well as in HEA23b.

Our study reveals that a sizable number of specimens have previously been mis-identified as *C. thysbe* prior to the analyses of TEA20. Specimens labelled as *C. thysbe mithras* and *C. thysbe whitei* were found in our analyses and those of TEA20 to group clearly outside the well supported clade of *bona fide C. thysbe* (*i.e.*, mt clade 2).These samples are assigned to their own species (*C.* mithras and *C.* whitei) in HEA23b. A few samples previously identified as *C. thysbe bamptoni* were found to group with *C. lyndseyae*, *C. perseus* and *C.* williami, with which *C. t. bamptoni* is sympatric. This suggests that *C. thysbe* has tended to serve as a "catch-all" name in the face of taxonomic uncertainty – understandable given the wide morphological variation it shows while being sympatric with those species. However, *C. t. bamptoni* has also been misidentified, most commonly as *C. lyndseyae*. Other misidentifications include *perseus* as *pan*, williami as *trimeni*, and *azurius* as *pan*; especially in places where they occur in sympatry. Most or all of these misidentifications involved damaged or female specimens which are more difficult to identify compared to fresh males.

Phylogenetic analyses: individual nuclear loci compared with COI

Table 1 shows whether and how well each species is phylogenetically supported (monophyletic, paraphyletic or polyphyletic) by each of the four gene trees. Outside the *thysbe* clade, most of the species with multiple samples were supported by all four genes. The total dataset tree (Fig. S2), in addition to showing all samples included in the molecular analyses, identifies the subset of samples for which data from nuclear genes is available.

The CAD, EF and H3 trees are shown in Fig. S3 (A, B, & C, respectively). The Eastern and Western Lineages are also found in the nuclear gene trees; although the H3 tree shows the Eastern Lineage to be paraphyletic, this likely stems from misplaced rooting in the analysis. The *thysbe* clade is recovered in the EF tree (97% bootstrap support) and in the CAD tree (99% bootstrap support). It was not found in the H3 tree owing to *C. chrysantas* emerging among *thysbe* clade samples. Most of the clades common to the COI tree and nuclear gene trees (shaded in green in the nuclear gene trees in Fig. S3) lie outside the *thysbe* clade.

All four genes differ in the relative placements of the following with respect to one another: *C. chrysantas*, *C. felthami*, *C. pyroeis*, the *thysbe* clade, and the lineage comprising *C. zeuxo* + *C. zonarius*. But all three nuclear genes place the lineage *C. zeuxo* + *C. zonarius* as sister to all the others whereas the COI tree places it as part of that group (and sister to *C. felthami*), thus the placement of this lineage in the COI tree may not reflect its true phylogenetic position. The grouping (*C. chrysaor*, (*C. lyncurium*, (*C. aethon*, *C. aureus*))) (written in Newick tree notation) occurs in all genes except H3 (compared with other loci, H3 tends to produce trees with decreased taxonomic alignment [S.-P. Quek, pers. obs.]). COI and H3 place *C. dicksoni* and *C. phosphor* as sister to each other but *C. phosphor* is missing from the CAD and EF datasets. The position of *C. chrysantas* with respect to the *thysbe* clade differs in all four loci.

Within the *thysbe* clade, the EF tree is comb-like, showing almost no structure that is well supported by bootstrap values, except for several small clusters along the spine. The H3 tree shows some structure in the *thysbe* clade + *C. chrysantas* but a majority of the nodes are poorly supported. In the EF tree, groups of samples with sequences identical to each other (and where no data are

Table 1 – Support provided for each *Chrysoritis* species by the four genes used in this study. Sample sizes are shown in columns "n". Abbreviations: i.s: insufficient sampling. M: monophyletic. M* monophyletic with root placement corrected. R: paraphyletic. R(?): equivocal, possibly paraphyletic. R(1): identical haplotype but includes one other species, R(2): identical in overlapping region but haplotype shared by two others. R(m): identical haplotype but includes many other species. R*: one specimen identical to an *aureus* specimen. L: polyphyletic. L?: equivocal, possibly polyphyletic. L(x): polyphyletic and x = number of lineages. **: identical to lycegenes specimen. Note: *zeuxo* and *zonarius* show ancestor – descendent relationship in all genes except CAD where their relationship is unclear.

Species	**COI**			**CAD**			**EF**			**H3**		
	support	**code**	**n**	**support**	**code**	**n**	**support**	**code**	**n**	**support**	**code**	**n**
adonis	i.s.		1	i.s.		1	i.s.		1	i.s.		1
amatola	**YES**	R	3	no	L	2	no	L	2	no	L	2
aridimontis	no	L	3	i.s.		1	i.s.		1	i.s.		1
aridus	**YES**	M	4	i.s.		1	i.s.		1	i.s.		1
azurius	**YES**	M	9	no	L	3	no	L	3	no	L	3
beaufortia	**YES**	M	6	no	L(2)	4	no	L	4	no	L?	4
beulah	no	L	3	**possibly**	R(1)	2	no	L?	2	no	L	2
blencathrae	i.s.		1	i.s.		1	i.s.		1	i.s.		1
braueri	**YES**	M	4	i.s.		1	i.s.		1	i.s.		1
brooksi	**YES**	M	2	**YES**	M	2	no	L	2	i.s.		1
daphne	**YES**	M	2	i.s.		1	i.s.		1	i.s.		1
endymion	i.s.		1	i.s.		1	i.s.		1	i.s.		1
irene	i.s.		1	i.s.		1	i.s.		1	i.s.		1
lyndseyae	**YES**	R	49	i.s.		1	no	L	2	no	L	2
lysander	**YES**	M	2	i.s.		0	i.s.		0	i.s.		0
mithras	**YES**	M	7	**possibly?**	R?	2	**YES**	M	2	no	L	2
nigricans	**YES**	M	3	i.s.		1	i.s.		1	i.s.		1
orientalis	i.s.		1	i.s.		1	i.s.		1	i.s.		1
palmus	i.s.		1	i.s.		1	i.s.		1	i.s.		1
pan	**YES**	M	19	no	L	5	no	L	5	no	L	5
pelion	**YES**	M	2	i.s.		1	i.s.		1	i.s.		1
penningtoni	**YES**	M	2	i.s.		1	i.s.		1	i.s.		1
perseus	no	L	37	no	L	4	no	L	4	no	L	4
plutus	**YES**	M	2	i.s.		1	i.s.		1	i.s.		1
pyramus	**YES**	M	4	no	L	2	no	L	2	no	L	2
rileyi	**YES**	R	2	i.s.		1	i.s.		1	i.s.		1
stepheni	**YES**	M	4	no	L	2	no	L	2	**possibly**	R(m)	2
swanepoeli	**YES**	M	3	**possibly?**	L	3	no	L	3	no	L	3
thysbe	**YES**	M	75	no	L	10	no	L	10	no	L	10
trimeni	no	L	14	**YES**	M	2	no	L	3	no	L	3
turneri	**YES**	M	4	no	L	2	no	L	2	no	L	2
whitei	**YES**	M	3	i.s.		1	i.s.		1	i.s.		1
wykehami	no	L	3	**possibly**	R(2)	2	i.s.		1	i.s.		1
uranus	i.s.		1	i.s.		1	i.s.		1	i.s.		1
violescens	**YES**	M	3	i.s.		1	i.s.		1	i.s.		1
zwartbergae	no	L	5	**YES**	M	3	no	L	3	no	L	3
chrysantas	i.s.		1	i.s.		1	i.s.		1	i.s.		1
felthami	**YES**	M	2	**YES**	M	2	**YES**	M	2	**YES**	M	2
pyroeis	**YES**	M	2	**YES**	M	2	**YES**	M	2	**YES**	M	2
zeuxo	**YES**	M	4	**YES**	R	2	**YES**	M	2	**YES**	M	2
zonarius	**YES**	R	5	**YES**	R	3	**YES**	R	3	**YES**	M	2
aethon	i.s.		1	i.s.		1	i.s.		1	i.s.		1
aureus	**YES**	M	2	i.s.		1	i.s.		1	i.s.	**	1
chrysaor	**YES**	M	40	**YES**	M	5	**YES**	M	4	**YES**	M*	5
dicksoni	i.s.		1	i.s.		1	i.s.		1	i.s.		1
lyncurium	**YES**	M	5	**YES**	M	2	**YES**	M	2	**YES**	R*	2
oreas	i.s.		1	i.s.		1	i.s.		1	i.s.		1
phosphor	i.s.		1	i.s.		0	i.s.		0	i.s.		1

missing) range up to ~480 km apart, and in the H3 tree they are up to ~960 km apart. In the H3 tree, some of these groups span the entire range of wing facies (with/without iridescence, and highly/minimally melanistic). In both EF and H3 trees, most of the clades or identical clusters in the *thysbe* clade are not reflected in the COI tree (shaded in pink in Fig. 1B). For the *thysbe* clade, the H3 tree has no clades or identical sequence clusters in common with the COI tree, the EF tree has one and the CAD tree has five (see groups marked in green in Fig. S3). The EF and H3 trees show an almost complete absence of lineage sorting (explained in Note S2 in HEA23b).

Compared to the other nuclear genes, the CAD tree shows more groupings in common with the COI tree (marked in green in Fig. S3A) as well as more structure, with several clades sporting high bootstrap values. Additionally, among the six species that are polyphyletic in the COI tree, the CAD topology provides positive or suggestive (but inconclusive) support for four of them, as well as confirming the hybrid status of one sample with wing facies that were intermediate between two species. They are as described below. The only COI-polyphyletic taxon that the CAD tree does not "resolve" is *C. perseus*, which is widely dispersed in the CAD tree. For the COI-polyphyletic *C.* aridimontis, only a single CAD sequence is available.

Chrysoritis trimeni

The two COI lineages of *C. trimeni* intermingle in the same Kleinsee location and both unequivocally display *C. trimeni* wing facies. However, the smaller COI lineage of *C. trimeni* is nested within *C. lyndseyae* (Fig. 1B). Among the possible explanations is past mitochondrial capture of *C. lyndseyae* mtDNA by *C. trimeni* (both species occur in the Namaqualand region). A mating between a *C. trimeni* male and a *C. lyndseyae* female (due to the maternal inheritance of mtDNA) with subsequent generations of female descendents backcrossing with *C. trimeni* males would result in a lineage of individuals with *C. trimen*i wing facies harbouring *C. lyndseyae* mtDNA in the population, resulting in polyphyly for *C. trimeni* in the COI tree. In the CAD tree (Fig. S3A), *C. trimeni* samples (n = 2, representing both COI lineages) are paired to the exclusion of others (99% bootstrap) and their sequences are identical where they overlap. Thus the CAD data are in agreement with the morphological data in uniting the separate COI lineages as *C. trimeni*.

Chrysoritis zwartbergae

Unlike *C. trimeni*, *C. perseus* and *C.* aridimontis, the two separate COI lineages of *C.* zwartbergae are not sympatric. The three CAD sequences of *C.* zwartbergae (representing both COI lineages and spanning ~186 km) are exclusively monophyletic, and supported by 90% of bootstrap trees (Fig. S3A). Thus the CAD data are in agreement with the classical data in uniting the separate COI lineages as *C.* zwartbergae.

Chrysoritis wykehami and *C. beulah*

For these two species, the CAD topology is suggestive of support but inconclusive due to the presence of other species/samples. *C.* wykehami is in a 93%-supported clade with 3 other samples/species, and *C. beulah* is in a 56%-supported clade with one other sample/species.

Chrysoritis rileyi – C. brooksi hybrid

The specimen (AH12C011) identified as a hybrid between *C.rileyi* and *C. brooksi* based on wing patterning, groups with *C. brooksi* in the COI tree but its CAD sequence is identical to those of the other two *C. rileyi* specimens in regions of overlap (see Fig. S3A). Thus it is very likely the product of a mating between a wandering *C. brooksi* female and a resident *C. rileyi* male.

The taxonomic designations for all samples (based on wing marking, MPT, and the presence/absence of distributional overlap with other species) were already in place prior to in-depth examination of the nuclear trees and data, thus the CAD data independently support the viability of the aforementioned taxa that presented as polyphyletic in the COI tree.

Although the CAD data provide some resolution for most of the taxa that are polyphyletic in the COI tree, we find that many species that are monophyletic in the COI tree are found to be scattered in the CAD tree: *C. thysbe, C. swanepoeli, C. azurius, C.* stepheni, *C. pyramus, C. beaufortia, C.* amatola, *C.* williami and *C. turneri* (comparing Fig. 1A with Fig. S3A). There are, however, a few species where the COI and CAD data are in agreement (*i.e.*, they are monophyletic in both, or monophyletic in COI and share identical sequences in regions of overlap in the CAD tree/data): *C. brooksi*, and *C.* mithras. Eighteen of the 37 species in the *thysbe* clade are represented by single samples in the CAD data thus comparisons between the two datasets for these species are not possible.

COI Haplotype Network

The statistical parsimony haplotype network construction for the *thysbe* clade (Fig. 2) resulted in seven separate networks: 1) a main network containing most of the species, 2) *C. beaufortia* specimens, 3) *C.* stepheni specimens, 4) *C. beulah* specimens, 5) *C. braueri* specimens, 6) *C. nigricans* specimens, and 7) *C. penningtoni* specimens. The main network shows a tangle of loops, reflecting a complex history of reticulating as well as bifurcating diversification, and hence the challenging nature of *Chrysoritis* taxonomy. The networks also reveal a large number of unsampled/hypothetical haplotypes (mostly found in the links between taxa), indicating that much of the genetic diversity of the *thysbe* clade has yet to be sampled or is no longer extant. Disregarding the non-monophyletic species and those represented by singletons, each species in the network forms a cohesive cluster wherein haplotypes are more closely related to conspecifics than to non conspecifics. Two exceptions are 1) *C.* zwartbergae ssp. from South Coast, where one haplotype of this taxon is equidistant between *C. perseus* and the other haplotypes within its cluster, 2) *C. swanepoeli*, where one haplotype of this taxon is equidistant between *C. irene* and the other haplotype within its cluster, and 3) *C. rileyi* where one of its samples (C010) is genetically closer to *C. endymion* than to its conspecific. The network presents a slightly differing view of species groupings compared to the COI

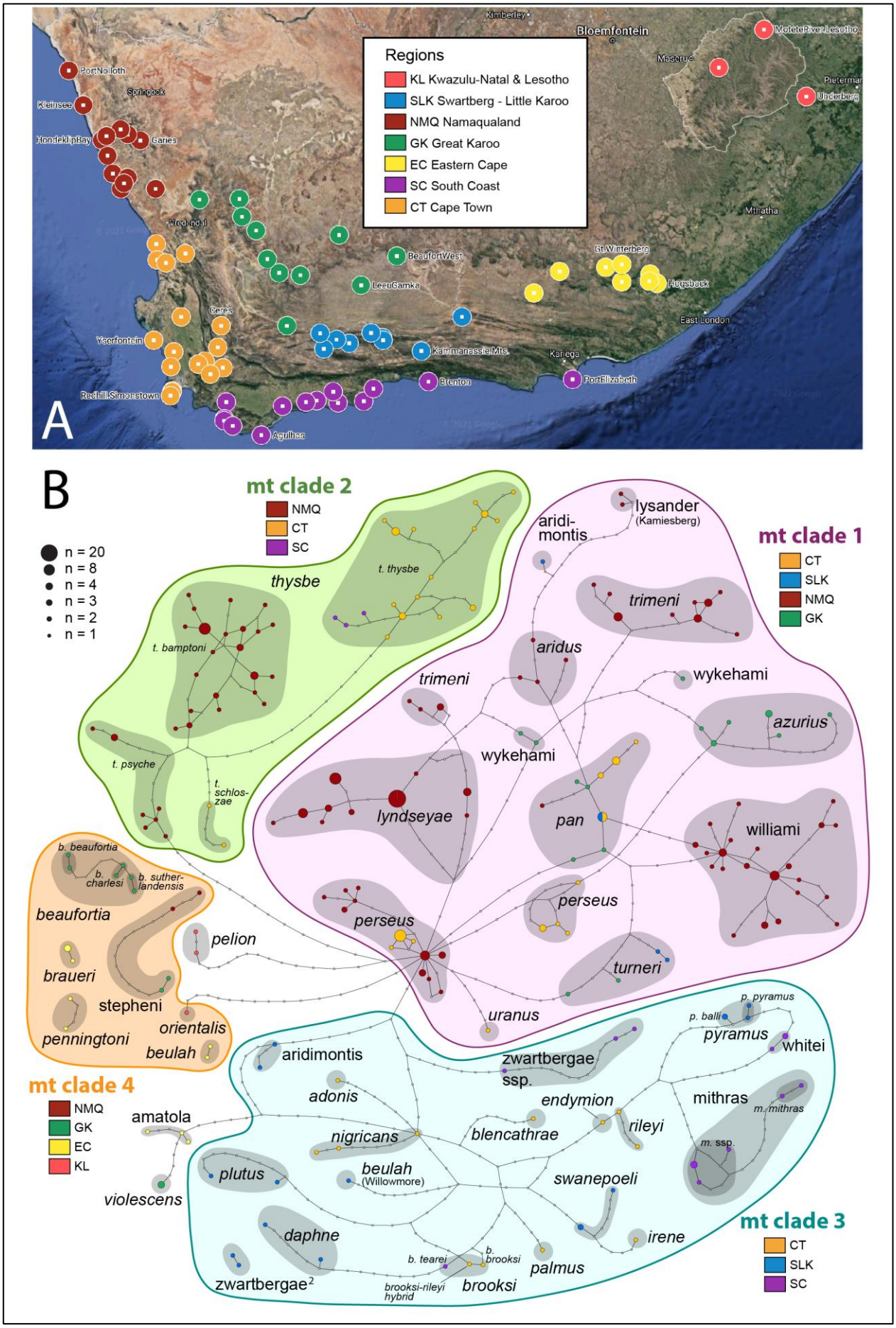

Figure 2 – **A** *thysbe* clade sampling locations (details in Table S2; map image from Google). **B** Statistical parsimony network of the 200 COI haplotypes in the *thysbe* clade. Each link represents a single nucleotide change, and circle sizes are proportional to the number of samples. See Table S1 for a list of samples sharing the same haplotype. The smallest nodes (uncolored circles) represent hypothetical/unsampled haplotypes. MtDNA clades from Fig. 1 are indicated.

ML phylogeny, with *C.* amatola + *C. violescens* connected to mt clade 3 (whereas it is sister to mt clade 1 in the ML tree), and *C.* zwartbergae, a member of mt clade 3, as separate to the main network. The five species unattached to the main network are the bulk of mt clade 4, perhaps reflecting the limited resolving power of the network analysis.

AMOVAs

AMOVA results indicate that for the *thysbe* clade as a whole, geography has a much smaller effect on genetic differentiation compared to taxonomy, but smaller geographic subdivisions accounted for more differentiation compared to larger geographic divisions (Φ_{ST} = 0.337 for 41 locality groups and Φ_{ST} = 0.192 for 7 regions; *p* values <0.00001 for both). Of note is that the four main clades in the *thysbe* clade showed some geographic differentiation at the regional level (Fig. 2). Notably, Clades 3 and 4 do not overlap geographically.

In comparison to geography, taxonomic assignment accounted for much greater genetic differentiation (Φ_{ST} = 0.769 when grouped by species and Φ_{ST} = 0.844 when grouped by subspecies; *p* values <0.00001 for both). The high Φ_{ST} values for the species and subspecies groupings do not support the *thysbe* clade *s.l.* as comprising just a single species. Pairwise Φ_{ST} values for all species pairs can be found in Table S4, that for all subspecies pairs in Table S5, and that for select taxa outside the *thysbe* clade in Table S6.

Exploratory analyses with species delimitation software

Exploratory GMYC (General Mixed Yule-Coalescent model: Pons *et al*., 2006; Fontaneto *et al*., 2007; Fujisawa & Barraclough, 2013) species delimitation analyses were previously done by G. Talavera for TEA20 (using methods described in Talavera *et al*. (2013) and based on the total dataset), but results remained unpublished. With kind permission from G. Talavera, these results are shown in Fig. S4. GMYC species delimitation suggested that the *thysbe* clade comprised a single species.

We further explored species delimitation using a Poisson Tree Processes model (PTP, Zhang *et al*., 2013, implemented on Exelixis Lab's bPTP web server at https://species.h-its.org/) for the COI data alone. This yielded a ML solution of 58 entities and a Bayesian solution of 102 entities for the *thysbe* clade (detailed results not shown); most of the entities suggested therein did not correspond with the species proposed here. The GMYC and PTP species delimitation software are designed to use only molecular data and do not take into account information typically used in classical taxonomy (morphology, ecology, distribution).

Trait comparisons between sister taxa

The COI tree afforded 11 species pairs (either sister pairs or ancestor – descendent pairs) and nine subspecies pairs for trait comparisons, namely, whether they differed or overlapped in MPT, wing facies, ant associate, elevation, and distribution (Table 2). Polyphyletic species were excluded. Twenty out of 21 comparisons showed non-overlapping distributions; the exception was *C. pan pan* v. *C.* pan atlantica. The main trait that distinguished sister species comparisons from sister *sub*species comparisons was MPT. Where MPT was relevant (i.e. in the *thysbe* clade), six of the nine species pairs differed in MPT. None of the *sub*species pairs differed in MPT. However, this is partly a consequence of MPT being used to help distinguish species level differentiation from *sub*species level differentiation; when other data were lacking or ambiguous, sister taxa that differed in MPT were interpreted to be species while sister taxa with similar MPT were interpreted to be subspecies. Of the three species pairs showing no clear differences in MPT, two of them (*pan* v. *aridus* and amatola v. *violescens*) were clearly differentiated on the basis of iridescence (presence/absense); for *beaufortia* v. stepheni, MPT and iridescence did not vary and the pair differed only by small but consistent differences in wing facies; for this pair we consulted pairwise AMOVA results (see *C.* stepheni in HEA23b) to determine rank assignment.

Ant associates tended to differ more between species pairs compared to subspecies pairs, but information on ant associates is incomplete.

DISCUSSION

The analyses of molecular data performed here allow for greater taxonomic resolution than that afforded by TEA20 using the same data. The use of IQTree for ML analyses likely resulted in better searches of tree space, and the implementation of ultrafast bootstrapping enabled greater node confidence in phylogenetic relationships. However, a large part of the improved resolution can be attributed to the separate analysis of each gene. In the young *thysbe* clade, while the COI topology supported the monophyly of most of the species, low levels of lineage sorting and of parsimony informative characters in each of the nuclear loci (Fig. S3 and Table S3) most likely diluted the phylogenetic signal from COI when all four loci were combined, resulting in a Total Dataset tree (Fig. S2) with less support for taxonomic hypotheses based on classical data (morphology, ecology, distribution) compared to the COI-only tree. Furthermore, while the nuclear loci in combination were not informative for taxonomy within the *thysbe* clade, for a few species that were polyphyletic in the COI tree, the CAD-only topology placed their respective samples as monophyletic or closely related; the same was noted for EF in one species. Thus, phylogenetic analyses of individual loci may be more useful than that of combined loci for inferring phylogenetic relationships in young radiations (this applies mainly to data obtained by Sanger sequencing).

The species circumscriptions proposed herein are based primarily on classical taxonomic traits cross validated with phylogenetic and network analyses of COI as the primary molecular data source, and secondarily, the CAD data. Differences in MPT and wing facies were particularly important in determining whether sister taxa should be considered as separate species or relegated to subspecies status. However, relying heavily on the COI genealogy for cross validation has caveats. A plethora of studies has now demonstrated that mitochondrial phylogenies may not accurately capture the histories of species or populations, especially those that are young (e.g., Shaw, 2002; Sullivan

Table 2 – Traits compared within pairs of closely related or sister species and subspecies in the COI tree. All but three species pairs are sister species, and the following pairs have ancestor descendent relationships: *pan* – *aridus*, amatola – *violescens* and *zonarius* – *zeuxo*. COI distances are uncorrected and were not used for taxonomic rank assignment. Polyphyletic species have been excluded. The column "min. km" indicates closest distance in km between known ranges. "s.b.c. difference" stands for small but consistent difference, comprising various degrees of small differences, qualitatively assessed. Symbols: * assumed. ** unrecorded for *C. phosphor*. ^ may have been sympatric until recently. Under "Wing facies": 1 = differ in presence/absence of iridescence. 2 = differ in size, behaviour. 3 = *aureus*' wings paler. 4 = differ in facies & ecology. 5 = differ in wing shape & female host preference. 6 = verso plain (or weakly marked) vs. well marked. 7. differ also in paleness of underside.

Taxon pair	**Elevation**	**Range overlap**	**min. km**	**MPT**	**Wing facies**		**Ant associates**	**Mean COI distance and range (%)**
pan - *aridus*	same; low to mid	**no**	8	very similar	**very diff.**	1	**diff.**	0.56 (0.33–0.91)
williami - *pan* (minus *aridus*)	partial overlap: low vs. low to mid	**no**	80	**diff.**	**s.b.c. diff.**		same	0.71 (0.33–1.39)
amatola - *violescens*	same; high	**no**	500	same*	**very diff.**	1	same*	0.83 (0.82–0.85)
daphne - *plutus*	partial overlap; high vs. mid to high	**no**	15	**diff.**	**s.b.c. diff.**		**diff.**	1.97 (1.33–2.61)
adonis - *nigricans*	partial overlap; high vs. low to high	**no**	90	**diff.**	**s.b.c. diff.**		**diff.**	1.18 (0.84–1.34)
endymion - *rileyi*	**diff.; high vs. low**	**no**	10	**diff.**	**s.b.c. diff.**		same	0.25 (0.08–0.41)
beaufortia - stepheni	same; high	**no**	17	same*	**s.b.c. diff.**		same	2.38 (1.98–2.88)
whitei - *pyramus*	**diff.; high vs. low**	**no**	245	**diff.**	**s.b.c. diff.**		same	0.54 (0.41–0.67)
swanepoeli - *irene*	partial overlap; low to high vs. mid to high	**no**	180	**diff.**	**underside**	6	**diff.**	0.58 (0.49–0.83)
zeuxo - *zonarius*	same; low to mid	**no^**	5	NA	**s.b.c. diff.**	2	**diff.**	0.83 (0.74–0.91)
aethon - *aureus*	same; low to mid	**no**	300	NA	**s.b.c. diff.**	3	same	1.11 (0.99–1.23)
dicksoni - *phosphor*	same; low	**no**	630	NA	**diff.**	4	?**	5.39 (singles)
pan pan - *pan henningi*	same; low	**no**	290	same	**s.b.c. diff.**	7	same	0.12 (0.0–0.25)
pan pan - p. atlantica	partial overlap; low vs. low to high	partial	0	same	**underside**	6	diff.	0.16 (0.0–0.41)
p. henningi - p. atlantica	partial overlap; low vs. low to high	**no**	100	same	**underside**	6	diff.	0.20 (0.0–0.41)
thysbe bamptoni - *t. psyche*	partial overlap; low vs mid to low	**no**	60	similar	**tiny diff.**		same	0.87 (0.16–0.99)
thysbe bamptoni - *t. schloszae*	**diff.; low vs mid to high**	**no**	250	similar	**s.b.c. diff.**		same	0.94 (0.49–1.32)
thysbe psyche - *t. schloszae*	partial overlap; mid to low vs mid-high	**no**	80	similar	**s.b.c. diff.**		same	0.95 (0.66–1.23)
t. thysbe - *all other thysbe ssp.*	partial overlap; low vs low to high	**no**	35	similar	none		same	1.52 (0.08–1.97)
p. pyramus - *p. balli*	same; high	**no**	93	same	**s.b.c. diff.**		same	0.21 (0.16–0.25)
b. brooksi - *b. tearei*	partial overlap; low to mid vs. low	**no**	202	same	**s.b.c. diff.**		same	0.25 (singles)
m. mithras - mithras ssp.	same; low	**no**	90	same	**s.b.c. diff.**		same	1.12 (0.98–1.25)
b. beaufortia - *other* ssp.	same; low	**no**	170	same	**s.b.c. diff.**		same	0.62 (0.49–0.74)
l. lyncurium - *l. lycegenes*	same; low to mid	**no**	100	NA	**s.b.c. diff.**		same	0.16 (0.16–0.16)
c. chrysaor - *c. midas*	**diff.; high vs. low to mid**	**no**	5	NA	**s.b.c. diff.**		**diff.**	1.14 (0.49–1.97)
c. chrysaor - *c. natalensis*	same; low	**no**	120	NA	**s.b.c. diff.**	5	same	1.18 (0.57–1.80)

et al., 2004; Quek *et al*., 2010; Ivanov *et al*., 2018; Hinojosa *et al.*, 2019 and Ahrens *et al.*, 2021). Indeed,agreements between the nuclear gene trees and the COI tree are predominantly seen in taxa outside the *thysbe* clade (Figs. 1A & B, S1 & S2). Reliance on this single non-recombining, uniparentally-inherited genome can lead to an overestimation of species numbers (Després, 2019), and infection by the intracellular bacterium *Wolbachia*,prevalent in the Lycaenidae, can distort mitochondrial divergence levels with respect to taxonomic divergences (Sucháčková Bartoňová *et al*., 2021). Despite these caveats, the mtDNA phylogeny, more often than not, supports taxonomic hypotheses based on morphology, distribution, and ecology. The generation of 37 species

from a single lineage in ~2 million years is a remarkable feat of radiation. The perhaps unsurprising absence of male genitalic variation in the *thysbe* clade given such a shallow divergence time, combined with the limited variation in wing ground plan present significant challenges for its taxonomy. Nonetheless, over ninety person-years of research, field observation, dissections, collecting and rearing (by A. Heath and E. Pringle combined) has resulted in species circumscriptions that, for the majority of species in the *thysbe* clade, are congruent with COI clades, and in several cases consistent with the CAD data.

However, the presence of several COI-polyphyletic species suggesting past mitochondrial capture, and the presence of a hybrid indicates that the *thysbe* clade is in a state of flux. Thus, some of the species circumscriptions proposed herein may not be robust. On the other hand, numerous recent studies have documented that gene flow between related lineages is common in most organisms, including butterflies (Edelman *et al*., 2019; Pazhenkova *et al*., 2021). Given the limited amount of molecular data applied to this taxonomy of *Chrysoritis* and its conserved phenotypes, our current hypotheses of species await testing with new data and tools. Increased sampling of the poorly sampled species and of geographic areas not represented here are likely to "fill in" the haplotype network and reveal more diversity and perhaps more taxa; the distributions indicated in Fig. 2A likely represent only partial ranges for many of the species (see further notes on geographic sampling in Note S3). Further sampling, especially of poorly represented species, may also reveal more species to be non-monophyletic at the COI locus. Application of data with higher resolving capabilities may well redraw species boundaries, reveal oversplitting, and/or perhaps even uncover cryptic species.

Mitochondrial DNA distance thresholds and species delimitation

Mitochondrial DNA distance thresholds have sometimes been used to determine whether closely related taxa should qualify as species or remain as subspecific taxa. This practice is based on the premise that mtDNA distance can predict degree of reproductive isolation between taxa. An accumulating body of literature shows that, more often than not, this premise is violated. Studies of North American birds have shown that speciation can occur rapidly under certain circumstances, with little or no concomitant divergence in mtDNA (Johnson & Cicero, 2004), and thus "mtDNA divergence is neither necessary nor sufficient as a criterion for delineating species" (Moritz & Cicero, 2004). For 39 pairs of avian sister species, Moritz and Cicero reported that mtDNA sequence divergences ranged from 0.0% to 8.2%, and within the genus *Empidonax*, the range was 0.7% to 4.6%. In *Chrysoritis*, the 11 pairs of sister species show a range of 0.25% to 5.39% in mean COI divergence (Table 2) and for the 10 subspecific pairs the range is 0.16% to 1.51%, overlapping with that for species pairs. Moritz and Cicero further note that "using some level of mtDNA divergence as a yardstick for species boundaries ignores the low precision with which coalescence of mtDNA predicts phylogenetic divergence at nuclear genes."

Cicero *et al*. (2021) report that "at one extreme, species may show weak genetic divergence but strong divergence in other traits. For example, Red-breasted and Red-naped sapsuckers …. have low levels of divergence in allozymes [ref], mtDNA [ref], and across thousands of single nucleotide polymorphisms [ref]. Nonetheless, these species are clearly diagnosable in plumage, and they maintain phenotypic boundaries in the face of extensive admixture…. At the other extreme, species may show deep molecular divergence but little phenotypic differentiation. One classic example involves sibling species of *Empidonax* flycatchers, which are renowned for their morphological stasis in the face of genetic, vocal, and ecological differences. Another involves morphologically cryptic species where molecular markers have been used to reveal divergent lineages that also differ in behavioural and ecological traits …." Hogner *et al*. (2012) and Pereira *et al*. (2011) also argue against the use of mtDNA divergence thresholds for species delineation.

Species boundaries do not answer to mtDNA distance thresholds because mtDNA is a bystander, rather than a player, in the speciation process (rare exceptions probably exist). For this reason we have avoided using genetic distance thresholds to infer species limits, relying instead on morphological or MPT/habitat differences – as supported by genetic data – as proxies for reproductive isolation.

Should *Chrysoritis dicksoni* be placed in its own genus?

Due to its highly divergent ecology compared with the rest of *Chrysoritis* (see life history of *C. dicksoni* in HEA23a), it has been suggested that *C. dicksoni* should be placed in its own genus. Twenty five years ago, a review of the Aphnaeinae (Heath, 1997) synonymised *Oxychaeta dicksoni* with *Chrysoritis*, based on the genitalia (see Fig. 176 in Heath, 1997, reproduced in HEA23a). This action was later supported by Rand *et al*. (2000); Boyle *et al*. (2014) and TEA20. The wing colour and markings, host ant, geographic region, venation and genitalia are all typical of the genus, thus similarities far outweigh distinctiveness. More importantly, those studies demonstrated that *Chrysoritis* as it currently stands is monophyletic. Extricating *C. dicksoni* would render the genus paraphyletic.

It was common practice in early taxonomic studies to erect genera to reflect highly distinct biologies, like those of *C. dicksoni* and *C. phosphor*. However, with the advent of molecular data and increasingly cheaper and faster ways of generating more of it for reconstructing evolutionary relationships of taxa, the idea that genera should reflect distinct biologies has been superseded by phylogenetic taxonomy – based on the principle of common descent, taxonomy should reflect evolutionary relationships. Of supraspecific taxa, De Queiroz and Gauthier (1992), wrote that "Under traditional interpretations, organisms belong to taxa because they possess certain characters. By contrast, taxa in phylogenetic taxonomy are historical entities resulting from the process of common evolutionary descent [….] Therefore, organisms are parts of taxa not because they possess certain characters, but because of their particular phylogenetic relationships" (references and bracketed text have been omitted from this quote). In other words, genera should be monophyletic. Donoghue and Cantino (1988) also argue that paraphyletic higher taxa "should be abandoned, as continued recognition of such

taxa will only serve to retard progress in understanding evolution."

Distinctive facies maintained in sympatry suggests multiple species in the *thysbe* clade

The analyses presented here do not support the proposition that the *thysbe* clade is a single species. Almost every species in the *thysbe* clade shares part or all of its distribution with one or more species in that clade, and in the zones of sympatry, distinctive wing facies have been maintained over multiple generations, with no intermediate forms observed. For example, eight km east of Hondeklip Bay, as many as four species from the *thysbe* clade (*C. thysbe bamptoni*, *C. perseus*, *C. lyndseyae*, *C.* williami, plus two outside the clade) occur within an area of about half a hectare. At that locality, all the species are distinct, with no intermediate forms of wing facies having been confirmed during more than 40 years of sampling by collectors (including over 90 person years of field observations and sampling by A. Heath and E. Pringle). Another such locality is the Kammanassie Mountain Range where *C. pyramus balli*, *C. daphne*, *C. swanepoeli*, and *C. z. zwartbergae* occur sympatrically with no intermediate wing facies observed. At the top of Swartberg Pass, *C. pyramus pyramus*, *C. swanepoeli* and *C. z. zwartbergae* also occur sympatrically with distinctive wing facies. Seventeen more such localities with multiple *thysbe* clade species can be found in Table S6 in TEA20 (see also Fig. 5 therein). Females at these localities shared by multiple species may be discriminating among males of the various species using visual cues; additionally, differences in MPT and specificity to host ants, hemiptera, or combinations of ant and hemiptera may present additional barriers to gene flow in sympatry.

Preliminary observations of the length of the upper row of lateral setae of first instars show that it varies considerably among species (see HEA23a: Table 2 & Fig. 9), as does the length and shape of the tentacular organ tubercles of the final instar (Fig. 10 in HEA23a). These characters further suggest that the *thysbe* clade comprises a multitude of species, but more work is needed to confirm their utility in diagnosing species.

Similar looking taxa are not more closely related

The COI phylogeny shows that similarities in wing markings fail to predict matrilineal relatedness. For example, taxa previously classified as subspecies of *C. turneri* (all without structural colouring) were found scattered on the tree, necessitating raising some of these to species level (*e.g.*, *C.* wykehami and *C.* amatola). By the same token, individuals of the same species (or even the same subspecies) can vary in appearance. For example, *C. thysbe thysbe* and *C. azurius* have wings that differ in the extent of melanistic scaling and structural colour (Fig. 3). Thus, for the *thysbe* clade, the extent of melanistic scaling and of structural colouring within a given species can be are highly labile traits (see also Table 1 in HEA23a) and their use alone for taxonomy can be misleading. Nonetheless, in combination with other data, they can be of value.

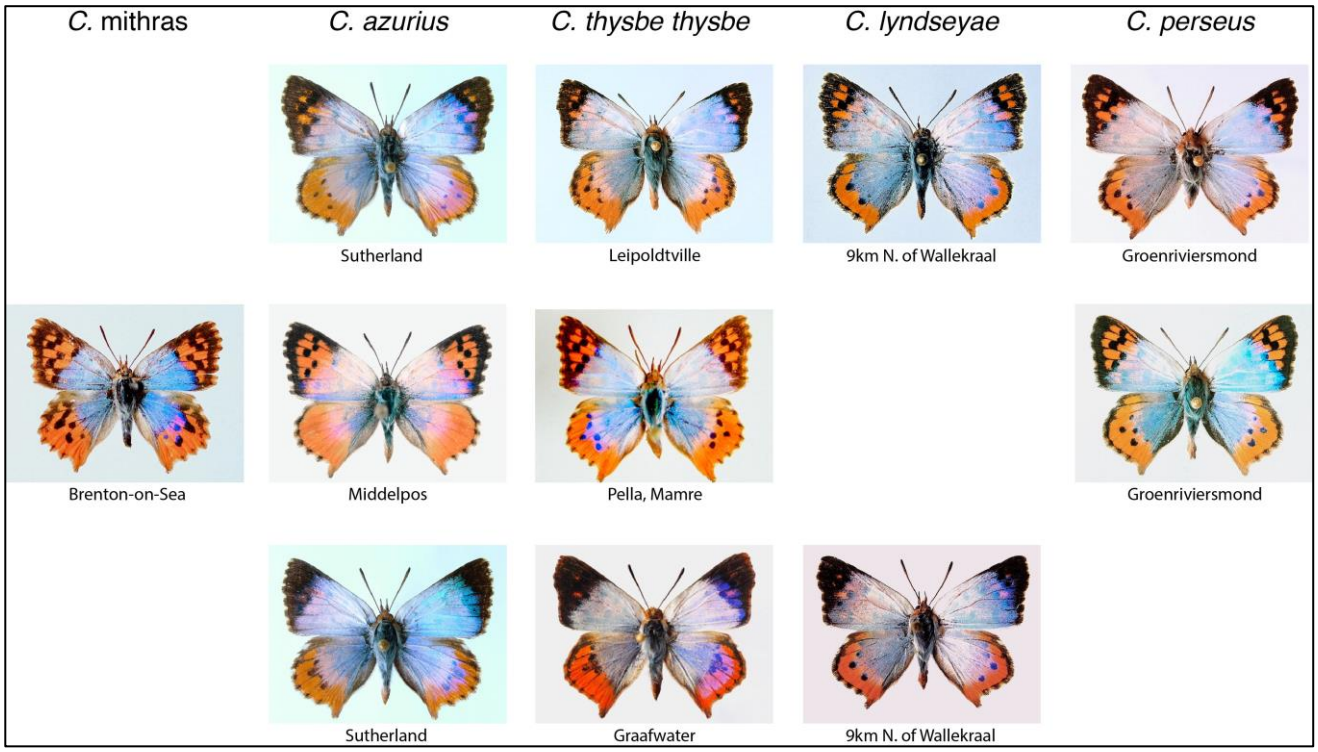

Figure 3 – Overlapping wing facies among species (in rows) and variation within species/subspecies (in columns) in the *thysbe* clade. Photos by A. Heath.

Diversification in the GCFR

Analysis by TEA20 (Fig. 4 therein) showed no tendency for geographic distribution or climate to predict phylogenetic proximity in *Chrysoritis*. Nevertheless, the *Chrysoritis* phylogeny shows a major geographic divide separating the Western Lineage and the Eastern Lineage. As sister lineages, they are equally aged, but differ markedly in various ways, not least in numbers of species (42 and 7, respectively) and biogeography. The centre of

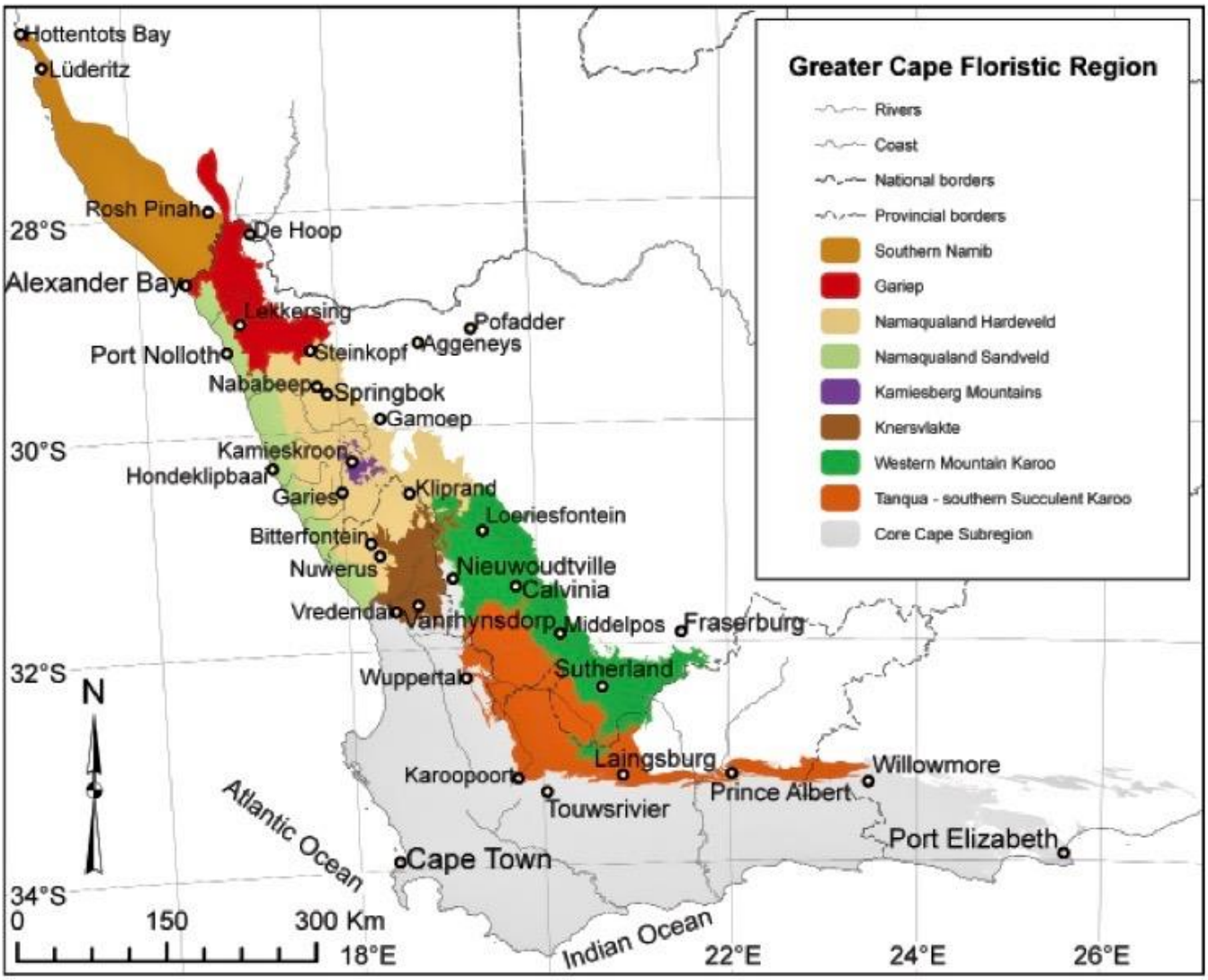

Figure 4 – The Greater Cape Floristic Region (GCFR). Reproduced from *Strelitzia: Plants of the Greater Cape Floristic Region.* Snijman, D.A. (ed.) 2013 by kind permission of the South African National Biodiversity Institute (SANBI).

diversification of the Western Lineage appears to be the Greater Cape Floristic Region (GCFR, Fig. 4; see also Table 1.1 in Bergh *et al*., 2014). This region covers the predominantly winter rainfall area in the west of southern Africa as well as a non-seasonal rainfall area in the extreme south and southeast (Born *et al.*, 2007; Snijman, 2013) and is renowned for its mega-diversity and endemism. The Eastern Lineage, on the other hand, occurs predominantly outside the GCFR (Fig. 2 in TEA20).

Iridescence is widespread in the Western lineage but absent in the Eastern lineage, and the extensive sympatry seen among species of the Western lineage is largely absent in the Eastern lineage with the exception of the very widespread species *C. chrysaor* which ranges throughout much of South Africa. In short, the Western Lineage is characterised by diversification in the GCFR, high species richness, iridescent wings, specificity in MPT and extensive range overlap, whilst the few species in the Eastern lineage diversified outside the GCFR, lack iridescence, have non-overlapping ranges (noting the exception of *C. chrysaor*), and, to our knowledge, do not show specificity in MPT. The Eastern and Western Lineages do however, have obligate ant association (primarily with *Crematogaster* species) in common.

Obligate ant association, including living in ant nests (referred to here as ant-dependency) was likely favoured by natural selection because it protected butterfly juvenile stages from wildfires in the Fynbos and from desiccation in the arid landscapes of the GCFR. Such environments threaten dependency on plants and require long periods of dormancy in safe places such as subterranean ant nests. Cottrell (1985) showed that *Chrysoritis* and *Thestor* (both ant-dependent genera) evolved mainly in Karoid (Karoo-like) environments that became increasingly arid, driving selection for reduced dependency on plants and increased dependency on ants whenever their host plants became unusable due to aridification. *Lepidochrysops*, on the other hand, probably evolved mainly in grassveld environments (Espeland *et al*., in press) where fire would have been the primary driver of ant-dependency. The larvae of *Aloeides, Lepidochrysops, Thestor* and *Trimenia* (all genera having clades with centres of diversification in the GCFR) have been found far underground in ant tunnels or brood chambers. *Chrysoritis* larvae are found at shallower depths close to the plant stem or in special shelters at the base of their host plants that are often reinforced by the host ants.

Concealment, especially below ground, provides a refuge from predation and desiccation, and importantly, in the

Fynbos biome, from veld fires. In some cases, the ants have provided a means to eliminate the need for food plants altogether. For example, there is evidence that larvae of *Thestor* species, some *Aloeides* and *C. dicksoni* depend wholly on food provided by their host ants (Edge, 2005; Bazin & Edge, 2015; Giliomee & Edge, 2015; Heath *et al.*, 2023a). Furthermore, it is entirely plausible that during their diapause periods, the larvae of herbivorous species might also solicit food directly from the host ants. In fact, food solicitation and ant trophallaxis could be more widespread in *Chrysoritis* than currently known (see Heath & Kaliszewska, 2012: 20). Such behaviour is difficult to observe in nature, and even less likely to be seen by a researcher after a fire or during drought. As it is, this behaviour is difficult to detect in captivity, even with special measures.

Via ant dependency, the flammable environment of the Fynbos and the aridity of the other biomes within the GCFR likely provided the niche for radiation, perhaps even adaptive radiation, in ant-dependent lineages such as *Chrysoritis, Aloeides, Trimenia, Lepidochrysops* and *Thestor*. It is noteworthy that, although the GCFR seems to be poor in butterfly genera, among the Lycaenidae occurring there, ant-dependent species vastly outnumber non-ant-dependent ones by a ratio of ~4:1 (Table S7).

Within the GCFR, *Chrysoritis* and their host ants were subjected to all the same forces that drove the prolific speciation of plants in that region – notably, topography (high mountains, deep valleys, various mountain aspects), climate (winter rainfall and hot, dry summers) and complex geology. Climatic oscillations of the Pleistocene (de Menocal, 2004) likely drove cyclic range expansions and contractions while the shifting coastline of the Agulhas Plain due to glacial-interglacial cycling (Compton, 2011) may also have facilitated periodic migrations, particularly when sea levels were low. Such climate driven range changes may help explain disjunct taxa (*e.g., C. pan henningi*, *C. dicksoni* and *Lepidochrysops bacchus*) or those now isolated on mountain tops (e.g. *C. beaufortia, C. uranus* and *C. penningtoni*); indeed, a warmer past climate may explain the origin of the predominantly high elevation clade in the CAD tree (marked by a blue diamond in Fig. S3A). Even through the Pleistocene climate fluctuations, the relative climatic stability of the region, particularly the stability of rainfall seasonality, over the last several million years is thought to have enabled the high diversity of the GCFR (Altwegg *et al*., 2014), presumably by lowering extinction rates.

A spatially complex and heterogeneous environment in combination with periodic climate change at times fostered divergence within species as populations contracted or fissured, and at times eroded that divergence as recently diverged taxa expanded and re-encountered one another and exchanged genes. For the *thysbe* clade, it appears that the net effect of these opposing processes against a backdrop of stability in rainfall seasonality was the rapid creation of diversity that, despite challenges, are now recognised as distinct species through decades of research with the aid of molecular data.

All species and subspecies pairs in the COI tree currently show non-overlapping distributions (Table 2; with the exception of the pair *C. pan pan* vs *C.* pan atlantica – discussed in HEA23b). However, due to the numerous challenges in attaining geographically comprehensive sampling (see Note S3) we do not know to what extent these distributions are actually allopatric or parapatric, and the possibility of sympatry in areas not yet sampled cannot be ruled out.

Comparison of the pair *pan–aridus* with the pair *pan*–williami offers some insights into possible modes of speciation. These three species form a clade and are closely related. For the *pan–aridus* pair, the nesting of *aridus* within samples of *pan* suggests peripatric speciation, and indeed, the distribution of *aridus* lies peripheral to that of *pan* (Fig. S1A in HEA23b), separated by ~10 km. This speciation event was accompanied by a loss of iridescence in *C. aridus*, possibly as a mechanism of reinforcement since both show similar MPTs (low to mid elevation gullies) and thus have the potential to hybridise. In the case of the allopatric sister pair williami–*pan* (excluding *aridus*), the converse pattern is seen, where the facies are similar, but MPT differs (ridgetops and upper slopes of hills and dunes in williami vs. low to mid elevation gullies in *pan*).

The following outlines a possible scenario by which some *Chrysoritis* speciated. Climate-driven habitat fragmentation may have isolated populations of a species. The ensuing drift, or adaptation to local biotic and abiotic conditions, may have engendered ecological, genetic and/or phenotypic divergence between populations. If sufficiently diverged, subsequent contact between populations may result in hybrids that are sterile or less fit, and this could subsequently drive divergence in MPT, facies, or female mate preference. Because of their requirements for specific ant, and possibly plant and hemipteran species (and, for *thysbe* clade species, their specificity to MPTs), the distribution of *Chrysoritis* species might be expected to be patchy (in line with anecdotes noted in Note S3) and thus vulnerable to fragmentation. Such ecological constraints faced by obligate myrmecophiles, along with drought- or fire-prone environments may help explain why the *thysbe* clade and other ant-associated butterfly groups have undergone extensive radiations in the GCFR.

Whether the phylogeographic patterns suggested by the mtDNA tree solely reflect female migration or can be extrapolated to species remains to be tested with genomic data. At face value however, the patterns point predominantly to allopatric speciation (Table 2).

Ecological divergence may have also played a role in generating some of the diversity in *Chrysoritis*. For example, the two species *C. zeuxo* and *C. zonarius* on the Cape Peninsula are morphologically different and associate with different ant species. *Chrysoritis zeuxo* associates with *Crematogaster* (abbreviated here as *Crem*.) *liengmei* ants and *C. zonarius* with *Crem. peringueyi*. These ants in turn appear to specialise on inhabiting different host plant species (perhaps via specificity to hemipterans which may themselves be host plant specialists) – *Osteospermum moniliferum* for *Crem. liengmei* and the geographically non-overlapping *O. incanum* for *Crem. peringueyi*. As *Chrysoritis* larvae use a wide range of host plant species and families, they are treated here as mostly polyphagous, thus it is less likely

that the larvae of these *Chrysoritis* species specialise on these host plants, but rather on their respective ant associates (Cottrell, 1984:41). As *C. zeuxo* is nested within *C. zonarius* in the COI tree (Fig. 1), the former could be an offshoot whose ancestors switched host ants from *Crem. peringueyi* (the presumed ancestral ant associate) to *Crem. liengmei*. Feeding on different host plants may have resulted in phenotypic differences which were then reinforced by their different emergence times, and/or different host ant species. Specialisation at each stage or trophic level (*i.e.*, butterflies to ants, ants to hemipterans, and hemipterans to host plants), with geographically non-overlapping host plants (see next paragraph) at the base of this chain might have driven the divergence of these two taxa.

The host plant species *Osteospermum incanum* occurs largely in the West Coast and *O. moniliferum* largely in the South Coast and regions eastwards. Cottrell (1978a: 55 & 59) provides accounts of these two host plants' distributions and their suspected sympatry on the Cape Peninsula. However, the taxonomy surrounding these two plant species, formerly in the genus *Chrysanthemoides*, is uncertain given the occurrence of many forms/subspecies (Jan Vlok, pers comm.).

The adults of *Chrysoritis* larvae hand-reared in captivity without ants are known to often differ in appearance from their parents (A. Heath, pers. obs.). Hence, the observed variation in the above examples may be due to developmental plasticity, perhaps responding to host plant chemistry, choice of ant, and/or environmental conditions such as aridity. Nevertheless, the morphological differences may over time be reinforced, for example, through mate choice or females' choice of host ants for oviposition.

Further investigations of *Chrysoritis'* ant hosts may reveal cryptic species (B. Blaimer, pers. comm.) that may provide more avenues for resource specialisation. Because of the incomplete record and potential for cryptic species in the host ants, this trait as it currently stands may contribute to underestimating diversity in *Chrysoritis*, particularly if specialisation on ants turns out to be a contributing factor in *Chrysoritis'* diversification (for this reason we have relied minimally on host ant species to help circumscribe *Chrysoritis* species). *Chrysoritis* or their host ants might also be specialised on different hemipteran taxa (on which the ants rely for food), or perhaps *Chrysoritis* species may specialise on particular combinations of ants and Hemiptera in ways that are presently undetectable (but see Giliomee & Edge, 2015). The presence of Hemiptera upon which the ants rely may serve to attract the ovipositing female butterfly to the host plant. However, little is known about these hemipterans and their role, if any, in stimulating butterfly oviposition but they may specialise on host plant species and hence dictate which ant species is likely to be found on a particular plant. These four-tiered trophic relationships (host plant, scale insect, host ant and butterfly) may have created numerous opportunities for a butterfly lineage to form a mosaic of isolated populations with varied ecologies. In concert with changing abiotic conditions, these webs of interactions may have facilitated divergence into new species, subspecies, or forms.

GCFR distribution and genitalic uniformity and simplicity

The *thysbe* clade's challenging taxonomy arises also from the virtual absence of morphological variation in male genitalia. In addition, genitalic morphology in *Chrysoritis* is relatively simple. This combination of traits is not restricted to *Chrysoritis*. A survey of lycaenid butterfly clades occurring in South Africa indicates a tendency for low genitalic variability and simple morphology to co-occur. Interestingly, as is seen in the *thysbe* clade, these traits tend to also be found in GCFR-associated clades, as follows: 1) the *brachycerus* (black) *Thestor* clade (Miletinae), 2) the *methymna* + *ortygia* clade of *Lepidochrysops* (Polyommatinae), 3) the *thyra* group of *Aloeides* (Aphnaeinae), and 4) *Trimenia* (Aphnaeinae) (Table S10); the *thyra* group of *Aloeides*, however, occurs inside and outside the GCFR).

The converse pattern of high genitalic variability across species and more complex genitalia tends to be seen in clades occurring exclusively outside the GCFR: 1) the Afrotropical clade of *Cigaritis* (Aphnaeinae), 2) *Axiocerces* (Aphnaeinae), and 3) *Iolaus* (Theclinae). *Aphnaeus* (Aphnaeinae) aligns with these three groups, with the exception that it has simple, rather than complex genitalic structure. Among the lycaenid groups mentioned in these paragraphs, all except *Iolaus* are obligately associated with ants (*Iolaus* species have facultative or no associations with ants), thus ant dependency *per se* is unlikely to explain the differences in these genitalic traits between GCFR and non-GCFR clades.

Male genitalic uniformity among species may reflect simplicity of structure and/or recent divergence. Simple genitalic structures tend to remain unaltered over time, perhaps because there is a lack of features for selection to shape into diverse forms. In this respect, simplicity and uniformity may be autocorrelated. Uniformity may also result from recent diversification. Indeed, the *brachycerus* clade of *Thestor*, the *methymna* + *ortygia* clade of *Lepidochrysops*, and the *thysbe* clade of *Chrysoritis* are all young, estimated at 3–4, 1.7 and 2–3.6 my respectively (Fig. 1.2 in Kaliszewska, 2015; Espeland *et al*., in press; TEA20); the ages of the other six groups are unknown, so the strength of the correlation between age and uniformity cannot be ascertained.

If genitalic uniformity does indeed result from recent diversification, then uniformity, simplicity and age may all be autocorrelated. Furthermore, if the various lycaenid groups of the GCFR represent young radiations in the region, it would suggest that the niche favouring ant-dependent lineages in the GCFR evolved only recently, perhaps in the Pliocene or younger. If so, then the genitalic uniformity in each of the GCFR clades is not unexpected.

A role for *Thesium* host plants in the diversification of the *thysbe* clade?

All species in the *thysbe* clade can or do feed on *Thesium*, a large genus of root parasites. *Thesium* is widespread in the Cape provinces, occurring abundantly in a variety of habitats but they are seldom noticed because they mostly lack leaves and often appear as yellowing grass to the

untrained eye (A. Heath, pers. obs.). *Chrysoritis* larvae develop well on *Thesium* species as long as *Crematogaster* ants are present (A. Heath, pers. obs.). Newly emerged first instar larvae of several species within the *thysbe* clade have been observed to readily feed on *Thesium*, and a *C. mithras* larva captured from Mossel Bay and raised on *Thesium* (albeit without *Thesium's* host plant) in captivity grew unexpectedly quickly (unpublished experiments conducted by A. Heath). Outside the *thysbe* clade, only *C. pyroeis* and *C. oreas* feed on *Thesium*, and interestingly, these are also the only *Chrysoritis* species associated with the droptail ant (*Myrmicaria* spp.). Outside *Chrysoritis*, only one other species in the Lycaenidae (*Eicochrysops messapus*) is known to feed on *Thesium* as larvae in southern Africa (Pringle *et al*. 1994: 636), suggesting that feeding on *Thesium* requires special adaptation (and may explain why *C. pyroeis* and *C. oreas* switched from *Crematogaster* to *Myrmicaria*). By evolving adaptations to feed on *Thesium*, the *thysbe* clade may have unlocked a niche which enabled its prolific diversification in the GCFR.

CONCLUSIONS AND FUTURE RESEARCH

Achieving a robust molecular framework for a taxonomic revision of *Chrysoritis* requires further research employing methods that sample variability throughout the entire genome, such as ddRADseq (Peterson *et al*., 2012) or even whole genome sequencing. Additionally, for the *thysbe* clade species at least, dense sampling within species (and even subspecies) covering the span of each taxon's distribution will be needed to properly circumscribe taxonomic boundaries. Recent advances in laboratory methods now allow the sampling of aged specimens, many of which reside in private and museum collections.

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