Evolution of cycloalexy in Neotropical chrysomeline beetles (Coleoptera: Chrysomelidae)

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ABSTRACT

The larvae of some insects arrange themselves in a tight, orderly circle at rest; a behaviour that Vasconcellos-Neto and Jolivet (1988b) first called "cycloalexy". The word was defined by Jolivet and collaborators in 1990 as a defensive behaviour adopted by insect larvae that form a tight circle with the best defended extremity at the periphery, either heads or abdomens. The formation is also associated with coordinated movements to repel threats. The term has steadily gained acceptance and expanded to include insect nymphs, adult insects and even vertebrates. We review reports of cycloalexy and find the behaviour is less common than suggested. Convincing examples are found only in sawflies, leaf beetles, caterpillars, one weevil and one midge species. We question reports of cycloalexy in penguins, an amphipod crustacean, nymphs of Hemiptera and larvae of Hymenoptera and Neuroptera. We argue that analogous behaviours in mammals are reactive rather than preventive and, hence, not cycloalexic. A molecular phylogeny was reconstructed to evaluate the relationships of 70 species of Neotropical Chrysomelinae and the evolution of cycloalexy. We sequenced five gene segments: nuclear protein-coding *CAD* and *28S* rDNA, mitochondrial *12S* rDNA and protein-coding *COI* and *COII*. The phylogeny was inferred using Bayesian and Maximum Likelihood (ML) methods and ancestral larval behaviour was reconstructed with ML and Maximum Parsimony methods. The ancestral behaviour reconstructions show five independent evolutionary origins of larval gregariousness in Neotropical chrysomeline beetles, two with maternal care and circular grouping of larvae and one accompanied by cycloalexy. Further, our phylogeny clarifies relationships within Chrysomelinae, and shows a revision is needed: the genus *Stilodes* is paraphyletic with *Zygogramma*, *Platyphora* is polyphyletic and divided into two clades; one with *Doryphora* nested within and the other with *Proseicela*.

RÉSUMÉ

Les larves de certains insectes forment un cercle serré et ordonné au repos, un comportement appelé « cycloalexie » pour la première fois par Vasconcellos-Neto et Jolivet) (1988b). Le mot a été défini par Jolivet et collaborateurs en 1990 comme étant un comportement défensif de larves d'insectes qui forment un cercle serré, avec l'extrémité la mieux défendue à la périphérie. soit leurs têtes ou leurs abdomens. La formation est aussi associée à des mouvements coordonnés du groupe pour repousser les menaces. Le terme a progressivement gagné en acceptation et s'est élargi pour inclure des nymphes d'insectes, des insectes adultes et même des vertébrés. Nous faisons la revue des rapports de cycloalexie et trouvons que le comportement est moins rependu que suggéré. Les exemples convaincants ne se retrouvent que chez les mouches à scie, les chrysomèles, les chenilles, une espèce de charançon et une de moucheron. Nous questionnons les rapports de cycloalexie chez les manchots, un crustacé amphipode, des nymphes d'hémiptères et des larves d'hyménoptères et de névroptères. Nous suggérons que les comportements analogues chez les mammifères sont réactifs plutôt que préventifs, et donc pas cycloalexiques. Une phylogénie moléculaire a été reconstruite pour évaluer les relations de 70 espèces de Chrysomelinae néotropicales et l'évolution de la cycloalexie. Nous avons séquencé des segments de cinq gènes : les gènes nucléaires *CAD* codant et *28S* ribosomal, et les gènes mitochondriaux *12S* ribosomal et *COI* et *COII* codants. La phylogénie a été inférée en utilisant des méthodes bayésienne et de Maximum de Vraisemblance (MV), le comportement ancestral des larves a été reconstruit à l'aide de méthodes de MV et de Maximum de Parcimonie. La reconstruction du comportement ancestral montre cinq origines évolutives indépendantes du grégarisme des larves chez les chrysomèles Néotropicales, deux avec soins maternels et regroupement circulaire des larves et une accompagnée de cycloalexie. De plus, notre phylogénie clarifie les relations à l'intérieur des Chrysomelinae, et montre qu'une révision est nécessaire : *Stilodes* est paraphylétique avec *Zygogramma*, *Platyphora* est polyphylétique et divisé en deux clades; l'un avec *Doryphora* imbriqué dedans et l'autre avec *Proseicela*.

RESUMEN

Las larvas de algunos insectos se disponen en un círculo cerrado y ordenado en reposo, un comportamiento llamado "cycloalexia" por primera vez por Vasconcellos-Neto y Jolivet (1988b). La palabra fue definida por Jolivet y colaboradores en 1990 como un comportamiento defensivo de larvas de insectos que forman un círculo cerrado, con la extremidad mejor defendida en la periferia, o cabezas o abdómenes. La formación es asociada de movimientos coordinados del grupo para repeler las amenazas. El término ha ido ganando aceptación y se ha ampliado para incluir ninfas de insectos, insectos adultos e incluso vertebrados. Revisamos los informes de cycloalexia y encontramos que el comportamiento es menos común de sugerido. Ejemplos convincentes se encuentran sólo en moscas de sierra, en crisomélidos, en orugas, en una especie de gorgojo y en una de ceratopogónido. Cuestionamos informes de cycloalexia en pingüinos, un crustáceo anfípodo, ninfas de hemípteros y larvas de himenópteros y de neurópteros. Sugerimos que los comportamientos análogos en mamíferos son reactivos en vez de preventivos, entonces no son cycloalexicos. Una filogenia molecular fue reconstruida para evaluar las relaciones de 70 especies de Chrysomelinae Neotropicales y la evolución de cycloalexia. Secuenciemos segmentos de cinco genes: los genes nucleares *CAD* codificante y *28S* ribosómico, y los genes mitocondriales *12S* ribosómico y *COI* y *COII* codificantes. La filogenia fue inferida usando métodos bayesianas y de Máxima Verosimilitud (MV), el comportamiento ancestral de las larvas fue reconstruido usando métodos de MV y Máxima Parsimonia. La reconstrucción de comportamiento ancestral muestra cinco orígenes evolutivos independientes de gregarismo de larvas en crisomélinos Neotropicales, dos con cuidado maternal y agrupamiento circular de las larvas y una acompañada de cycloalexia. Además, nuestra filogenia clarifica relaciones dentro de Chrysomelinae, y muestra que una revisión es necesaria: *Stilodes* es parafiletico con *Zygogramma*, *Platyphora* es polyfiletico y dividido en dos clados; uno con *Doryphora* imbricado a dentro y el otro con *Proseicela*.

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CONTRIBUTIONS OF AUTHORS

This is a manuscript-based thesis where the literature review in Chapter I and original research in Chapter II represent co-authored papers to be submitted. I collected insects from the field in Panama, Ecuador, Canada and Mongolia, and observed their behaviour. I conducted the experiments and performed the analyses. I was primarily responsible for writing the manuscripts. The co-authors, Dr. Jacquie Bede, Dr. Don Windsor for chapters I and II, and Dr. Barb Sharanowski for chapter II have revised and corrected the manuscript.

1.0. GENERAL INTRODUCTION

1.1.PROBLEM DEFINITION

Many insects form aggregations; these can benefit for the members of the group. Possible benefits include feeding faciliation, thermoregulation, reproduction and defence (Costa, 2006). Gregariousness in larvae is favoured by mutual defence, at least ancestrally; living in groups can reduce predation by simple dilution effects (e.g. Wcislo, 1984), stronger warning signals (e.g. Sillén-Tullberg, 1988, 1993) or stronger chemical and behavioural defences (e.g. Vulinec, 1990). The larvae of some herbivorous insects show a remarkable behaviour: they rest in a tight circle, presumably for defence (Jolivet et al., 1990). This was called cycloalexy and first described in the larvae of leaf beetles of genera *Coelomera* and *Platyphora* in Brazil and sawflies of genus *Perga* in Australia (Vasconcellos-Neto and Jolivet, 1988a, 1988b, Weinstein, 1989). However, there is no single convincing explanation for the evolution of circular grouping in these insects or why this behaviour occurs in some species but not others. Cycloalexy was defined by Jolivet and collaborators in 1990 as a defensive behaviour of insect larvae forming a tight circle, with heads or abdomens at the periphery, depending on which functions defensively. The definition also states the formation is often accompanied by movements to repel threats (Jolivet et al., 1990). Within the Chrysomelinae, while most species have solitary larvae, some are gregarious, with or without cycloalexy. As well, in some species, the female extends maternal care to its offspring (Jolivet et al., 1990, Grégoire, 1988, Windsor et al., 2013). Cycloalexy has been reported in insect larvae of several sawflies, leaf beetles, moths, butterflies, owlflies, flies, hemipteran insect nymphs, fully developed insects, millipedes, an amphipod crustacean and even mammals (Santiago-Blay et al., 2012, Costa, 2006, Jolivet and Verma, 2011).

Although it is clear from the onset that several reports do not meet the criteria of the original definition, it remains unclear how many do meet the criteria. Strict application of the original definition will maximize the usefulness of the term, and ascertain that the behaviours described are convergent. We review the reports of cycloalexy and attempt to determine if they meet all the criteria of Jolivet et al.'s (1990) definition. That is a necessary first step to studying the evolution of this behavioural trait.

Notwithstanding the taxonomic restriction of the original definition, we find reports of cycloalexy in hemipteran nymphs, fully developed insects, crustaceans and vertebrates are deficient in one or several criteria; circular formation may not be defensive, or is taken while feeding, or is taken in reaction to a threat. Polydesmid millipedes may be considered cycloalexic, if they were insect larvae. There is convincing evidence of cycloalexy for larval aggregations of sawflies (Tenthredinoidea: Pergidae and Argidae), leaf beetles (Chrysomelidae: Cassidinae, Galerucinae, Criocerinae and Chrysomelinae), caterpillars (Saturniidae: Hemileucinae: *Lonomia* and tentatively Papilionidae), one weevil (Curculionidae: *Phelypera distigma*) and midges (Ceratopogonidae: *Forcipomyia*). Tight defensive groups of owlfly larvae (Neuroptera: Ascalaphidae: *Ascaloptynx furciger*) are unidirectional rather than circular and used at rest but also while feeding.

We, then, determine the evolutionary history of larval gregariousness and cycloalexy in chrysomeline leaf beetles. Cycloalexy has been more frequently reported in species of sawflies and leaf beetles than any other insect groups (Jolivet et al., 1990). Four subfamilies of leaf beetles have been reported to display cycloalexic behaviour: Galerucinae, Criocerinae, Cassidinae and Chrysomelinae. Cycloalexy has been only reported for the genus *Coelomera* within the Galerucinae. In comparison, cycloalexy occurs in multiple genera of Cassidinae and Chrysomelinae (Jolivet et al., 1990, Vasconcellos-Neto and Jolivet, 1994, Santiago-Blay et al., 2012).

To reconstruct the ancestral behaviour of larvae, we collected specimens and observed the behaviour of larvae in Panama, Ecuador, Bolivia, French Guiana and Brazil. Some specimens and behavioural observations were obtained in Canada, France, Mongolia and Australia. To infer a molecular phylogeny, we selected five gene segments. Three segments from mitochondrial DNA: the protein-coding cytochrome oxidase I (*COI*) and cytochrome oxidase II (*COII*) and the ribosomal *12S*, all three were previously used in insects or specifically leaf beetles (Simon et al., 1994, Termonia et al., 2002). Within mitochondrial DNA, protein-coding regions evolve faster than ribosomal regions, and thus *COI* and *COII* are more useful in differentiating closely related species in a phylogeny (Moritz et al., 1987). We chose two nuclear genes, a segment of the protein-coding gene region of carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase (*CAD*) and a segment of the ribosomal *28S*. Nuclear genes evolve slower than mitochondrial genes, making them generally more useful for older divergences (Li et al., 1985). *CAD* has not been used to infer phylogenies of Chrysomelinae, but has been used in several other insect groups, including Coleoptera (Moulton and Wiegmann, 2004, Polihronakis and Caterino, 2010, Wiegmann et al., 2009, Wild and Maddison, 2008, Winterton et al., 2010). *CAD* is especially useful at differentiating shallower divergences, tribe, subtribe and supra-generic divergences and was the highest performing segment tested by Wild and Maddison (2008). The protein coding genes, *COI*, *COII* and *CAD*, were aligned directly. *28S* sequences were aligned using a secondary structure model; ambiguous regions were excluded (Gillespie et al., 2004). Ribosomal gene *12S* was aligned using with an the alignment algorithm MUSCLE (Edgar, 2004). The phylogeny was inferred using Bayesian and Maximum Likelihood (ML) methods (Ronquist et al., 2012, Stamatakis, 2006). Ancestral larval behaviour was reconstructed with ML and Maximum Parsimony methods (Maddison and Maddison, 2006, 2011). The reconstructions show five independent evolutionary origins of larval gregariousness in Neotropical chrysomeline beetles, two with maternal care and circular grouping of larvae in *Doryphora paykulli* Stål and *Proseicela* and one accompanied by cycloalexy in *Platyphora*, two with neither cycloalexy nor maternal care in subtribe Chrysomelina (*sensu* Daccordi, 1982) and *Platyphora eucosma* (Stål). Further, our phylogeny clarifies relationships within Chrysomelinae, and shows a revision is needed: the genus *Stilodes* is paraphyletic with *Zygogramma*, *Platyphora* is polyphyletic and divided into two clades; (((*Desmogramma* + *Elytrosphaera*) + (*Doryphora* + *Platyphora*)) and (*Labidomera* + *Leptinotarsa* + *Platyphora* + *Proseicela*).

1.2.RATIONALE FOR RESEARCH

Most insects are solitary. In the spectrum of social behaviours, group living is the first step following solitary life. Group living, cycloalexy and maternal care of offspring are interesting in themselves, but understanding their evolution will help clarify the ecological conditions, if any, that favoured group living over solitary life. Ultimately, these studies seek to explain how eusociality evolved. The first and often neglected step in this progression is to clarify the evolutionary history of these behaviours.

Convergence in leaf beetles complicates the creation of a phylogeny using only morphological characters. For this reason, DNA sequence data is particularly useful in clarifying the phylogenetic relationships in the Chrysomelinae.

1.3. OBJECTIVES

Broadly, we aim to build upon the knowledge of cycloalexy and its evolution, by summarizing current knowledge and adding to it. We have two specific objectives:

Objective 1. Review the reported cases of cycloalexy and determine if they meet the criteria of the original definition by Jolivet et al. (1990).

Objective 2. Determine the evolutionary history of cycloalexy and larval gregariousness in Neotropical Chrysomelinae.

  a. Infer a robust molecular phylogeny of Neotropical Chrysomelinae.
  b. Observe and determine the behaviour of larvae and differentiate cycloalexic species from gregarious non-cycloalexic species.
  c. Reconstruct ancestral larval behaviour onto the inferred phylogeny.

1.4. THESIS FORMAT

This thesis is manuscript-based. As such, chapters I and II will be submitted to scientific journals. The contributions of authors are mentioned at page viii.

2.0. CHAPTER I: LITERATURE REVIEW - Cycloalexy: definition and occurrences revisited

G.J. Dury, J.C. Bede and D.M. Windsor

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2.1. ABSTRACT

Cycloalexy was coined by Vasconcellos-Neto & Jolivet (1988b) and further defined by Jolivet & collaborators (1990) as a defensive behaviour taken at rest where immature insects align in a circle with their most defended body part, the heads or abdomens but not both, at the periphery of the circle. Some larvae may remain inside the circle. This resting formation is defensive and when under attack is accompanied by coordinated movements to repel predator or parasitoid threats. With either the original or revised definition, to date, in all true examples of cycloalexy larvae break their circle when moving or feeding. Since the publication of the definition in 1990, cycloalexy has been applied to an increasing number of organisms, including insect nymphs, fully developed insects and even vertebrates. However, reports of cycloalexy are often missing one or more key attributes of this behaviour; i.e. were the animals observed at rest or during feeding? Was it a defensive behaviour? Were the best defended extremities on the periphery? Here, we review a suite of reports of this behaviour and provide suggestions and justifications to improve the original definition. True cycloalexy as defined by Jolivet et al. (1990) is less common than suggested in the literature. We find convincing evidence only for sawflies (Tenthredinoidea: Pergidae and Argidae), leaf beetles (Chrysolemidae: Galerucinae, Cassidinae, Chrysomelinae and Criocerinae), caterpillars (Saturniidae: Hemileucinae: *Lonomia* and tentatively Papilionidae), weevils (Curculionidae: *Phelypera distigma*) and midges (Ceratopogonidae: *Forcipomyia*). We question reports of cycloalexy in an amphipod crustacean (*Phronima sedentaria*), in several Hymenoptera, several Chrysomelinae, Hemiptera nymphs, owlfly larvae (Neuroptera: Ascalaphidae), Antarctic penguins, and vertebrates.

KEY WORDS: cycloalexy, insect, defence, behaviour, aggregation.

2.2. INTRODUCTION

Some insect larvae show a remarkable behaviour: they group in a tight circle for defence (Jolivet et al., 1990). This behaviour is reminiscent of Carl von Clausewitz's 1812 *Principles of War*: "In strategy (…) the side that is surrounded by the enemy is better off than the side which surrounds its opponent, especially with equal or even weaker forces" (von Clausewitz and Gatzke, 1942). Many animals species employ this strategy as well. For example, among vertebrates, muskoxen (*Ovibos moschatus*) form a circle enclosing the young calves when attacked by wolves, their principal natural predators (Tener, 1954, in Wilson, 1975). This circular formation allows the most vulnerable body parts to be covered while the extremity that is best defended or involved in attack is at the periphery. In nature, there are examples of insect species that engage in these circular defensive formations, however, Vasconcellos-Neto & Jolivet (1988b) recognized a related behaviour in immature insects to which they gave the name "cycloalexy":

> "Cycloalexy (kuklos = circle, alexo = defend) is defined here as 'the attitude adopted at rest by some insect larvae, both diurnal and nocturnal, in a tight circle where either the heads or ends of the abdomen are juxtaposed at the periphery, with the remaining larvae at the center of the circle. Coordinated movements such as the adoption of threatening attitudes, regurgitation, and biting, are used to repel predators or parasitoids.'" (Jolivet et al., 1990)

There are clear distinctions between general circular defensive formations and cycloalexic behaviour; however, these distinctions have become blurred in the literature resulting in a weakening of the concept of cycloalexy. In this review, we analyzed reported examples of cycloalexy. We question whether all of these examples are truly cycloalexic behaviour or simply different forms of aggregation. Incorrect or diffuse usage of cycloalexy may result in the loss of distinction of this term to define a specific behaviour. If applied strictly, the definition will yield more interesting comparisons and examples of convergent evolution, presumably arising from similar evolutionary pressures. Therefore, a strict definition is most useful for the study of insect behaviour and behavioural evolution.

We feel that there are critical distinctions between cycloalexy and other forms of defensive aggregations. These distinctions were stated in the original definition: 1) It is adopted at rest. 2) It is associated with larval stages of holometabolous insects, though, as will be discussed later, the formation is sometimes maintained during pupation. 3) The larvae are arranged “in a tight circle where either the heads or ends of the abdomen are juxtaposed at the periphery”, sometimes with larvae at the centre. This means that peripheral individuals in a given group face outwards or inwards, but not both. 4) The group uses coordinated movements to repel predators or parasitoids (Jolivet et al., 1990). The definition should reflect the intended etymology “to defend by forming a circle” (Santiago-Blay et al., 2012). Though not specified in the original definition, we suggest adding the distinction that the behaviour is for the defence of the "group" itself, as opposed to defending a resource or nest. As well, we think that the definition should be expanded to include immature arthropods.

Table 2.1: Reported cases of cycloalexy and how they fit within the definition criteria.

Organism	**Criteria**							**Reference**
	Insect larvae	**Circular formation**	**Uniformity (best defended extremity outwards)**	**At rest only**	**Group reactions to threats**	**Aggregation is defensive**	**Cycloalexy?**	
Coleoptera: Chrysomelidae: Galerucinae								
Coelomera spp.; e.g. *C. ruficornis* Baly, 1865; *C. helenae* Jolivet, 1987; *C. raquia* Bechyně, 1956; etc.	Yes	Yes	Inwards	Yes	Yes	Yes	Yes	(Vasconcellos-Neto and Jolivet, 1988b, 1989, 1994, Jolivet et al., 1990, Vasconcellos-Neto and Jolivet, 1988a)
Dircema spp.	Yes	Not observed	Not observed	Not observed	Not observed	Not observed	None observed	(Vasconcellos-Neto and Jolivet, 1989, 1994)
Coleoptera: Chrysomelidae: Criocerinae								
Lema sp.; *Lema apicalis* Lacordaire, 1845 and *L. reticulosa* Clark, 1866	Yes	Yes	Outwards	Yes	Yes	Yes	Yes	(Fig. 2.1a this study, Medeiros et al., 1996, Medeiros, 1991)
Lilioceris nigropectoralis (Pic, 1928), *Lilioceris formosana* Heinze, 1943	Yes	Yes	Outwards	Yes	Not specified	Yes	Yes	(Fig. 2.1b this study, Chi-Feng Lee personal communication in Santiago-Blay et al., 2012)
Coleoptera: Chrysomelidae: Chrysomelinae								
Agrosteomela chinensis (Weise, 1922)	Yes	Not observed	Not observed	Not observed	Not observed	Not observed	None observed	(Jolivet and Verma, 2011, Santiago-Blay et al., 2012, Bontems and Lee, 2008)

Chrysophtharta obovata (Chapuis, 1877)	Yes	Yes	Inwards	Yes	Yes	Probably	Yes	(Santiago-Blay et al., 2012, Simmul and de Little, 1999)
Doryphora paykulli (Stal, 1859), *D. reticulata* Fabricius, 1787	Yes	Yes	Inwards	Yes	No	Yes	No	(Windsor et al., 2013)
Eugonycha melanostoma (Stål, 1859)	Yes	Yes	Inwards	Not specified	Not specified	Not specified	Tentatively	(Vasconcellos-Neto and Jolivet, 1994, Santiago-Blay et al., 2012)
Gonioctena sibirica Kimoto, 1994	Yes	Roughly	Mostly inwards	Mostly	Probably	Unclear	Unclear	(Santiago-Blay et al., 2012, Jolivet and Verma, 2011, Kudô and Hasegawa, 2004)
Labidomera suturella Guérin-Méneville, 1838	Yes	Not observed	Not observed	Not observed	Not observed	Not observed	None observed	(Daccordi et al., 1999, Santiago-Blay et al., 2012, Daccordi, 1993, Reid et al., 2009)
Paropsis spp.; e.g. *P. aegrota* Boisduval, 1835, *P. maculata* (Marsham, 1908), *P. atomaria* Olivier, 1807 and *P. tasmanica* Baly, 1864	Yes	No	Both	No	Yes	Perhaps, unclear	No	(Santiago-Blay et al., 2012, Vasconcellos-Neto and Jolivet, 1994, Selman, 1994, Simmul and de Little, 1999, Carne, 1966)
Paropsisterna spp.	Yes	Not specified	Not specified	Not specified	Yes	Not specified	Not enough information	(Santiago-Blay et al., 2012, Jolivet and Verma, 2011, Simmul and de Little, 1999, Selman, 1994)
Plagiodera spp. e.g. *P. versicolora* (Laicharting, 1781)	Yes	No	Both	No	Yes	No	No	(Vasconcellos-Neto and Jolivet, 1994, Wade, 1994, Wade and Breden, 1986, Costa, 2006, Breden and Wade, 1987)
Phratora spp.	Yes	Not observed	Not observed	Not observed	Not observed	Not observed	None observed	(Santiago-Blay et al., 2012, Vasconcellos-Neto and Jolivet, 1994)
Phyllocharis undulata (Linnaeus, 1763)	Yes	Roughly	Mostly inwards	No	Not observed	Not observed	No	(Mohamedsaid, 2008, Santiago-Blay et al., 2012)
Platyphora selva Daccordi, 1993, *Pl. microspina* (Bechyně, 1954)	Yes	Yes	Inwards	Yes	No	Yes	No	(Choe, 1989, Windsor et al., 2013)

Platyphora conviva (Stål, 1858), *Pl. anastomozans* (Perty, 1832) and *Pl. nigronotata* (Stål, 1857), *Pl. nitidissima* (Stål, 1857) *Pl. fasciatomaculata* (Stål, 1857), *Pl. vinula* (Stål, 1858)	Yes	Yes	Outwards	Yes	Yes	Yes	Yes	(Medeiros and Vasconcellos-Neto, 1994, Vasconcellos-Neto and Jolivet, 1994, Medeiros et al., 1996, Medeiros, 1991)
Proseicela vittata (Fabricius, 1781), *Pr. bicruciata* Jacoby, 1880, *Pr. spectabilis* (Baly, 1858)	Yes	Yes	Inwards	Yes	No	Yes	No	(Fig. 2.6, this study, Windsor et al., 2013)
Proseicela crucigera (Sahlberg, 1823)	Yes	Yes	Inwards	Yes	Not specified	Yes	Probably	(Vasconcellos-Neto and Jolivet, 1994, Medeiros et al., 1996)
Pterodunga mirabile Daccordi, 2000	Yes	Yes	Inwards	Not specified	Not specified	Not specified	Tentatively	(Santiago-Blay et al., 2012, Jolivet and Verma, 2011, Reid et al., 2009)
Coleoptera: Chrysomelidae: Cassidinae								
Acromis sparsa (Boheman, 1854)	Yes	Yes	Inwards	Yes	Not specified	Yes	Probably	(Santiago-Blay et al., 2012, Vasconcellos-Neto and Jolivet, 1994, Windsor, 1987)
Aspidomorpha puncticosta Boheman, 1854, *A. miliaris* (Fabricius, 1775)	Yes	Yes	Inwards	Yes	Yes	Yes	Yes	(Heron, 1992, Verma, 1992, Vasconcellos-Neto and Jolivet, 1994, Fig. 155 and 156 in Świętojańska, 2009)
Chelymorpha informis Boheman, 1854, *C. alternans* Boheman, 1854, *C. cribraria* (Fabricius, 1875)	Yes	Yes	Inwards	Yes	Probably	Yes	Yes	(Vasconcellos-Neto and Jolivet, 1988a, 1994)
Cistudinella foveolata (Champion, 1894)	Yes	Yes	Inwards	Yes	Probably	Yes	Yes	(Fig. 2.2a, this study)
Conchyloctenia punctata (Fabricius, 1787)	Yes	Yes	Inwards	Yes	No	Yes	Tentatively	(Heron, 1999, 1992, Vasconcellos-Neto and Jolivet, 1994)

Coptocycla dolosa Boheman, 1855	Yes	Yes	Inwards	Yes	Probably	Yes	Yes	(Fig. 2.5, this study)
Eugenysa columbiana (Boheman, 1850), *E. coscaroni* Viana, 1968	Yes	Yes	Inwards	Yes	Yes	Yes	Yes	(Fig. 2.2b, Chaboo, 2002, Windsor and Choe, 1994)
Echoma flava Linnaeus, 1758	Yes	Yes	Inwards	Yes	Probably	Yes	Yes	(Vasconcellos-Neto and Jolivet, 1988a)
Nuzonia sp.	Yes	Yes	Inwards	Yes	Yes	Yes	Yes	(Fig. 2.2c, this study)
Ogdoecosta biannularis (Boheman, 1854)	Yes	Yes	Inwards	Yes	Yes	Yes	Yes	(Santiago-Blay et al., 2012, Vasconcellos-Neto and Jolivet, 1994, Romero-Nápoles, 1990)
Omaspides tricolorata (Boheman, 1854), *O. pallidipennis* (Boheman, 1854), *O. sobrina* (Boheman, 1854), *O. bistriata* (Boheman, 1854) and *O. convexicollis* Spaeth, 1909	Yes	Yes	Inwards	Yes	Yes	Yes	Yes	(Gomes et al., 2012, Vasconcellos-Neto personal communication in Santiago-Blay et al., 2012, D. Windsor's obervations, Windsor and Choe, 1994, Rodriguez, 1994, Frieiro-Costa, 1995, Vasconcellos-Neto and Jolivet, 1994, Fig. 159, 160, 165 and 166 in Świętojańska, 2009)
Physonota alutacea Boheman, 1854	Yes	Yes	Inwards	Yes	Probably	Yes	Yes	(Fig. 2.2d, this study)
Polychalma multicava (Latreille, 1821)	Yes	Yes	Inwards	Yes	Probably	Yes	Yes	(Fig. 2.2e, this study)
Stolas sp., *Stolas xanthospila* (Champion, 1893)	Yes	Yes	Inwards	Yes	Probably	Yes	Yes	(Fig. 2.2f, this study, Vasconcellos-Neto and Jolivet, 1994)
Coleoptera: Curculionidae: Hyperinae								
Phelypera distigma (Boheman, 1842)	Yes	Yes	Outwards	Yes	Yes	Yes	Yes	(Costa, 2006, Jolivet and Maes, 1996, Fitzgerald et al., 2004)

Diptera: Ceratopogonidae: Forcipomyiinae								
Forcipomyia fuliginosa (Meigen, 1818)	Yes	Yes	Inwards	Yes	Probably	Yes	Yes	(Jolivet et al., 1990, Santiago-Blay et al., 2012, Young, 1984, Saunders, 1924, Hinton, 1955)
Hemiptera								
Not specified	No	Not specified	Not specified	Not specified	Not specified	Not specified	Not enough information	(Jolivet and Verma, 2011, Santiago-Blay et al., 2012)
Ceroplastes sp. (Coccidea), *Potnia* sp. (Membracidae), *Nephesa rosea* (Spinola, 1839) (Flatidae), *Derbe* sp. (Derbidae)	No	No to roughly	Both or neither	No	In some spp.	Probably not	No	(Fig. 11A, B, D and E in Santiago-Blay et al., 2012)
Antiteuchus tripterus (Fabricius, 1787) (Pentatomidae)	No	Yes	Inwards	No	No	No	No	(Eberhard, 1975)
Parastrachia japonensis (Scott, 1880) (Parastrachiidae)	No	Yes	Inwards	No	No	No	No	(Filippi et al., 2001)
Hymenoptera: Symphyta: Tenthredinoidea								
Bergiana sp. (Cimbicidae)	Yes	Yes	Not specified	Not specified	Not specified	Not specified	Not enough information	(Jolivet et al., 1990)
Perga dorsalis Leach, 1817, *P. affinis* Kirby, 1882 (Pergidae)	Yes	Yes	Outwards	Yes	Yes	Yes	Yes	(Fig. 2.3, this study, Weinstein, 1989, Santiago-Blay et al., 2012, Vasconcellos-Neto and Jolivet, 1994, Jolivet and Verma, 2011, Carne, 1962)
Pseudoperga guerini (Westwood, 1880) (Pergidae)	Yes	Yes	Outwards	Yes	Yes	Yes	Yes	(Morrow et al., 1976)
Themos olfersii (Klug, 1834) (Argidae)	Yes	Yes	Outwards	Yes	Yes	Yes	Yes	(Jolivet et al., 1990, de Souza Dias, 1975)
Dielocerus diasi Smith, 1975 (Argidae)	Yes	Not specified	Not specified	Not specified	Yes	Unclear	No	(Jolivet et al., 1990, de Souza Dias, 1975)

Hymenoptera: other families								
Trigona sp. (Apidae: Meliponinae)	No	Yes	Outwards	No	Yes	Yes	No	(Santiago-Blay et al., 2012, Vasconcellos-Neto and Jolivet, 1994, Jolivet et al., 1990, Wittmann, 1985)
adult Hymenoptera, bees (Apidae), wasps (Vespidae), *Conomyrma* spp. and numerous other ants (Formicidae)	No	Yes and no	Usually outwards	No	Yes	Yes	No	(Santiago-Blay et al., 2012)
Apoica sp. (Vespidae: Polistinae)	No	Yes	Outwards	Yes	Yes	Yes	No	(van der Vecht, 1972, Pickett et al., 2009, Neto and Andena, 2011)
"Parasitic Hymenoptera larvae and pupae [on] their host"	Yes	Yes	Inwards	No	No	No	No	(Santiago-Blay et al., 2012)
Lepidoptera: Papilionidae: Papilioninae								
Papilio laglaizei Depuiset, 1877	Yes	Yes	Outwards	Yes	Not specified	Not specified	Tentatively	(Costa, 2006, Straatman, 1975)
Lepidoptera: Saturniidae								
Hylesia spp. (Hemileucinae)	Yes	Unclear	Not specified	Yes	Yes	Probably	Not enough information	(Vasconcellos-Neto and Jolivet, 1994)
Lonomia spp. (Hemileucinae)	Yes	Yes	Outwards	Yes	Yes	Probably	Yes	(T. Fitzgerald personnal communication in Santiago-Blay et al., 2012, Fig. 4, this study, Lorini et al., 2007, Fig. 56 in Ministério da Saúde, 1998)
Arsenura spp. (Arsenurinae)	Yes	No	Both	Yes	No	Probably not	No	(Jolivet et al., 1990, Fig. 17A-B in Santiago-Blay et al., 2012).

Lepidoptera: other families								
Noctuidae and Sphingidae	Yes	No	Both	Not specified	Not specified	Probably not	No	(Santiago-Blay et al., 2012)
Neuroptera: Ascalaphidae								
Ascaloptynx furciger (McLachlan, 1891)	Yes	Yes, around twig	Both	No	Yes	Yes	No	(Jolivet and Verma, 2011, Santiago-Blay et al., 2012, Henry, 1972, Jolivet et al., 1990)
Thysanoptera: Phlaeothripidae								
Unidentified species and *Anactinothrips gustaviae* Mound & Palmer, 1983	No	Yes	Inwards	Yes	Probably	Yes	Yes*	(Fig. 7, this study)(Kiester and Strates, 1984)
Non insect arthropods								
Phronima sedentaria (Forskål, 1775) (Crustacea: Amphipoda: Hyperiidea)	No	Yes	Outwards?	Yes	No	No	No	(Costa, 2006, Laval, 1978, 1980)
Unidentified Platydesmidae (Myriapoda)	No	Yes	Inwards	Not specified	Not specified	Not specified	Tentatively analogous	(Costa, 2006, Hoffmann, 1982)
Vertebrates								
Some ungulates, e.g. muskoxen *Ovibos moschatus* (Zimmermann, 1780)	No	Yes	Outwards	No	Yes	Yes	No	(Santiago-Blay et al., 2012, Jolivet and Verma, 2011, Jolivet et al., 1990)
Antarctic penguins	No	Yes	Inwards	Yes	Not specified	No	No	(Jolivet and Verma, 2011, Gilbert et al., 2006)

* With the definition of cycloalexy expanded from strictly insect larvae to any immature insect.

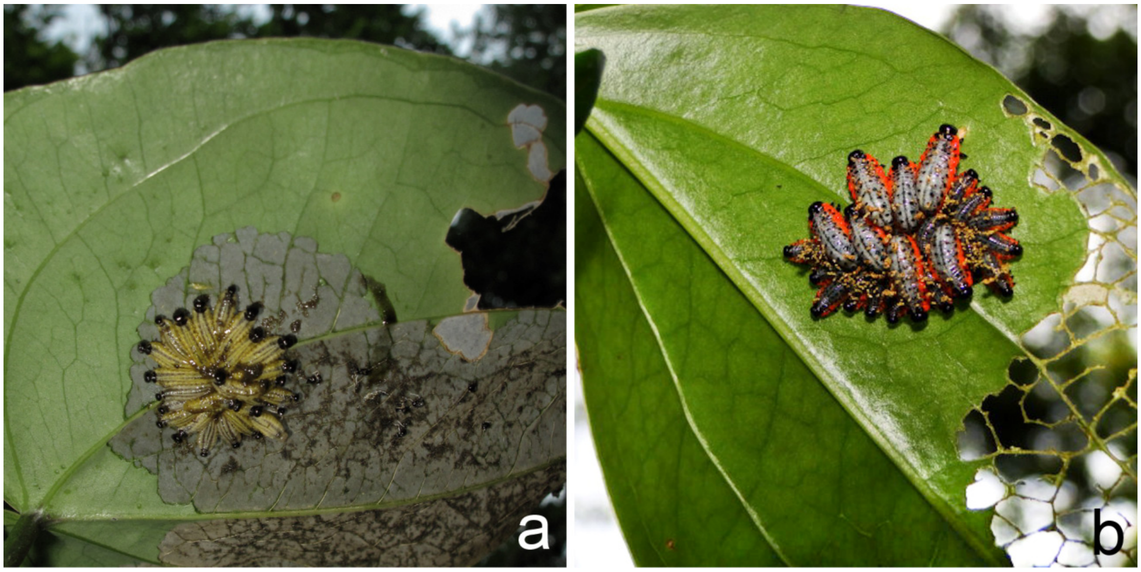

Figure 2.1: Cycloalexy with heads outwards in larvae shining leaf beetles (Criocerinae). (a) Larvae of *Lema* sp. at rest, photograph in Potrerillos del Guendà, Dept. Santa Cruz, Bolivia, © D. Windsor. (b) *Lilioceris nigropectoralis* larvae in Taiwan. Seven larvae are distinctly larger and appear to be from a different cohort than the other twelve. Photograph taken in Yangmingshan National Park on 2 August 2011, by 劉達偉 (Liu Dá Wěi) and licensed under the Creative Commons 3.0 Taiwan (CC BY-NC 3.0 TW). Available online from http://taibnet.sinica.edu.tw/chi/taibnet_addpicture3.php?name_code=332248&id=4449 (accessed 29 March 2013).

2.4. STRICT CYCLOALEXY

Some species demonstrate behaviour that precisely fit the definition and criteria of cycloalexy. This is the case of *Coelomera* spp. (Coleoptera: Chrysomelidae: Galerucinae) and spitfire grubs *Perga dorsalis* (Hymenoptera: Symphyta: Pergidae) (Jolivet et al., 1990).

Approximately 35 species of genus *Coelomera* are cycloalexic and feed on *Cecropia* sp. (Urticaceae). These *Cecropia* plants are myrmecophytes protected by mutualistic *Azteca* ants (Formicidae: Dolichoderinae). The gregarious leaf beetle larvae feed during the day and rest in a tight circular cluster at night with heads inside and abdomens at the periphery (Vasconcellos-Neto and Jolivet, 1988a, 1994). The circle is broken for the day. Their rear end is protected by a supra-anal shield and, when threatened, these larvae excrete a nauseating fluid from the anus. Therefore, the better protected part of the insect, the posterior, is facing outwards in the circle, with the more vulnerable head inside (Vasconcellos-Neto and Jolivet, 1988a, 1994). The collective behaviour help larvae protect each other.

Spitfire grubs *Perga dorsalis* feed on *Eucalyptus* during the night, rest during the day in a circular formation, with heads outwards (Weinstein, 1989). The larvae rest with their heads at the periphery of the circle, with some larvae in the middle of the aggregation. When threatened, the larvae rear their heads and abdomens and regurgitate oils sequestered from their host *Eucalyptus* (Weinstein, 1989). The oils is an effective deterrent of potential predators, including ants, birds and mice (Morrow et al., 1976). Although in this species it is the heads, again the better protected part of the insects form the periphery of the circle.

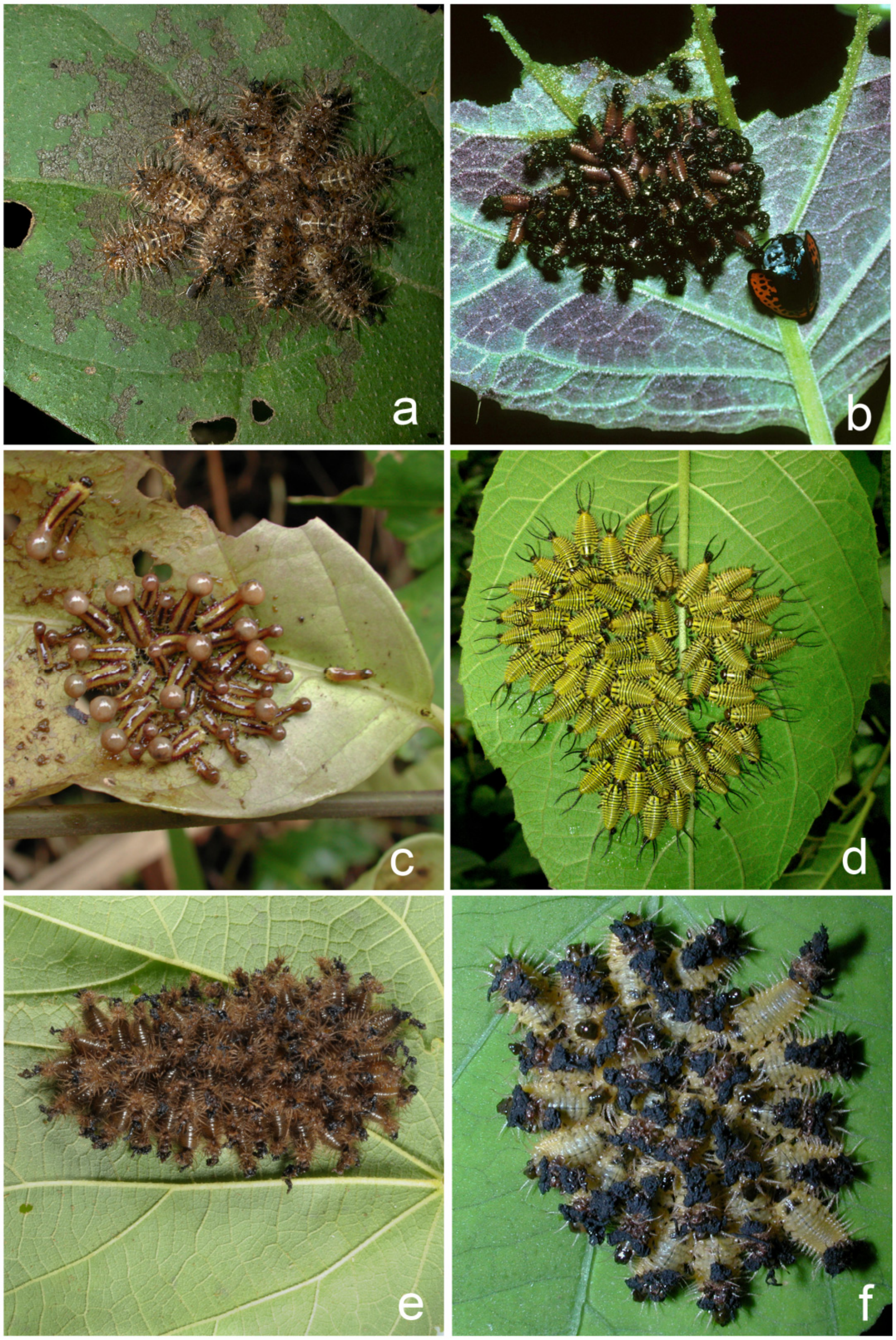

Figure 2.2: Cycloalexy in tortoise beetle larvae.
(a) *Cistudinella foveolata* (Ischyrosonychini) larvae on host *Cordia alliodora* (Ruiz & Pav.) Oken.; (b) *Eugenysa coscaroni* (Eugenysini) larvae and mother on host *Mikania guaco* Bonpl. (Asteraceae); (c) *Nuzonia* sp. on host *Maripa nicaraguensis* Hemsl.; (d) *Physonota alutacea* (Ischyrosonychini) larvae on host *Cordia spinescens* L.; (e) *Polychalma multicava* (Goniocheniinae) larvae on host *Helicteres guazumaefolia* Kunth. (Sterculiaceae); (f) *Stolas xanthospila* (Mesomphaliini) larvae on host *Turbina corymbosa* (L.) Raf. (Convolvulaceae); All photographs © D. Windsor.

Figure 2.3: Larvae of *Perga* sp. (Pergidae) rest aggregated in a cycloalexic formation. Even around a stem of their host plant, *Eucalyptus* sp., spitfire larvae rest with their heads outwards, Black Mountain, Canberra, ACT, Australia. Photograph by Donald Hobern on 24 May 2010, licensed under the Creative Commons Attribution 2 (CC BY 2.0) available online from http://www.flickr.com/photos/25401497@N02/4633828450 (accessed 31 March 2013).

2.5. EXAMPLES OF REPORTED CYCLOALEXY THAT DO NOT MEET THE DEFINITION CRITERIA

2.5.1. The oxymoron of non-circular cycloalexy

Gregarious caterpillars of genus *Arsenura* (Saturniidae: Arsenurinae) are reported to “show a kind of cycloalexy when resting on tree trunks during the day” (Jolivet et al., 1990). The caterpillars, align side-by-side or head-to–abdomen or both, in an elongated oval cluster (Fig. 17A-B in Santiago-Blay et al., 2012). The posture of these caterpillars with their heads, sides, and abdomens at the periphery in a linear mass rather than a circle does not satisfy the strict definition of cycloalexy. The tight circle formation with heads either in the centre or the outside is an important characteristic of cycloalexic behaviour, where the better-protected extremities of the immature insect form the periphery of the rosette. *Arsenura* are gregarious and rest in a tightly aggregated mass, but they are not cycloalexic. Santiago-Blay et al. (2012) suggest that on a tree trunk "the available background surface makes the shape of the larval aggregation distorted". This statement is perplexing because on a caterpillar scale and depending on the diameter of the tree, a tree trunk can be quite large and nearly flat. In addition, cycloalexy on tree trunks has been observed in *Lonomia* sp. (Fig. 2.4). We suggest the caterpillar aggregations described by Santiago-Blay et al. (2012) are less circular and compact not because of the shape of tree trunks but because these caterpillars' resting positions are not cycloalexic.

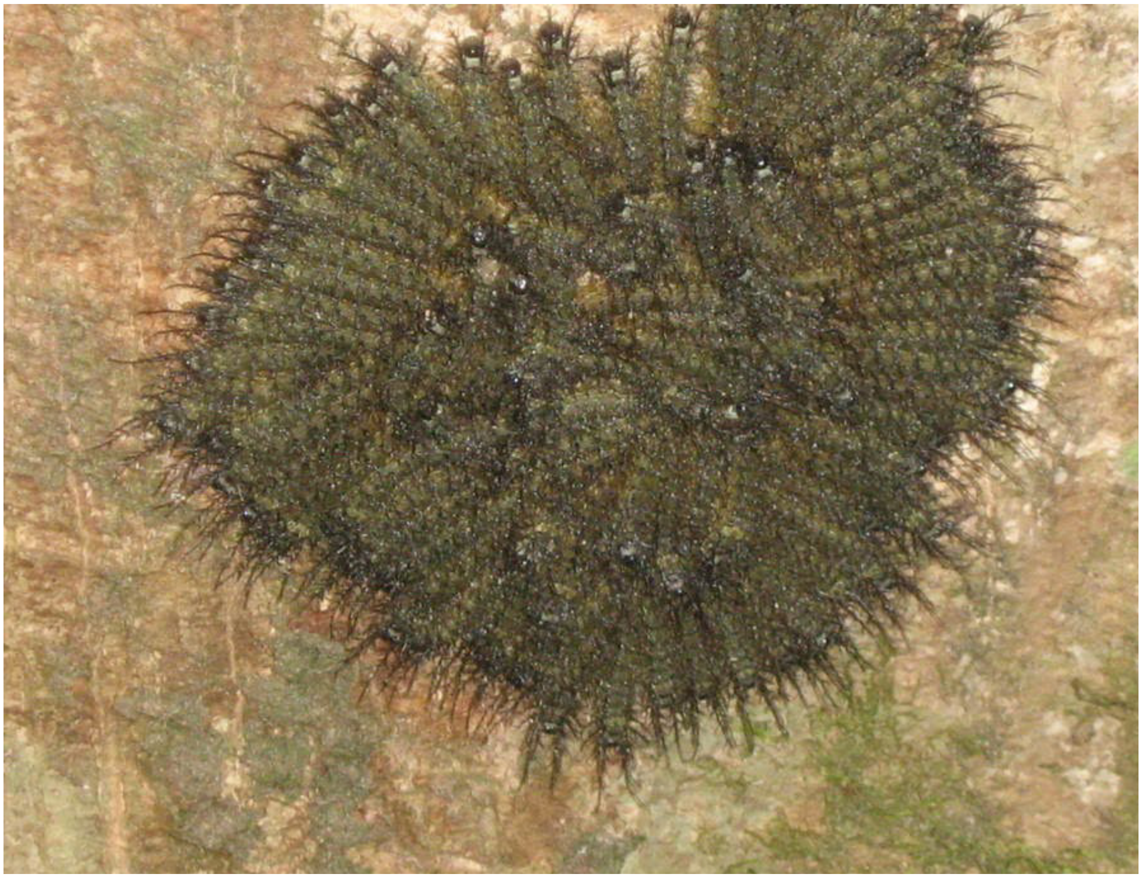

Figure 2.4: Cycloalexy with heads pointing outwards in caterpillars of *Lonomia* sp. (Saturniidae: Hemileucinae) on tree trunk in Peru.
Photograph taken near Pongo de Caynarachi, Lamas, San Martin, Peru, reproduced with the author’s permission © Marc Díaz Rengifo (Universidad Nacional Federico Villarreal - Lima-Perú).

2.5.2. Mixed head orientations

Larvae of the owlfly *Ascaloptynx furciger* (McLachlan) (Neuroptera: Ascalaphidae) are gregarious. After eclosion and their first meal of abortive eggs, they settle head-downwards on and around the twig on which they were laid (Henry, 1972). Jolivet et al. (1990) deem the behaviour of *A. furciger* is 'not strictly cycloalexy but related to it' since the owlfly larvae all point in the same downward direction. We agree with Jolivet & Verma (2011) cycloalexy exists around twigs and is not restricted to flat surfaces. However, even on twigs, cycloalexic larvae collectively orient their heads outward or inward, but not both. This is true for larvae of *Perga* sp. (Hymenoptera: Symphyta: Pergidae) (Fig. 2.3), *Omaspides tricolorata* (Boheman) (Frieiro-Costa, 1995), and this arrangement is retained in the pupae of *Omaspides pallidipennis* (Boheman) (Chrysomelidae: Cassidinae) (Fig. 4 in Gomes et al., 2012). For owlfly larvae, heads form the periphery at the bottom of the aggregation and abdomens are at the periphery on top, but unlike Cassidinae or *Coelomera* larvae, their abdomens are more vulnerable. It is more accurate to describe the behaviour as unidirectional defence rather than circular defence; larvae are only protected from predators walking up to the group. Secondly, larvae also feed while in this position, making it a passive hunting formation and not only a resting position (Henry, 1972). For these reasons, we question reports of cycloalexy in Neuroptera (Santiago-Blay et al., 2012, Jolivet and Verma, 2011, Vasconcellos-Neto and Jolivet, 1994, Jolivet, 2008).

2.5.3. Non-defensive behaviour

Cycloalexy is a defensive behaviour; it protects the individuals from predation or parasitism. Yet, some reported behaviours are not defensive. This is the case for huddling in Antarctic penguin, the huddle is a resting behaviour, usually with heads inwards, but it is for heat-conversation rather than defence (Gilbert et al., 2006). For these reasons, we disagree with Jolivet & Verma (2011); penguins are not cycloalexic.

2.5.4. *Non-resting behaviours*

Cycloalexy is a behaviour taken at rest. When the immature insects are active, when they, feed for example, the formation is broken and when they rest again, the circle regroups (Fig. 2.5). Larvae of *P. versicolora* and other *Plagiodera* species form a lose circle when feeding and at rest, with individual larvae not consistently facing outwards or inwards (Wade, 1994, pers. obs.). The grouping is not specifically taken at rest, and the shape of the formation is often influenced by feeding and the shape of the leaf, with multiple 'feeding rings' on larger leaves (Wade, 1994). Santiago-Blay et al. (2012) state that "in *Plagiodera versicolora* (Laicharting, 1781) chrysomelines, as well as in sawflies, cycloalexy appears to facilitate feeding (Costa, 2006)." However, Costa (2006) never suggests such a link. These larval aggregations facilitate feeding, likely through increased feeding efficiency (Breden and Wade, 1987, Wade and Breden, 1986, Costa, 2006). In *P. versicolora*, the size of the group does not influence survival of larvae, but helps with feeding (Breden and Wade, 1987). Thus, aside from being an active behaviour rather than at rest, it seems that grouping in *P. versicolora* is not defensive: two criteria are missing for strict cycloalexy.

Figure 2.5: *Coptocycla dolosa* larvae, Potrerillos del Guendà, Dept. Santa Cruz, Bolivia. (a) when active, feeding or moving; (b) at rest. © D. Windsor.

2.5.5. Circular formations that do not react to threats

Larvae of several chrysomelines rest in tight circular groups with the heads pointing inwards: *Doryphora paykulli*, *D. reticulata*, Platyphora *microspina*, Pl. selva, *Proseicela vitatta*, *Pr. spectabilis*, *Pr. bicruciata* and *Pr.* sp. nov. “Yasuni" (Windsor et al., 2013). All these species also have maternal care but when disturbed, larvae do not have coordinated defensive reactions, instead the mother assumes this role (Fig. 2.6, Windsor et al., 2013). Is this behaviour still cycloalexy? Is there a relationship between maternal care and a disintegration from the strict definition of the behaviour? Coordinated movements to repel threats or parasitoids are part of the strict definition proposed by Jolivet et al. (1990). However, in these aggregations, larval grouping makes guarding more efficient, but the grouping and larvae do not defend themselves. We adhere to the strict definition proposed by Jolivet et al. (1990) that defensive posturing when under threat is a key component of cycloalexic behaviour. The observation that truly cycloalexic *Platyphora* face outwards in their defensive circle supports this interpretation.

The distinction becomes less clear with larvae that have maternal care and passive protection, like the exuvial or exuvio-fecal shields of tortoise beetles. *Conchyloctenia punctata* (Cassidinae) is a good example of this; the larvae are actively protected by their mother but also passively protected by their shield (Heron, 1992). In our opinion, despite the lack of reactive defences, the larvae's passive defences makes this behaviour tentatively cycloalexic.

Figure 2.6: Larvae of *Proseicela spectabilis* Baly (Chrysomelidae: Chrysomelinae) at rest encircling the stem of their host plant.
The larvae rest with heads inwards on a stem of their host plant, *Solanum* sp. section *Dulcamara*. Note the tachinid fly at the bottom left of the cluster and the adult female beetle on the opposite side, at the extreme right of the larval group. Photograph taken in Reventador, Napo Province, Ecuador, © G. Dury.

2.5.6. Adult insects

We use *Apoica* as an example even though cycloalexy was not explicitly reported in this genus. During the day, these nocturnal wasps rest on the circular or nearly circular lower surface of their nests (van der Vecht, 1972, Pickett et al., 2009, Neto and Andena, 2011). The wasps rest facing outwards and this results in a circular formation that could loosely be termed cycloalexy. This behaviour meets all criteria of the original definition, except one: adult wasps are not larvae. Thus, we argue that the behaviour is not cycloalexic because it is observed in fully-developed insects and, more importantly, the shape of the nest or nest entrance explains the circular formation. In a similar fashion, stingless bees of genus *Trigona* (Apidae: Meliponinae) are not cycloalexic as suggested by Vasconcellos-Neto & Jolivet (1994). In this case, fully-developed individuals are not even at rest: in most Meliponinae, the nest entrance is protected by bees positioned in or around the entrance tube and, at night, the entrance is closed (Wittmann, 1985). The bees are not resting but are actively guarding and the ring formation is an artefact of the nest entrance shape.

These examples motivate amending the definition of cycloalexy to a formation taken by individuals for mutual defence, thus excluding formations taken for defence of a nest, brood or food stores. We argue that evolution of circular nests and resource guarding have little to do with the evolution of cycloalexy.

2.5.7. Circular defence in vertebrates

Several authors compare cycloalexy to the 'circle-the-wagons' formation employed by American pioneers to defend themselves against Native Americans (Costa, 2006, Jolivet et al., 1990, Jolivet and Verma, 2011, Santiago-Blay et al., 2012). In Jolivet et al. (1990) and Jolivet & Verma (2011), the authors discuss behaviours analogous to cycloalexy in vertebrates: muskoxen (*Ovibos moschatus*), eland (*Taurotragus oryx*), elk (*Cervus canadensis*) and penguins. The authors do not provide citations for the behaviour in elands or elk, and cite Wilson (1975) for descriptions of this behaviour in muskoxen and penguins. Wilson (1975) does not mention penguins in this manner, but does mention similar behaviours in elands, water buffalo (*Bubalus bubalis*), red deer (*Cervus elaphus*), and even killer whales (*Orcinus orca*) (Tener, 1954, Kruuk, 1972, Eisenberg and Lockhart, 1972, Darling, 1937, Martinez and Klinghammer, 1970; all p.45 in Wilson, 1975). Wilson (1975) describes elk grazing in a "windrow" formation, but does not mention circular defence (Altmann, 1956; p.45 in Wilson, 1975). We agree that several vertebrates (muskoxen, elands, water buffalo, red deer and killer whales) employ defensive circular formations perhaps analogous to cycloalexy. However, we disagree with Santiago-Blay et al. (2012) to broaden the definition to include these behaviours. Circular defence in mammals is not a passive resting behaviour, it is a formation that is taken when the animals are threatened. In cycloalexic species, the circular formation is the main resting position. The circular defence of vertebrates is reactive, while cycloalexy is preemptive.

2.6.CYCLOALEXY IN IMMATURE HEMIMETABOLOUS INSECTS

We report cycloalexy in thrips (Thysanoptera) [specimens have been sent to Dr. Mound of CISRO for identification]. We observed a group of 14 thrips, in their pupal instar, forming a tight circle with abdomens outwards on the leaf of the woody vine *Maripa panamensis* Hemsl. (Convolvulaceae) (Fig. 2.7). When disturbed, the threatened individuals and those beside them waved their abdomen. When disturbance continued, a brown liquid was exuded and formed a droplet at the end of the abdomen. The group was then further disturbed and the individuals dispersed. Approximately an hour later, the thrips had reassembled in a circular resting formation. In the lab, after the final molt, the adult thrips dispersed in the container they were kept. Our observations support those of *Anactinothrips gustaviae* Mound & Palmer, thrips that rest in bivouacs and exude a defensive liquid from their abdomen when disturbed (Kiester and Strates, 1984).

Although thrips are hemimetabolous and, thus, do not have larvae, the behaviour was observed in immature stages. It meets all other criteria of the original definition of the term. Thus, we propose to broaden the definition to include all immature insects.

Figure 2.7: Circular resting position in Panamanian thrips on *Maripa panamensis* Hemsl. (Convolvulaceae).
Photograph taken 24 April 2013, on Cerro Campana, Panama. © G. Dury.

2.7.COMMON TRAITS OF ALL CYCLOALEXIC SPECIES

When the definition of cycloalexy is strictly applied, a set of traits common to all species becomes apparent. Foremost, all cycloalexic species are gregarious. This may seem obvious, but gregarious lifestyles have implications in terms of cooperative feeding and continued group cohesion through chemical, tactile or acoustic communication (Costa, 2006).

To date, all cycloalexic species use chemical defence. The cycloalexic larvae in genera *Lema* (Criocerinae) and *Platyphora* (Chrysomelinae) regurgitate when threatened (Medeiros et al., 1996, Vasconcellos-Neto and Jolivet, 1994). The larvae of *Forcipomyia* have paired setae on the head, thorax and abdomen that exude hygroscopic substances that repel ants (Hinton, 1955). The chemical defences of gregarious *Lonomia* caterpillars is so potent that the resulting trauma caused by venom injected from their setae can be lethal to humans (Veiga et al., 2001). Most tortoise beetle larvae carry an exuvial or exuvio-fecal shields on the furca of their last abdominal segment, this shield serve as a mechanical or chemical barrier against predators (Gómez et al., 1999, Olmstead and Denno, 1993, Vencl et al., 1999). In all cases, the best protected extremity faces outwards.

To date, all the species that exhibit cyclolaexic behaviour are miniature grazers, most feed on leaves. This is the case for cycloalexic caterpillars, and larvae of sawflies (Weinstein, 1989), weevils and leaf beetles (Jolivet and Maes, 1996, Vasconcellos-Neto and Jolivet, 1994). Some feed on fungal hyphae, such as *Forcipomyia fuliginosa* midge larvae (Young, 1984), and the rest graze on lichen, like *Anactinothrips gustaviae* thrips (Kiester and Strates, 1984).

Gregarious lifestyles, chemical defence and grazing groups of immature insects are all traits of Costa's (2006) 'larval herd' syndrome of group living. Like cycloalexy, parental care is only present in some of these larval herds (Costa, 2006). Possibly, the slow-moving and exposed lifestyle of these immature insects makes them more vulnerable to predators and parasitoids (Cornell and Hawkins, 1995, Costa, 2006). Increased threats probably explain the multiple defences of herbivore insects, including chemical defence whose evolution generally precedes that of aggregation (Ruxton and Sherratt, 2006).

2.8. CONCLUSION

Several insect larvae exhibit cycloalexy, a behaviour whose definition we have amended to: ‘the attitude adopted at rest by immature insects, diurnal or nocturnal, in a tight circle where their best defended extremity, either head or abdomen, is exposed at the periphery. Sometimes remaining individuals rest at the centre of the circle. Individuals maintain this position when threatened and employ coordinated movements such as threatening attitudes, regurgitation, and biting, to repel predators or parasitoids.’

In leaf beetles (Chrysomelidae), cycloalexy with abdomens oriented outward is found in one genus of skeletonizing leaf beetles (Galerucinae: *Coelomera* spp.), at least fifteen tortoise beetle genera (Cassidinae), two genera of shining leaf beetles (Criocerinae: *Lema* and *Lilioceris*) and several genera of broad-shouldered leaf beetles (Chrysomelinae: *Platyphora*, *Eugonycha*, *Chrysophtharta* and perhaps *Pterodunga*). The behaviour with heads outwards, is found in some sawflies (Tenthredinoidea: Pergidae: *Perga* spp. and Argidae: *Themos olfersii*) of Australia and Brazil. Social caterpillars often form aggregations, but these aggregations are rarely cycloalexic. However, caterpillars of *Lonomia* spp. (Saturniidae: Hemileucinae) are cycloalexic and *Papilio laglaizei* (Papilionidae) are tentatively cycloalexic. One weevil *Phelypera distigma* (Curculionidae) and one midge *Forcipomyia fuliginosa* (Ceratopogonidae) also exhibit cycloalexy. We propose that some immature thrips are also cycloalexic. Platydesmid millipedes sometimes aggregate in a tentatively analogous way.

Several reports of cyclalexy do not meet the definition criteria. For example reports of cycloalexy in feeding aggregations of Hemiptera and larvae of Hymenopteran parasitoids. The behaviour has also been mistakenly attributed to adult Hymenoptera; for example, stingless bees (Apidae: Meliponinae), ants (Formicidae) and wasps (Vespidae), guarding their nest. This is active protection of a nest and not cycloalexy. Similarly, the term has been applied to the circular assembly of an amphipod crustacean which helps the mother herd the larvae. Owlfly larvae (Neuroptera: Ascalaphidae: *Ascaloptynx furciger*) form unidirectional defensive groups (not cycloalexy) allowing larvae to feed without changing position. While defensive circles are sometimes observed in some mammals: muskoxen, eland, water buffalo, red deer and killer whales. Contrary to cycloalexy, the defensive formations in these mammals are a reaction to imminent threat. Other vertebrates, like penguins, huddle to reduce heat loss.

Application of a more precise definition of cycloalexy provided by Jolivet et al. (1990) and amended here, may make unravelling the evolutionary pressures leading to the convergent origins of this form of collective defence in immature arthropods more tractable. Much remains to be learned about whether larval aggregation, cycloalexy, sequestration of plant metabolites and maternal care are alternative defensive strategies or are honed evolutionary responses to particular threats. Chrysomeline leaf beetles are an ideal group for phylogenetic reconstruction and character analysis of these behaviours to unravel the number of independent evolutionary origins of cycloalexy and larval aggregation.

3.0. CONNECTING TEXT

In chapter I, I review the literature on cycloalexy and argue not all aggregating insects exhibit the behaviour. Cycloalexy has specific criteria that differentiate it from other aggregations. Cycloalexy is a defensive attitude taken at rest by insect larvae in a tight circle, with either heads or abdomens at the periphery. The behaviour is accompanied under threats from predators and parasitoids by either coordinated behavioural movements or the production of defensive secretions.

In chapter II, I study the evolution of solitary, aggregated and, cycloalexic larvae within Neotropical Chrysomelinae. After inferring a molecular phylogeny I reconstruct ancestral behavioural states of larvae and discuss taxonomic and evolutionary implications of the phylogeny and ancestral state reconstruction.

4.0. CHAPTER II: Evolution of gregariousness and cycloalexy in the Neotropical Chrysomelinae (Coleoptera: Chrysomelidae) inferred from a molecular phylogeny

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4.1. ABSTRACT

Cycloalexy is a defensive behaviour in which groups form a circle at rest. We investigate this unique behaviour and the phylogenetic relations of beetles predominately of Neotropical origin. For the molecular phylogeny, we sequenced five gene amplicons: *CAD* (*Carbamoyl-phosphate synthetase 2, Aspartate transcarbamylase and Dihydroorotase*), *28S* rDNA, mitochondrial *cytochrome oxidase I* and *II* (*COI* and *COII*) and *12S* rDNA. Bayesian and Maximum Likelihood (ML) analysis methods inferred the phylogeny. Ancestral larval behaviour was reconstructed with ML and Maximum Parsimony. Taxonomic subtribe Chrysomelina (*sensu* Daccordi 1982) was monophyletic. The Pan-American Doryphorina (*sensu* Daccordi 1982) was also monophyletic and divided into two sister clades; one with ((*Stilodes* + *Zygogramma*), (*Calligraphra*) and (*Cosmogramma*)), the second with ((*Platyphora* + *Desmogramma* + *Elytrosphaera* + *Doryphora*) and (*Platyphora* + *Labidomera* + *Leptinotarsa* + *Proseicela*)). The reconstruction of ancestral larval behaviour shows five evolutionary origins of larval gregariousness, one accompanied by cycloalexy and two accompanied by maternal care. Cycloalexy evolved in *Platyphora anastomozans* (Perty) and *Platyphora nigronotata* (Stål), and maternal care in *D. paykulli* (Stål), *Platyphora microspina* Bechyně and multiple species of genus *Proseicela*.

4.2. INTRODUCTION

The larvae of some herbivorous insects show a remarkable behaviour: they group in a tight circle, presumably for defence (Jolivet et al., 1990). This uncommon behaviour, called cycloalexy, has been observed in diverse insect groups; such as sawflies (Hymenoptera: Pergidae and Argidae), moths (Lepidoptera: Saturniidae), midges (Diptera: Ceratopogonidae) and beetles (Coleoptera: Chrysomelidae and Curculionidae) (Jolivet et al., 1990, Jolivet and Maes, 1996). Cycloalexy, has been frequently reported in species of sawflies and leaf beetles than any other insect groups (Jolivet et al., 1990). Four subfamilies of leaf beetles have cycloalexic behaviour: Galerucinae, Criocerinae, Cassidinae and Chrysomelinae. Reports of cycloalexy are only known for the genus *Coelomera*, within the Galerucinae. In comparison, cycloalexy occurs in multiple genera of Cassidinae, Criocerinae and Chrysomelinae (Jolivet et al., 1990, Vasconcellos-Neto and Jolivet, 1994, Santiago-Blay et al., 2012).

Cycloalexic species share a set of traits. They live in groups and are gregarious or subsocial during their larval stage (Costa and Fitzgerald, 1996). Larval groups feed together on leaves and move from leaf to leaf as nomadic foragers (Costa et al., 2004, Jolivet et al., 1990). When not feeding, the larvae usually arrange themselves in a rosette-shaped formation. When threatened, cycloalexic larvae use coordinated group movements to threaten, regurgitate, bite or otherwise repel predators or parasitoids (Costa, 2006, Jolivet et al., 1990).

The use of chemical secretions is another common defensive trait of cycloalexic larvae (Jolivet et al., 1990, Costa, 2006). For example, sawfly larvae in the genus *Perga* (Hymenoptera: Pergidae) sequester, regurgitate and spit *Eucalyptus* oils (Jolivet et al., 1990). The fluid regurgitated by *Platyphora conviva* (Stål) (Chrysomelidae: Chrysomelinae) larvae is probably repellant or toxic (Vasconcellos-Neto and Jolivet, 1994). Larvae of *Coelomera* (Chrysomelidae: Galerucinae) eject a nauseating fluid from the anus (Vasconcellos-Neto and Jolivet, 1994, Jolivet et al., 1990). The exuvio-fecal shield of cycloalexic tortoise beetle larvae (Chrysomelidae: Cassidinae) contain defensive compounds (Gómez et al., 1999, Vencl et al., 1999). The compounds used for defence are often derived from either directly or metabolized from plant percursors (Pasteels et al., 2003b, 2003c, 2003d, Termonia et al., 2002). Therefore, there may be links between host plants and cycloalexic behaviour. Similarly, maternal care may arise in species with less potent chemical defences.

For example cycloalexic beetle species in at least three genera of Chrysomelinae in the subtribe Doryphorina (*sensu* Seeno and Wilcox, 1982): *Platyphora*, *Proseicela* and *Eugonycha* are closely associated with Solanaceae host plants (Vasconcellos-Neto and Jolivet, 1994). Cycloalexy is often associated with other behavioural traits, including ovoviviparity and maternal care, however, the evolutionary relationships between these traits remain unknown.

The larvae of all *Proseicela* species observed to date (5 spp.) are gregarious and form a tight cluster with heads towards the inside (Windsor et al., 2013). In contrast, cycloalexy is known to occur in only some *Platyphora* species; the remainder lead solitary lives. *Platyphora* species with gregarious larvae vary in how offspring are first delivered, with some species laying eggs in closely spaced clutches, while other species quickly deposit well developed larvae in distinct cohorts (Costa, 2006, Vasconcellos-Neto and Jolivet, 1994). Size at birth appears to vary considerably among live-bearing species, although this variation has not been well documented. These species-level differences in reproductive behaviour facilitate evolutionary studies of cycloalexy in the genera *Platyphora* and *Proseicela* (Costa, 2006). Species in both genera have large, overlapping ranges across Central and South America with numerous sympatric species (Blackwelder, 1957). This enables comparisons at the genus level, while reducing ecological differences due to location.

Existing phylogenies do not address the relative position of the two genera or their behaviour (Termonia et al., 2002, Hsiao, 1994, Gómez-Zurita et al., 2007, 2008). Because there is no extensive molecular phylogeny for the genera *Platyphora* or *Proseicela*, the evolutionary pattern of cycloalexy has not been tested. The frequency and evolutionary positions of the origins and losses of cycloalexy and how species with this behaviour are related to gregarious or solitary species is unknown. Understanding the evolutionary pattern of larval grouping will help direct ecological tests hypotheses on the evolutionary processes leading to this behaviour.

We hypothesize that cycloalexy has multiple origins in the Chrysomelinae. Given the large number of genera in the subfamily, and the relative rarity of species with gregarious larvae, several independent origins of cycloalexy are more likely than the alternative hypothesis of many losses; several independent origins result in a more parsimonious scenario. To test this hypothesis, we made observations, photographed and collected specimens from 63 species and 12 genera in Central and South America, plus 7 species and 4 genera in North America, Asia, Europe and Australia. Observations for all but two Brazilian species were directly made by us. There are 37 genera of Chrysomelinae in the Neotropical region according to Daccordi (1994) We observed beetle behaviour to determine if larvae occurred on host plants as solitary individuals or were arranged in groups and in the latter, whether they grouped in a circle or not. Segments from five genes, three mitochondrial and two nuclear, were amplified and compared to infer a strong phylogeny at different taxonomic levels (Parker et al., 1998, Gómez-Zurita and Galian, 2005). Ancestral behavioural states were reconstructed to determine the minimum number of independent evolutionary origins of active larval grouping.

4.3.MATERIALS AND METHODS

Beetles used in this study were collected primarily in the Neotropical region (Panama, Ecuador, Bolivia, French Guiana and Brazil) with a small number of species collected in Nearctic (Canada) and Palearctic (France and Mongolia) regions, and one species in Australia. Beetles were located visually and by inspecting known host plants. Observation sites were documented through Global Positioning System (GPS) co-ordinates. Photographs and herbarium samples of both insects and host plants were curated and kept in the working collection of D.W. at the Smithsonian Tropical Research Institute, Tupper Research and Conference Center, Panama City, Panama.

Once beetles were located in the field, behaviour was observed to determine whether larval groups demonstrated cycloalexy as defined by Jolivet et al. (1990). Cycloalexy is a circular formation taken at rest, with either heads or abdomens at the periphery, and the individuals in the group repel threats together. Larvae found in a tight circle with the majority touching each other, at rest (i.e. not feeding), that reacted to a threat with defensive movements (biting, regurgitation, etc.), were deemed cycloalexic. Larval groups were physically disturbed by poking them with a twig or moving the leaf on which they feeding. If individuals in the larval group did not react to this treatment or did not maintain a roughly circular formation; the species was classified as gregarious but not cycloalexic. For subsocial species (with maternal care), the adult female was collected and used for species identification. An attempt was made to rear larvae to maturity in the laboratory on their host plant, although this was not always successful. Adult beetles found without larvae were reared in the laboratory to obtain eggs or larvae, and corroborate larval behaviour observed in the field. Beetle specimens were preserved in absolute ethanol and stored at -20°C.

Total genomic DNA was extracted from muscle tissue in the thorax or from legs using the DNeasy Blood & Tissue kit (Qiagen Inc., Valencia, CA, U.S.A.), following the supplementary protocol on insect DNA extraction (Qiagen, 2006). Polymerase chain reaction (PCR) was used to amplify five target DNA fragments from three mitochondrial and two nuclear genes (Table 4.1), for a total of ~3690 bp, using an Eppendorf epgradient S or Mastercycler thermal cycler (Eppendorf, Hamburg, Germany). Each reaction contained 1 x standard *Taq* Buffer (New England Biolabs, Ipswich, MA, U.S.A.) (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$), 80 µM dNTPs (New England Biolabs), 400 nM of each primer, 0.4-1.2 ng/µL of sample DNA and 0.03 Unit/µL of *Taq* DNA polymerase (New England Biolabs). The total reaction volume was usually 25 µL, but was doubled for low yielding amplifications and was decreased by two thirds for the first amplification of nested PCR (Table S1, supplementary material).

Table 4.2: Target regions for amplification and primers used in this study

Gene segment (length)		Primer (length)	Primer sequence (5' to 3')	Source
12S mt rDNA (504-550 bp)		SR-N-14756 (21-mer)	GAC AAA ATT CGT GCC AGC AGT	Simon *et al.*, (1994)
		SR-J-14233 (20-mer)	AAG AGC GAC GGG CGA TGT GT	
28S nu rDNA (440-482 bp)		D2 UP-4 (23-mer)	GAG TTC AAG AGT ACG TGA AAC CG	Gillespie *et al.*, (2003)
		D2 DN-B (21-mer)	CCT TGG TCC GTG TTT CAA GAC	
COI mt protein-coding (472 bp)		C1-J-1718F (26-mer)	GGA GGA TTT GGA AAT TGA TTA GTT CC	Simon *et al.*, (1994)
		C1-N-2191 (26-mer)	CCC GGT AAA ATT AAA ATA TAA ACT TC	
COII mt protein-coding (617 bp)		modTL2-J-3037 (20-mer)	ATG GCA GAT TAG TGC AWT RG	Termonia *et al.*, (2001)
		modC2-N-3661 (24-mer)	CCA CAA ATT TCW GAA CAT TGA CCA	
CAD nu protein-coding* (1636 bp)		CD439F (29-mer)	TTC AGT GTA CAR TTY CAY CCH GAR CAY AC	Wild and Maddison (2008)
	640 bp	CD465F (30-mer)	ACC YAA RAA ART KYT VAT AAT TGG TTC WGG	This study
		CD688R (35-mer)	TGT ATA CCT AGA GGA TCD ACR TTY TCC ATR TTR CA	Wild and Maddison (2008)
	535 bp	CD667F (26-mer)	GGA TGG AAG GAA GTD GAR TAY GAR GT	Wild and Maddison (2008)
		CD851R (32-mer)	GGA TCG AAG CCA TTH ACA TTY TCR TCH ACC AT	
	556 bp	CD821F (29-mer)	AGC ACG AAA ATH GGN AGY TCN ATG AAR AG	Wild and Maddison (2008)
		CD1030R (30-mer)	CWS RGC AYA CCA RTC RAA CTC WAC WGA GCT	This study
		CD1098R2 (29-mer)	GCT ATG TTG TTN GGN AGY TGD CCN CCC AT	Wild and Maddison (2008)

* *CAD* (*Carbamoyl-phosphate synthetase 2, Aspartate transcarbamylase and Dihydroorotase*) was amplified in three partially overlapping segments using fully nested PCR and primers CD439F or CD465F and CD1098R2 or CD1030R (Figure S4.1 in the supplementary material).

PCR products were separated on a 1% agarose gel and stained with either ethidium bromide (Sigma-Aldrich Corp., St. Louis, MO, U.S.A) or SYBR Safe (Invitrogen, Carlsbad, CA, U.S.A.). For *12S* mtDNA, a 2% gel was used. Desired bands were excised from the gel under UV illumination and DNA extracted using the QIAquick gel extraction kit (Qiagen Inc.) following the manufacturer's instructions except that the elution buffer was heated to 70°C to increase yield at the final step. PCR products were sequenced in both directions by the Institut de Recherche Clinique de Montréal using a Genetic Analyzer 3130xl (Applied Biosystems, Foster City, CA, U.S.A.). Forward and reverse sequencing chromatograms were compiled into contigs, reconciled and edited in Geneious version 4.8.5 (Biomatters Ltd., 2009). Sequences were deposited in GenBank under accession numbers [submission to GenBank to be done].

The majority of sequences obtained in this study were amplified using DNA from single insect specimens. Amplification and sequencing failed for four amplicons but worked using DNA from a second insect specimen of the same species: *28S* and *CAD* for *Platyphora microspina*, *COI* for *Proseicela bicruciata* and *COII* for *Chrysolina coerulescens*. Two sequences were obtained from Genbank: *12S* for *Platyphora tangolita* (accession number AY055560) and *COI* for *Leptinotarsa decemlineata* (DQ649098).

Outgroup sequences, basal to the entire ingroup (Gómez-Zurita et al., 2008), were chosen among the closest taxa available in Genbank: *COI* and *28S* from *Timarcha tenebricosa* (Fabricius) (Chrysomelidae: Chrysomelinae: Timarchini; AY171412 and AY171439); *COII* from *Timarcha geniculata* Germar (AJ236336); and *12S* from *Prosopodonta limbata* (Chrysomelidae: Hispinae; AF097125). The outgroup sequence for *CAD* was a combination 1030 bp from *Prosopodonta limbata* (this study) and 606 bp from *Strangalia bicolor* (Swederus) (Cerambycidae: Lepturinae; GQ265599).

Protein coding genes, *COI*, *COII* and *CAD*, were aligned in Geneious by hand using translated segments. Alignment was trivial as there were no insertions or deletions in these gene regions. Sequences from the ribosomal gene *28S* were aligned in BioEdit version 7.1.7 (Hall, 1999) using the secondary structure model for Galerucinae and alignment methods developed by Gillespie *et al.* (2004). Regions of ambiguous alignment (RAAs) and regions of expansion and contraction (RECs) were excluded from the analysis, resulting in ~14% of each *28S* sequence being excluded. Ribosomal gene *12S* was aligned using 10 iterations of MUSCLE (Edgar, 2004) in Geneious with default settings. Bayesian phylogenies were inferred with MrBayes v.3.2.1 (Ronquist et al., 2012) on WestGrid high-performance computing facilities (Western Canada Research Grid). Analyses of single gene datasets (Figures S4.2 to S4.6 in the supplementary material) were done with 1 million generations, and the five-gene concatenated dataset was analysed with 10 million generations (Fig. 4.1). Convergence of runs and a suitable burn-in were determined using Tracer v.1.5 (Rambaut and Drummond, 2009): 5% for the concatenated dataset analysis and 10% for single-gene analyses. Maximum Likelihood (ML) phylogenies and 100 bootstrap iterations were inferred using RAxML-HPC (Stamatakis, 2006) on supercomputers of the Cyberinfrastructure for Phylogenetic Research (CIPRES) Science Gateway v.3.3 (Miller et al., 2010). For single gene analyses, the models of nucleotide substitution were determined with MrModeltest v.2.3 (Nylander, 2008) in Paup* v.4.0b10 (Swofford, 2003) using the PaupUP graphical interface (Calendini and Martin, 2005). For the dataset containing all concatenated sequences, the ideal partitioning strategy and models of nucleotide substitution were determined using PartitionFinder v.1.0.1 (Lanfear et al., 2012). This partition scheme was used for both the Bayesian and ML analyses. Twelve character sets were pre-defined as follows: all codon positions of all coding genes (*CAD*, *COI* and *COII*) (9 character sets total) and the stems of *28S*; non-pairing loops and arcs of *28S*, and the complete *12S* gene region. The ideal partitioning scheme determined using PartitionFinder divided the dataset in four partitions that included the following gene segments: Partition (1): first and second codon positions of *CAD*, *COI*, and *COII* and all regions of *28S* (except the excluded RAAs and RECs); Partition (2): third codon positions of *CAD*; Partition (3): third codon positions of *COI* and *COII*; Partition (4): *12S*. Data files (including alignments in nexus format) are available at the Dryad digital repository (http://datadryad.org) under DOI: [submission to Dryad will be done at a later date].

Ancestral states of larval behaviour and host plants were reconstructed using the StochChar module v.1.1 in Mesquite v.2.75 (build 564) (Maddison and Maddison, 2006, 2011). Characters states were reconstructed using the maximum *a posteriori* probability (MAP) Bayesian tree from the analysis of the concatenated gene dataset, since this represents the most probable and best resolved tree. Natural history traits were was reconstructed using ML under a Mk1 model of evolution and maximum parsimony (MP) methods. Characters we all pairs of independent categorical traits: 1) 'Gregarious' or 'not actively grouping'. 2) 'Cycloalexix' or 'not cycloalexic'. 3) 'Maternal care' or 'no maternal care'. 4) 'Ovoviviparous' or 'oviparous'. 5) Host plants as ten plant families or subfamilies.

4.4.RESULTS

Table 4.3: Species used in this study, including location of collection and the genes that were amplified for each taxon.

Species	**Database name**	***COI***	***COII***	***12S***	***28S***	***CAD 1***	***CAD 2***	***CAD 3***	**Country collected**
Calligrapha nupta Stål, 1859	Calligd57	×	×	×	×	×	×	×	Bolivia
Calligrapha suboculata Stål, 1859	Cgutogd31	×	×	×	×	×	×	×	Panama
Calomela sp.	Calom3973	×	×	×	×	×	×	×	Australia
Chrysolina coerulans (Scriba, 1791)	Ccoergd11	$\times^{1a}$	$\times^{1b}$	$\times^{1a}$	$\times^{1a}$	–	$\times^{1a}$	$\times^{1a}$	France
Chrysolina sp. 1	Chrysgd83	×	×	×	×	–	×	×	Mongolia
Chrysolina sp. 2	Chrysgd86	–	×	–	×	×	×	×	Mongolia
Chrysomela collaris Linnaeus, 1758	Ccollgd84	×	×	–	–	–	×	–	Mongolia
Cosmogramma kinbergi (Boheman,1858)	Ckinbgd34	×	×	×	×	×	×	×	Ecuador
Desmogramma subtropica Bechyně, 1946	Dsubtgd08	×	×	×	×	×	×	×	Panama
Doryphora paykulli (Stål, 1859)	Dpayk4569	×	×	×	×	×	×	×	Panama
Elytrosphaera xanthopyga Stål, 1858	Exant3925	×	–	×	×	–	×	–	Brazil
Labidomera clivicollis (Kirby, 1837)	Lclivgd44	×	×	–	×	×	×	×	Canada
Leptinotarsa decemlineata (Say, 1824)	Ldecegd45	$\times^{2}$	×	×	×	×	×	×	Canada
Leptinotarsa panamensis Tower, 1906	Lpanagd09	×	×	×	×	×	×	×	Panama
Phaedon semimarginatus Latreille, 1811	Psemigd15	×	×	×	×	×	×	×	Ecuador
Phaedon semimarginatus Latreille, 1811	Psemigd33	×	×	×	×	×	×	×	Ecuador
Plagiodera praecincta Erichson, 1847	Plagi8016	–	×	×	×	×	×	×	Bolivia
Plagiodera suturata (Stål, 1859)	Psutugd26	–	×	×	×	×	×	×	Ecuador
Plagiodera viridimaculata Jacoby, 1891	Pvirigd04	–	×	×	×	×	×	×	Panama
Platyphora (*Dorysterna*) *monticola* (Weise, 1916)	Pparagd59	×	×	×	×	×	×	–	Ecuador
Platyphora (*Dorysterna*) *paradoxa* (Achard, 1914)	Ewagngd24	×	×	×	×	×	×	×	Ecuador
Platyphora aestuans (L., 1758)	Paest3924	–	×	×	×	×	×	×	French Guiana
Platyphora aestuans (L., 1758)	Paestgd12	×	×	×	×	×	×	×	French Guiana
Platyphora anastomozans (Perty, 1832)	Panas3926	×	×	×	×	×	×	×	Brazil
Platyphora anastomozans (Perty, 1832)	Panas3927	×	×	×	×	–	×	–	Brazil
Platyphora adaequata (Bechyně, 1954)	Pastagd90	×	×	×	×	×	×	×	Panama
Platyphora aulica (Olivier, 1907)	Pauli3907	×	×	×	×	×	×	×	French Guiana
Platyphora aulica (Olivier, 1907)	Pauli7900	×	×	×	×	×	×	×	French Guiana
Platyphora bianca (Bechyně, 1954)	Pbian5232	–	×	×	×	×	×	×	Bolivia

Table 4.2. Continued.

Species	Database name	*COI*	*COII*	*12S*	*28S*	*CAD 1*	*CAD 2*	*CAD 3*	Country collected
Platyphora bifasciata (Fabricius, 1787)	Pbifagd14	×	×	×	×	–	×	×	French Guiana
Platyphora boucardi (Jacoby, 1883)	Pboucgd76	×	×	×	×	×	×	×	Panama
Platyphora decens (Stål, 1859)	Pdecegd48	×	×	×	×	×	×	×	Panama
Platyphora euchalca (Stål, 1859)	Pgrmegd63	×	×	×	×	×	×	–	Bolivia
Platyphora eucosma (Stål, 1858)	Peucogd47	×	×	×	×	×	×	×	Panama
Platyphora flavoannulata (Jacoby, 1903)	Pflavgd37	×	×	×	×	×	×	×	Panama
Platyphora flexuosa (Baly, 1858)	Pspecgd23	×	×	×	×	×	×	×	Ecuador
Platyphora fulgora (Stål, 1858)	Pfulggd43	×	×	×	×	×	×	×	Panama
Platyphora fulvicornis (Guérin-Méneville, 1855)	Pfulvgd02	×	×	×	×	×	×	×	Ecuador
Platyphora heliogena	Pheligd80	–	×	×	×	×	×	×	Ecuador
Platyphora imitans (Jacoby, 1903) "blue"	Pmeta7922	×	×	×	×	×	×	×	Bolivia
Platyphora imitans (Jacoby, 1903) "red"	Predagd58	×	×	×	×	×	×	×	Bolivia
Platyphora ligata (Stål, 1858)	Pligagd01	×	×	×	×	×	×	×	Ecuador
Platyphora ligata (Stål, 1858)	Pligagd82	×	×	×	×	×	×	×	Ecuador
Platyphora limbata (Guérin-Méneville, 1844)	Pboli7923	×	×	×	×	×	×	×	Bolivia
Platyphora luteipennis (Steinheil, 1877)	Plutegd32	–	×	×	×	×	×	×	Ecuador
Platyphora luteipennis (Steinheil, 1877)	Psalvgd29	×	×	×	×	×	×	×	Panama
Platyphora microspina Bechyně, 1954	Pmicr1456	×[1a]	×[1a]	×[1a]	×[1b]	×[1b]	×[1b]	×[1b]	Panama
Platyphora near *albovirens*	Palbogd07	×	×	×	×	×	×	×	Bolivia
Platyphora near *punctatissima*	Pbolwgd70	×	×	×	×	×	×	×	Bolivia
Platyphora nigronotata (Stål, 1857)	Pnigr3918	–	×	×	×	×	×	×	Brazil
Platyphora nigronotata (Stål, 1857)	Pnigr3919	×	×	×	×	×	×	×	Brazil
*Platyphora opima (*Stål, 1858)	Popimgd77	×	×	×	×	×	×	×	Panama
Platyphora punctipennis (Jacoby, 1878)	Ppuncgd78	×	×	×	×	×	×	×	Panama
Platyphora rubropunctata (Degeer, 1773)	Pbategd81	×	×	×	×	×	×	×	Ecuador
Platyphora sp. "checkered"	Pchecgd71	×	×	×	×	×	×	×	Bolivia
Platyphora tangolita (Bechyně, 1954)	Ptanggd40	×	×	×[2]	×	×	×	–	Panama
Platyphora testudo (Demay, 1838)	Pmegagd62	×	×	×	×	×	×	×	Bolivia
Platyphora transversoplagiata Jacoby, 1883	Ptrangd89	×	×	×	×	×	×	×	Panama
Platyphora vespertina (Baly, 1858)	Pvespgd46	–	×	×	×	–	×	×	Ecuador
Proseicela antenallis (Kirsch, 1883)	Pantegd21	×	×	×	×	×	×	×	Ecuador
Proseicela bicruciata Jacoby, 1880	Pbicrgd19	×[1b]	–	–	×[1a]	×[1a]	×[1a]	×[1a]	Ecuador

Table 4.2. Continued.

Species	Database name	*COI*	*COII*	*12S*	*28S*	*CAD 1*	*CAD 2*	*CAD 3*	Country collected
Proseicela flavipennis (Erichson, 1847)	Pflavgd17	×	×	×	×	×	×	×	Ecuador
Proseicela sp. nov. "Yasuni"	Pspnogd20	×	×	×	×	×	×	×	Ecuador
Proseicela spectabilis (Baly, 1858)	Pspecgd18	×	×	–	×	×	×	×	Ecuador
Proseicela vittata (Fabricius, 1781)	Pbivigd16	×	×	×	×	×	×	×	French Guiana
Stilodes (*Linographa*) *musicalis* (Stål, 1859)	Sbworgd65	×	×	×	×	–	×	×	Bolivia
Stilodes annuligera (Erichson, 1847)	Sannugd10	×	×	×	×	×	×	×	Bolivia
Stilodes duodecimmaculata (Stål, 1859)	Sduodgd13	×	×	×	×	–	×	×	Bolivia
Stilodes duodecimmaculata (Stål, 1859)	Sduodgd28	×	×	×	×	–	×	–	Bolivia
Stilodes fuscolineata (Stål, 1865)	Sfuscgd05	×	×	×	×	×	×	×	Panama
Stilodes motschulskyi (Stål, 1865)	Sleopgd68	×	×	×	×	×	×	×	Bolivia
Stilodes quatuordecimpustulata (Stål, 1859)	Sredggd56	×	×	×	×	–	×	×	Bolivia
Stilodes sladenae (Gahan, 1903)	Slarggd66	×	×	×	×	×	×	×	Bolivia
Stilodes sp. nov. "Campana"	Sspnogd36	×	×	×	×	×	×	×	Panama
Stilodes sp. nov. "Potrerillos 1"	Sjagugd74	×	×	×	×	×	×	×	Bolivia
Stilodes sp. nov. "Potrerillos 2"	Snrno8029	×	×	×	×	×	×	×	Bolivia
Zygogramma alternata (Kirsch, 1876)	Ssmalgd67	×	×	×	×	×	×	×	Bolivia
Zygogramma arcuigera (Stål, 1859)	Zarevgd30	×	×	×	×	×	×	×	Panama
Zygogramma hexagramma (Stål, 1859)	Zexaggd06	×	×	×	×	–	×	×	Panama
Percentage obtained (%)		87	97	94	99	85	100	91	

[1a] and [1b]: sequences obtained from two specimens;

[2]: sequences obtained from GenBank.

2.5.8. Concatenated gene analyses

Bayesian and Maximum Likelihood (ML) analyses of the complete concatenated gene dataset (except *28S* regions of ambiguity) generated similar topologies (Fig. 4.1 and S4.9). Subtribe Chrysomelina (clade A in Fig. 4.1) is monophyletic and separate from Chrysolinina + Doryphorina (*sensu* Daccordi, 1982). Subtribe Chrysomelina (*sensu* Daccordi, 1994) is polyphyletic with the Australian *Calomela* sp. placed in Chrysolinina (*sensu* Daccordi, 1994).

Genus *Chrysolina* (Chrysolinina) is sister to Doryphorina (*sensu* Daccordi, 1982) (clades B and D in Fig. 4.1) with high support values (Posterior Probability (PP) and bootstrap = 100). Subtribe Doryphorina (*sensu* Daccordi, 1982) is split in two clades, clade B contains the genera *Cosmogramma*, *Calligrapha*, *Stilodes* and *Zygogramma*, clade D contains the genera *Desmogramma*, *Doryphora*, *Elytrosphaera*, *Labidomera*, *Leptinotarsa*, *Platyphora* and *Proseicela* (PP and bootstrap ≥ 99).

Inside clade B, *Cosmogramma kinbergi* (Boheman) is sister to a paraphyletic genus *Calligrapha*, with *Zygogramma arcuigera* (Stål) nested within it (PP and bootstrap = 100). Genus *Stilodes* is paraphyletic with the rest of *Zygogramma* species nested within it, although the specific topology of these clades differed in ML and Bayesian analyses; this was the case in both analyses.

Clade D is composed of the polyphyletic genus *Platyphora* and genera *Desmogramma*, *Doryphora*, *Elytrosphaera*, *Labidomera*, *Leptinotarsa* and *Proseicela*. Clade D is divided into clades C and E (PP and bootstrap = 100), the first of these two clades, clade C, contains (*Desmogramma* + *Elytrosphaera*) sister to (18 spp. pf *Platyphora* + *Doryphora paykulli*). Clade E contains a polytomy of three branches: (*Platyphora aestuans*), (*Platyphora transversofasciata*, *Labidomera clivicollis, Leptinotarsa decemlineata* + *Leptinotarsa panamensis*) and (13 *Platyphora* spp. + 6 *Proseicela* spp.). This polytomy is resolved in the single-gene analysis of *CAD*, and places *Labidomera* and *Leptinotarsa* within that clade of *Platyphora* (Fig. S4.3). Clade F (Fig. 4.1) contains only Solanaceae-feeding species. *Proseicela* spp. form a monophyletic clade with *Platyphora microspina* (PP = 99 and bootstrap = 75), sister to *Platyphora anastomozans* (Perty) and *Platyphora nigronotata* (Stål) from Brazil (PP = 91 and bootstrap = 76). Together with *Leptinotarsa* spp., clade F represents the all Solanaceae-feeding Chrysomelinae sampled.

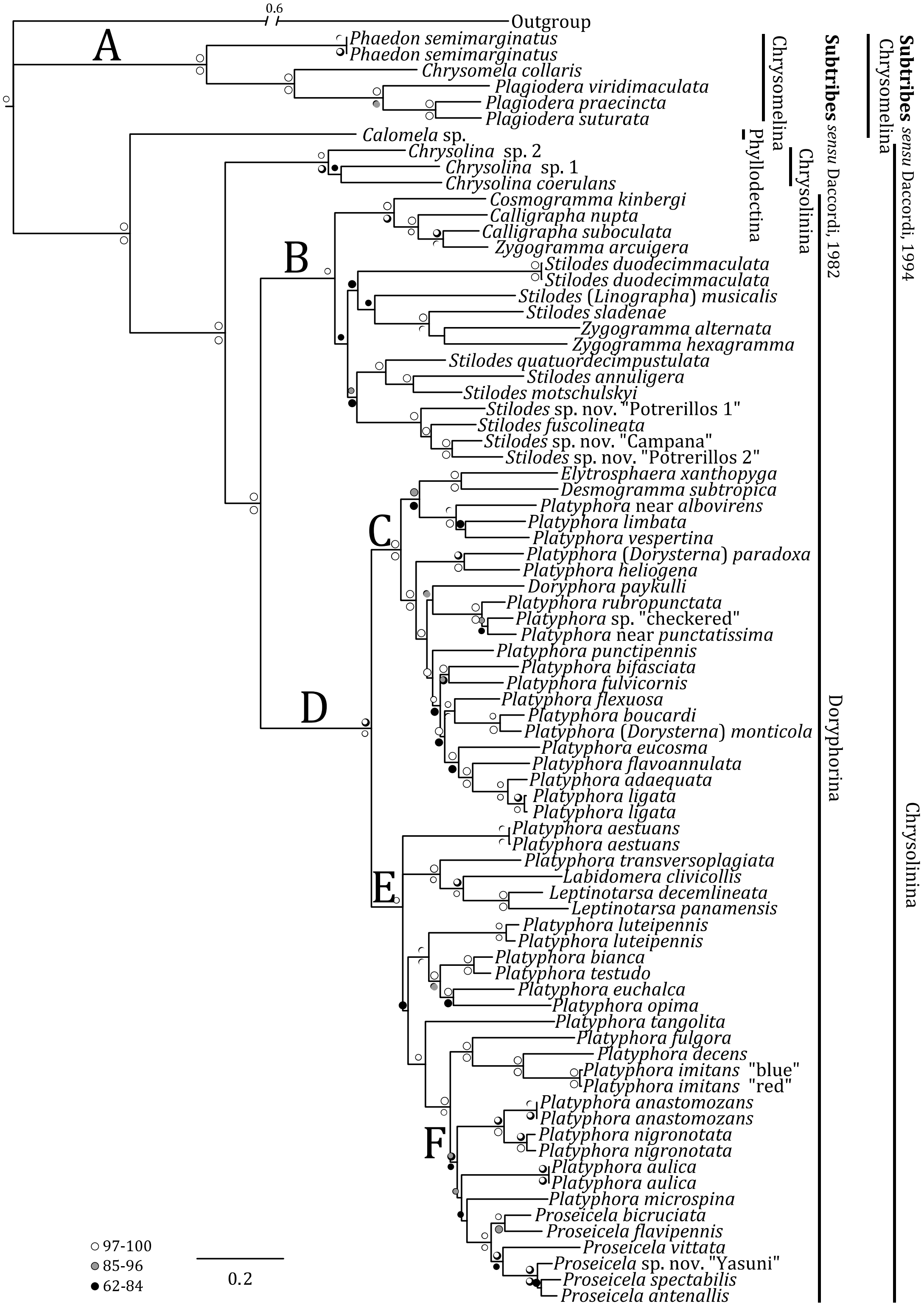

Figure 4.1: Topology and support values of the inferred phylogeny of Neotropical Chrysomelinae through Bayesian analysis of all genes concatenated (with regions of ambiguity from *28S* removed).

Posterior probabilities are represented by the circles on top of the nodes and bootstrap values from the likelihood analysis are represented by the circles below the nodes.

Individual gene analyses

The five gene segments were analyzed individually using Bayesian inference (Fig. S4.2 to S4.6). General topologies agreed between all gene segments. The topology of *COII* was most different (Fig. S4.5). Removal of *COII* segments from the concatenated dataset analysis did not significantly change the topology and do not affect the conclusions.

2.5.9. Ancestral larval behaviour reconstruction

Maximum Parsimony (MP) and ML reconstructions of ancestral larval behaviour indicate four independent origins of gregariousness (Fig. 4.2). If analyzed differently, namely if cycloalexy and maternal care are analyzed separately, there are five independent origins and no loss of gregariousness rather than four accompanied by one secondary loss.

The first origin of gregariousness is in the subtribe Chrysomelina (*sensu* Daccordi, 1982) represented in our study by genera *Phaedon*, *Chrysomela* and *Plagiodera*. The ancestor at the base of this branch likely had solitary larvae (ML: 0.73 solitary versus 0.27 gregarious or 0.64 and 0.36 if the outgroup is considered gregarious), while the common ancestor at the node was almost certainly gregarious (ML: 0.09 solitary versus 0.91* gregarious). The second independent origin of gregarious larvae is in *Doryphora paykulli* Stål; this species has maternal care and larvae that rest together tightly with heads towards the inside of the aggregation. This represents a clearly independent origin as the ancestor at the node has a high likelihood of having been solitary (ML: >0.99* solitary). *Doryphora paykulli* Stål also represents the first independent origin of maternal care (Fig. S4.7). *Platyphora eucosma* (Stål) larvae are gregarious. This represents the third evolutionary origin of gregariousness in our analysis, closely related species all have solitary larvae and larvae of the ancestor at the node were also predicted to have been solitary (ML: >0.99* solitary).

The next origin or origins of gregariousness are in a Solanaceae-feeding clade (clade F in Figure 4.1); the MP analysis is unclear as to whether this represents one or two origins of gregariousness. The ML analysis considers it is more likely a single origin followed by a loss (Fig. 4.2). *Platyphora anastomozans* (Perty) and *Platyphora* *nigronotata* (Stål) are the first of these two possible origins. The ancestor at the base of this clade's branch was likely gregarious (ML: 0.35 solitary versus 0.65 gregarious). These species have cycloalexic behaviour with heads pointing outwards, without maternal care and represent the only evolutionary origin of cycloalexy in our ingroup (Fig. S4.8) (Vasconcellos-Neto and Jolivet, 1994, Medeiros, 1991). The second possible origin of gregarious larvae is in the clade composed of *Platyphora microspina* Bechyně and 6 spp. of the genus *Proseicela*. The ancestor at the base of this clade was likely gregarious (ML: 0.17 solitary versus 0.84 gregarious), all these species rest in a tight circle with head pointing inwards and with maternal care. This clade represents the second independent origin of maternal care in our in-group (Fig. S4.7). In between these two possible origins of larval gregariousness is *Platyphora aulica* Olivier, a species with solitary larvae, whose ancestor at the base of the branch was likely gregarious (ML: 0.26 solitary versus 0.74 gregarious) (Fig. 4.2).

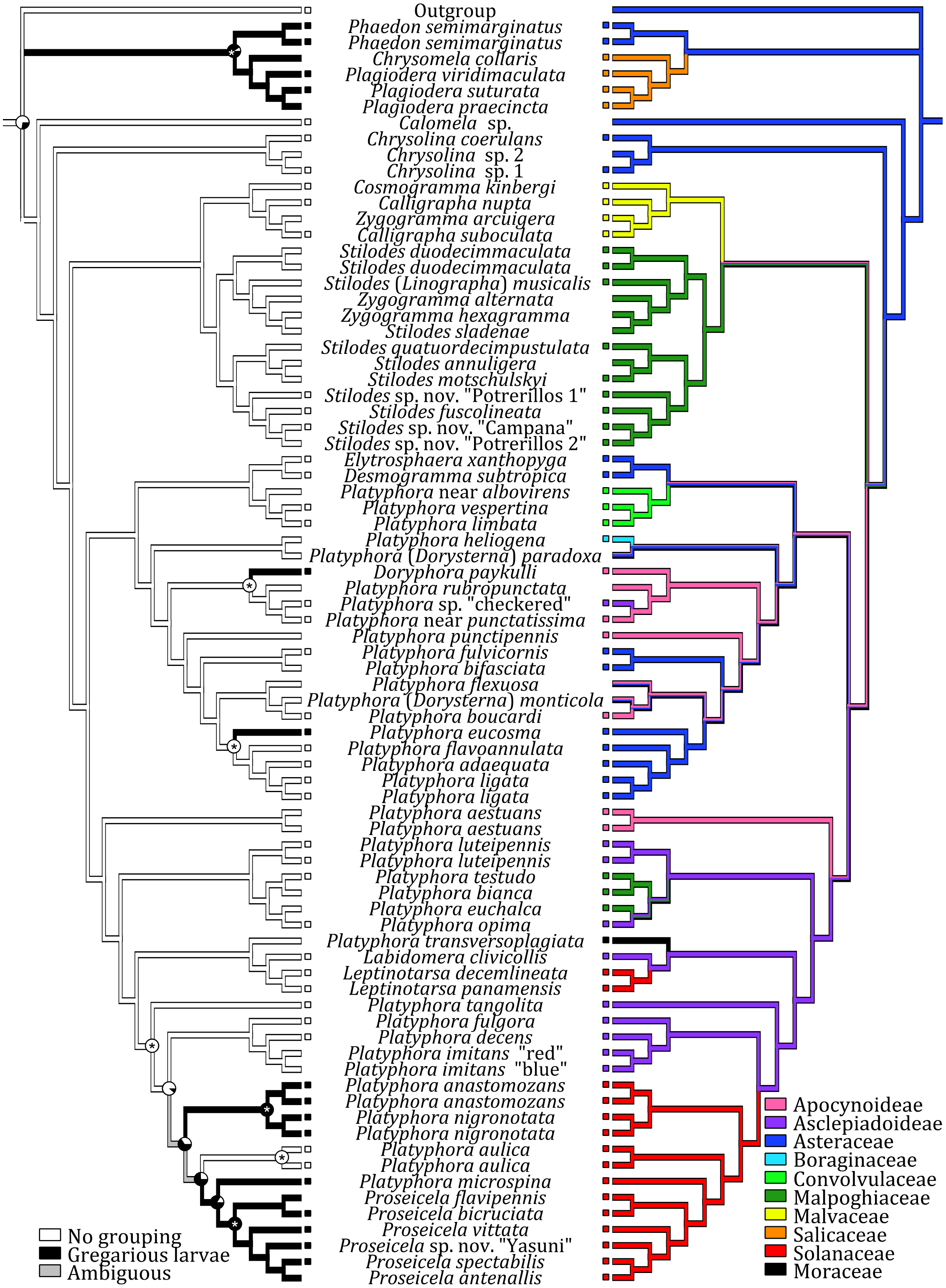

Figure 4.2: Cladogram representing the reconstruction of ancestral larval behaviour on maximum a posteriori probability (MAP) Bayesian tree from analysis of the concatenated gene dataset.

Colours on branches are from Maximum Parsimony reconstruction, pie charts at nodes represent Maximum Likelihood with Mk1 model of either state in the ancestor.

4.5. DISCUSSION

4.5.1. Taxonomic implications

The most recent taxonomic treatment of the Chrysomelinae divides the subfamily into two tribes: (1) the monotypic Timarchini and (2) the Chrysomelini, that is further divided into four to twelve subtribes. Daccordi (1982) divided the Chrysomelini in twelve subtribes: Barymelina, Doryphorina, Chrysolinina, Monarditina, Chrysomelina, Hispostomina, Dicranosternina, Paropsina, Gonioctenina, Phyllodectina, Phyllocharina and Entomoscelina. Daccordi (1994) merged several of the subtribes a proposed to keep only four: Entomoscelina, Paropsina, Chrysolinina and Chrysomelina. Our study has included taxa from four of these subtribes (*sensu* Daccordi, 1982): Phyllodectina, Doryphorina, Chrysomelina, and Chrysolinina (Fig. 4.1).

Chrysomelina (*sensu* Daccordi, 1982) is monophyletic, with *Phaedon* sister to (*Chrysomela* + *Plagiodera*) (clade A, Fig. 4.1). This agrees with prior phylogenetic analysis of several species and genera of this subtribe, which was done using segments of *COI*, *COII*, *12S* and *16S* (Termonia et al., 2001). Since our specimens of *Chrysomela collaris* were from Mongolia and the specimens of *Phaedon* and *Plagiodera* were all South-American, from Bolivia and Ecuador, this suggests a global range of Chrysomelina that later split through vicariance events. This idea is also supported by some of the maps in Daccordi (1994). On the other hand, subtribe Chrysomelina (*sensu* Daccordi, 1994) is paraphyletic because of the Australian taxon (*Calomela* sp.), found to be sister to the subtribe Chrysolilina. Before being placed in Chrysomelina, most of the Autralian taxa, including *Calomela*, were placed in the subtribe Phyllodectina (*sensu* Daccordi, 1982). Daccordi (1994) and Weise (1915) both indicated that the analysis of more Australian taxa will prove Phyllodectina artificial—not based on evolutionary history but on convergent traits. Both may be correct, however, Daccordi's (1994) proposition is found to be imperfect. Further analyses of Palearctic Chrysolinina and Australian Phyllodectina (*sensu* Daccordi, 1982) specimens will allow clarification of the different subtribes of Chrysomelini.

Subtribe Chrysolinina (*sensu* Daccordi, 1982), represented in our study by the genus *Chrysolina*, is monophyletic and sister to Doryphorina (*sensu* Daccordi, 1982). This subtribe was merged into Chrysolinina by Daccordi (1994). In our study

Doryphorina (*sensu* Daccordi, 1982) is found to be monophyletic (clade D, Fig. 4.1); based on this, we suggest that Doryphorina maintain its subtribe status.

Doryphorina is separated into clades B and D (Fig. 4.1). Clade B contains (*Cosmogramma* + *Calligrapha*) sister to (*Stilodes* + *Zygogramma*). Our results strongly suggest *Zygogramma arcuigera* (Stål) should be *Calligrapha arcuigera* (Stål), which would result in a monophyletic *Calligrapha*. Though Bayesian support for these branches is relatively low (75 ± 5), our analysis suggests that *Stilodes* is paraphyletic with *Zygogramma* nested within. Clade D is itself divided into two clades: clade C ((*Desmogramma* + *Elytrosphaera*) + (*Doryphora* + *Platyphora*)) and clade E (*Labidomera* + *Leptinotarsa* + *Platyphora* + *Proseicela*) (Fig. 4.1). In its current state, *Platyphora* is polyphyletic.

These results are not surprising, since Daccordi (personal comm. in Pasteels et al., 2004) already noted that the genera of Neotropical chrysomelines are badly in need of revision. However, this task in hindered by frequent convergent evolution in the subfamily (Daccordi *et al.*, 1999). An example of this is in the genera *Stilodes* Chevrolat and *Leptinotarsa* Stål. Based on morphological criteria, Flowers (2004) proposed to synonymise them. This hypothesis is rejected based on molecular evidence from nine *Stilodes* species and two *Leptinotarsa* species, which shows that *Stilodes* and *Leptinotarsa* clearly fall in different subclades of Doryphorina. This means that *Stilodes decemlineata* (Say) is not a valid combination and, fortunately for the applied entomological literature, *Leptinotarsa decemlineata* (Say) remains valid. Another example of convergence is the presence or absence of a mesosternal horn. The mesosternum of *Platyphora* and *Doryphora* is modified into a forward projecting horn or spine (Bechyně and Springlova de Bechyně, 1965, Flowers, 2004). Our phylogeny suggests that this horn is ancestral in the clade, but was secondarily lost in (*Desmogramma* + *Elytrosphaera*), in (*Labidomera* + *Leptinotarsa*), and in *Proseicela*. The mesosternum of *Proseicela* species is produced into a short lobe (Daccordi *et al.*, 1999). It is, thus, logical that *Platyphora microspina* Bechyně, named for the diminutive size of its sternal horn, is sister to *Proseicela*. The function of the sternal horn is still unclear but Eberhard (1981) has observed male *Doryphora* sp. near *punctatissima* using it in male aggressive behaviours. Females also have a horn, which could help them compete for food plants, although this has not been observed (Eberhard, 1981).

Chemical analysis of Chrysomelinae defensive secretions may also be an important trait in understanding Chrysomelinae evolution, as suggested by Pasteels (1993). The defensive secretions of several of the insect taxa studied here have been characterized. Species in subtribe Chrysomelina (*sensu* Daccordi, 1982) secrete nitropropanoic acid and isoxazolinone glucosides; this is true for *Plagiodera* spp., including *Plagiodera viridimaculata* Jacoby, *Phaedon semimarginatus* (Latreille) and *Chrysomela* spp. (Pasteels et al., 1994, 2004). Cardenolides, or polyoxygenated steroids, are secreted by *Calligrapha*, *Chrysolina* (*sensu* Daccordi, 1994), *Cosmogramma*, *Stilodes*, and *Zygogramma* (Daloze et al., 1991, 1995, Pasteels et al., 1982, 2003a, Timmermans et al., 1992). All of these species are in the monophyletic clade B of the phylogeny (Fig. 4.1). All other species of clade D studied secrete triterpene saponins (Fig. 4.1) (Timmermans et al., 1992, Pasteels et al., 2001, 2003a, 2004, Plasman et al., 2000a, 2000b). Preliminary analysis of *Proseicela antennalis* (Kirsch) secretions tentatively detected cardenolides and no triterpene saponins (Pasteels et al., 2004). In the light of *Proseicela*'s phylogenetic placement, *Proseicela* secretions should be studied again when sufficient quantities are available (J. Pasteels, personal comm. March 2013).

The defensive secretions were studied in several *Platyphora* species (Termonia et al., 2002). A clear clade was inferred in which most beetle species sequester pyrrolizidine alkaloids (PA) and metabolize pentacyclic triterpene saponins from sequestered plant amyrins (Termonia et al., 2002). We identified a similar topology for the clade with five of the same species included in our study (*P. vespertina*, *P. heliogena, P. boucardi*, *P. adaequata* = their *P. ligata* and *P. eucosma*). Our topology for species that do not sequester PA is distinct from the one they obtained (*P. transversoplagiata* = their *P. salviny*, *P. tangolita* = their *P. decorata*, *P. microspina* and *P. opima*) (Fig. 4.1). Although the topologies of the trees differ for species that do not secrete PA, the main findings of Termonia et al. (2002) remain valid: "dual sequestration could be the key mechanistic means by which transitions among ecological specializations (i.e. restricted host-plant affiliations) are made possible." Overall, these chemical traits support the molecular taxonomic classifications in the Chrysomelinae.

4.5.2. Behaviour evolution

Gregariousness is found in several clades of Neotropical Chrysomelinae (Vasconcellos-Neto and Jolivet, 1994). Some of these gregarious species form tight rosettes at rest, with either heads or abdomens at the periphery; these species are considered cycloalexic (Jolivet et al., 1990). However, it is sometimes difficult to discern strict cycloalexy from general gregarious behaviour. In this study we define cycloalexy as per Jolivet et al. (1990) as "the attitude adopted at rest by some insect larvae, both diurnal and nocturnal, in a tight circle where either the heads or ends of the abdomen are juxtaposed at the periphery, with the remaining larvae at the centre of the circle. Coordinated movements such as the adoption of threatening attitudes, regurgitation, and biting, are used to repel predators or parasitoids." Through reconstruction of ancestral behavioural states of larvae, we find that active grouping, including but not limited to cycloalexy, has evolved at least four times in Neotropical Chrysomelinae (Fig. 4.2).

The most ancient origin of active grouping is found in the subtribe Chrysomelina, represented in our analysis by species of *Plagiodera*, *Phaedon* and *Chrysomela* (Fig. 4.2). The species we included are not cycloalexic and provide no maternal care. In *Plagiodera versicolora*, group size did not affect survival, but larger groups have larger larvae, likely through feeding facilitation (Breden and Wade, 1987). This suggests that predation is the evolutionary pressure that led to gregariousness in this clade and points to feeding facilitation instead.

Doryphora paykulli Stål and *D. reticulata* (Fabricius, 1787) larvae group in a tight circle at rest, with heads pointing inwards (Windsor et al., 2013). Larvae of these species do not as a group repel parasitoid or predator threats, thus, their behaviour does not qualify as cycloalexic. Both species feed on *Prestonia* species (Apocynaceae: Apocynoideae), are oviparous, and have maternal care (Windsor et al., 2013). Therefore, maternal care and gregarious larvae have evolved in *Doryphora paykulli* Stål and both represent independent origins (Fig. 4.2 and S4.7).

Larvae of *Platyphora eucosma* (Stål) are also gregarious from the time they hatch until they pupate. The females lay clusters of eggs on large Asteraceae (*Critonia morifolia* (Mill.) or *Koanophyllon* sp.). Gregariousness in *Pl. eucosma* evolved independently (Fig. 4.2). At rest, they sometimes form relatively circular aggregations with heads pointing inwards; the larvae are definitely gregarious but their behaviour is not clear cycloalexy because the grouping is diffuse and not clearly defensive.

In genus *Platyphora*, *Pl. anastomozans* and *Pl. nigronotata*, are Solanaceae-feeding species and exhibit cycloalexy with heads outwards (Vasconcellos-Neto and Jolivet, 1994, Medeiros, 1991). This represents and an origin of larval gregariousness and the only origin of cycloalexy in our ingroup species (Fig. 4.2 and S4.8). Adult females of these species are ovoviviparous, depositing groups of larvae and abandoning them (Vasconcellos-Neto and Jolivet, 1994, Medeiros, 1991). Several other *Platyphora* species share similar natural history traits of cycloalexy, Solanaceae-feeding, ovoviviparity and absence of maternal care: *Pl. conviva* (Stål, 1858), *Pl. nitidissima* (Stål, 1857) *Pl. fasciatomaculata* (Stål, 1857) and *Pl. vinula* (Stål, 1858) (Medeiros and Vasconcellos-Neto, 1994, Vasconcellos-Neto and Jolivet, 1994). This suggests that these are closely related to *Pl. anastomozans* and *Pl. nigronotata*, since no other species of *Platyphora* sampled share these characteristics. From our ancestral state reconstruction, it is clear that cycloalexy has an evolutionary origin in Solanaceae-feeding *Platyphora*. It remains unclear whether there are one or two evolutionary origins of larval gregariousness in this clade; ML reconstruction suggests one origin accompanied by a secondary loss is most likely (Fig. 4.2).

The second potential origin of gregariousness is in the (*Platyphora microspina* + *Proseicela* spp.) clade. These species are all ovoviviparous and feed on Solanaceae but, unlike the cycloalexic *Platyphora* spp., the larvae rest with their heads inwards and are protected by their mother (Windsor et al., 2013). The (*Pl. microspina* + *Proseicela*) clade represents an independent origin of maternal care (Fig. S4.7). We favour the evolution of gregariousness as independent in this clade because the types of gregariousness associated with the two clades differ: the larvae of (*Pl. anastomozans* + *Pl. nigronotata*) and receive no care from their mother, while (*Pl. microspina* + *Proseicela*) larvae rest with heads inwards and receive maternal care. In all other ingroup taxa, cycloalexy and maternal care do not appear labile and both are always associated with gregarious larvae.

Platyphora aulica is sister species to (*Pl. microspina* + *Proseicela*). All these species, (*Pl. aulica* (*Pl. microspina* + *Proseicela*), are sister to (*Pl. anastomozans* + *Pl.*

nigronotata), however, *Pl. aulica* females do not care for their larvae which are not cycloalexic. The placement of *Pl. aulica* and their behaviour support two separate evolutionary origins of larval gregariousness.

More Solanaceae-feeding species have cycloalexy or maternal care, but could not be added to our ingroup: *Platyphora selva* Daccordi, 1993 feeds on *Lycianthes* (*Witheringia*) *heteroclita* Sendtm. in the Atlantic lowlands of Costa-Rica (Choe, 1989, Daccordi, 1993). This species has maternal care and larvae group tightly with heads inwards and abdomens at the periphery of the ring (Choe, 1989). This set of behaviour is also found in (*Pl. microspina* + *Proseicela*), suggesting that *Pl. selva* may be closely related to the clade. *Proseicela crucigera* (Sahlberg) is reported to be cycloalexic and, like other species of *Proseicela* larvae, point their heads inwards (Vasconcellos-Neto and Jolivet, 1994, Medeiros, 1991). Contrary to all other known *Proseicela crucigera*, females do not care for their larvae in this species (J. Vasconcellos-Neto, personal communication, Windsor et al., 2013). This probably represents a secondary loss of maternal care in the clade since the species lacks a mesosternal horn while *Pl. microspina*, sister to all *Proseicela* sampled (Fig. 4.1), has both maternal care and a short sternal horn.

Larvae of *Eugonycha melanostoma* (Stål) exhibit cycloalexy by forming a tight rosette with heads inward, the larvae cover each other with faeces and trichomes from their host plant *Solanum lycocarpum* A.St.-Hil. (Solanaceae) (Vasconcellos-Neto and Jolivet, 1994). The only other cycloalexic species known to use trichomes is *Platyphora conviva* (Stål) (Vasconcellos-Neto and Jolivet, 1994). Unfortunately lacking fresh DNA, we could not include *Pl. conviva* or *E. melanostoma* or other species in the genus *Eugonycha*. Based on taxonomic, morphological and behavioural differences, cycloalexy in *E. melanostoma* probably represents an independent evolutionary origin. Taxonomically *Eugonycha* is a separate genus from *Platyphora* and *Proseicela*. Morphologically it lacks a mesosternal horn as in *Platyphora* spp. or a short lobe as in *Proseicela*. Behaviourally unlike cycloalexic *Platyphora*, *E. melanostoma* larvae rest with heads inwards, and unlike *Proseicela* the larvae do not receive maternal care (Vasconcellos-Neto and Jolivet, 1994, Medeiros, 1991, Windsor et al., 2013).

The occurrence of three probable independent origins of gregariousness in Solanaceae-feeding taxa suggests that eating Solanaceae exerts selective pressures that favour increased defence of the larvae—leading to cycloalexy or maternal care. Preemptive defensive formations of chemically defended larvae presumably have a positive effect on survival in the presence of predators, especially ants and bugs, and parasitoids (Weinstein, 1989, Vasconcellos-Neto and Jolivet, 1994). The occurrence of cycloalexy in broad shouldered leaf beetles (Chrysomelinae) is sometimes linked to ant-plant mutualisms (Vasconcellos-Neto and Jolivet, 1994, Choe, 1989). Since ants are among the main predators of leaf beetle larvae, this suggests that cycloalexy may be linked to defence (Medeiros et al., 1996, Costa, 2006, Vasconcellos-Neto and Jolivet, 1994, Choe, 1989). For example, if the circle of *Platyphora conviva* larvae is broken, predatory ants readily attack the larvae (Jolivet et al., 1990). One possible explanation for the selective pressures on Solanaceae-feeding taxa would be the presence of ants that visit Solanaceae to feed from extrafloral nectaries and opportunistically hunt any herbivores on the plants, including leaf beetle larvae (Vasconcellos-Neto and Jolivet, 1994). Ecological and behavioural studies are still needed to prove this, keeping in mind that the related *Leptinotarsa* feed on Solanaceae, sometimes have gregarious larvae but are not cycloalexic and are not defended by adult females (Hsiao, 1988).

Larval gregariousness has evolved five times in the Neotropical Chrysomelinae. Once in the subtribe Chrysomelina, twice in genus *Platyphora*, once in genus *Doryphora* and once in genus *Proseicela* (Fig. 4.2). Clear Chrysomelinae cycloalexy has one independent origin in the Neotropical genus *Platyphora* (Fig. S4.7), but a minority of species in the genus are cycloalexic. *Eugonycha* is also reported to show cycloalexy, likely a second evolutionary origin. Cycloalexy has appeared in species whose close relatives have solitary larvae; the sister species to clade F are the solitary (*Pl. imitans* + *Pl. decens* + *Pl. fulgora*), inside clade F, *Pl. aulica* has solitary larvae, while (*Pl. microspina* + *Proseicela* spp.) have gregarious larvae with maternal care, this suggests that a gregarious ancestor may not be a requirement for cycloalexy to evolve. Circular grouping which we consider is not cycloalexy and maternal defence are found in the genera *Doryphora* and *Proseicela*. Both represent a separate evolutionary origin of maternal care (Fig. S4.8).

4.6. CONCLUSION

Using a five-gene molecular phylogeny, we revealed the phylogenetic relationships among 80 ingroup taxa of largely Neotropical Chrysomelinae. The resulting phylogenetic hypothesis identified problems with the most recent arrangement of Chrysomelinae genera and subfamilies. *Stilodes* was paraphyletic with respect to *Zygogramma*. Genus *Platyphora* was polyphyletic and clearly divided into two clades, one with *Doryphora* nested within and sister to *Desmogramma* and *Elytrosphaera*; the other clade a polytomy between *Labidomera, Leptinotarsa* and other *Platyphora* species in which the genus *Proseicela* was nested. Our phylogeny underscores the need for a revision of New World Chrysomelinae genera, especially *Platyphora*.

ML and MP reconstructions of ancestral larval behaviour showed five independent evolutionary origins of larval gregariousness, two origins of maternal care and one origin of cycloalexy (Fig. 4.2, S4.7 and S4.8). Leaf beetles often use chemicals for defence (Pasteels et al., 1994, Pasteels et al., 2003d); these chemicals may be sequestered from plant compounds, metabolized, or both. Chemical defence is often linked to group defensive behaviour. A clear relationship between host plant and cycloalexy or maternal care is not evident from this data. Further ecological studies are needed to clarify the evolutionary pressures leading to cycloalexy and maternal care, and to show if and how predator and parasitoid pressures influence behaviour.

SUPPLEMENTARY MATERIAL

Table S4.1: Polymerase chain reaction (PCR) cycling conditions used to amplify the selected gene segments.

All amplifications began with an initial 2 minute denaturation step at 94°C and ended with a final extension step of 5 minutes at 72°C.

Gene	Cycling conditions			
	Steps*	Temperature (°C)	Time (s)	Cycles
12S	Denaturation	93	35	45
	Annealing	55	35	
	Extension	72	60	
CAD amplicon for nested PCR	Denaturation	94	35	45
	Annealing	53	60	
	Extension	72	120	
28S & *CAD* segment 2 (a) *CAD* segments 1 & 3 (b)	Denaturation	95	30	45
	Annealing	53 (a) or 52 (b)	60	
	Extension	72	60	
COII	Denaturation	94	25	45
	Annealing	52	60	
	Extension	72	60	
COI	Denaturation	94	30	10
	Annealing	46	30	
	Extension	72	30	
	Denaturation	94	30	30
	Annealing	48	30	
	Extension	72	40	

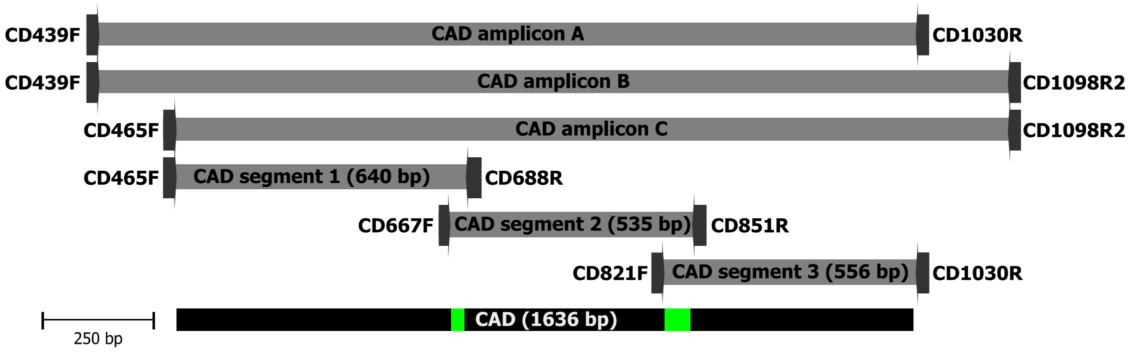

Figure S4.1: *CAD* (*Carbamoyl-phosphate synthetase 2, Aspartate transcarbamylase and Dihydroorotase*) segments used in this study and their respective primers (to scale).
The three different amplicons used for fully-nested PCR amplification are represented in pale grey; three *CAD* gene segments are in dark grey; the combined *CAD* sequence is represented in black, with internal segment overlap in green.

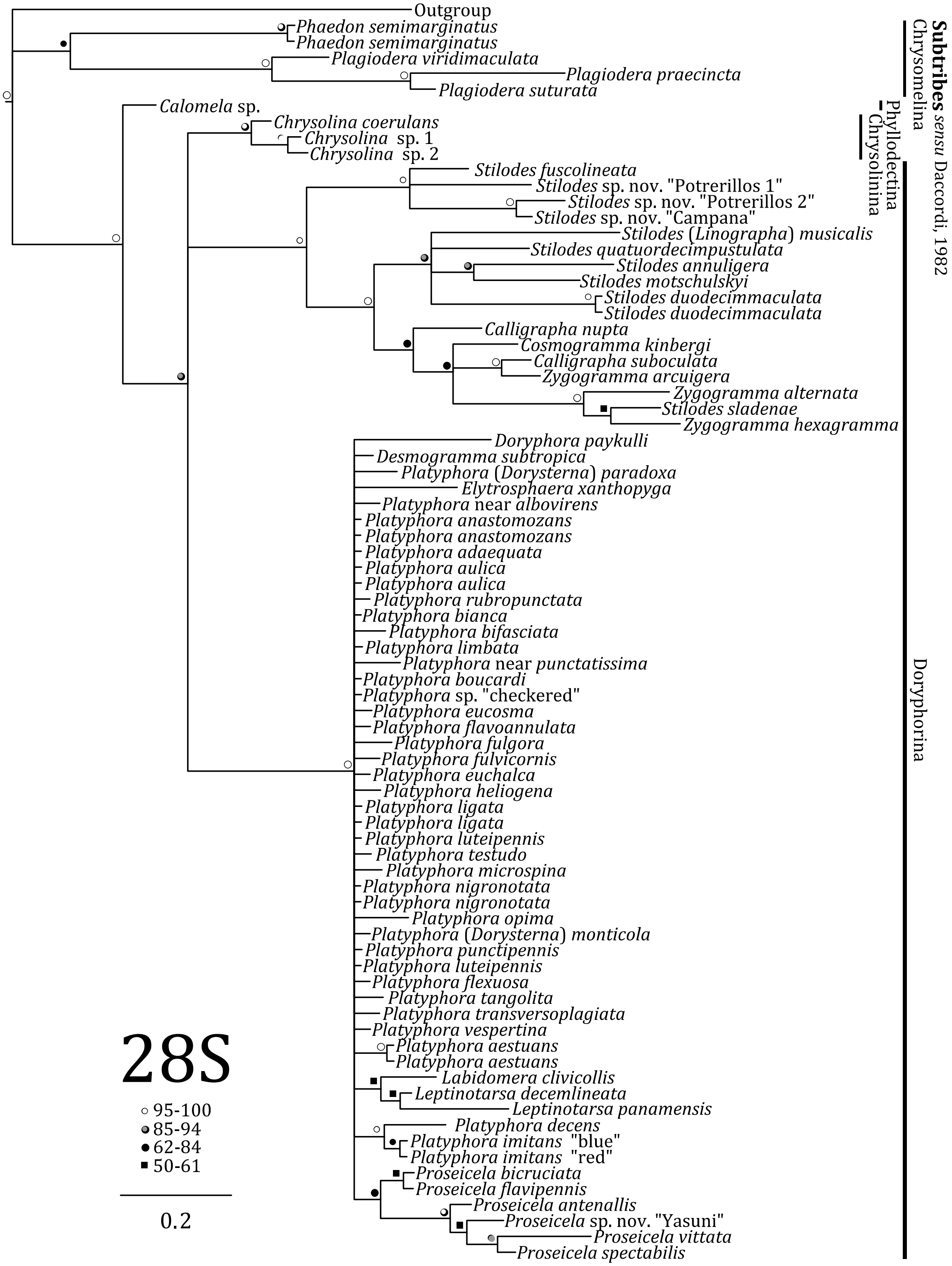

Figure S4.2: Topology and posterior probabilities values of the inferred phylogeny of Neotropical Chrysomelinae through Bayesian analysis of nuclear *28S* ribosomal DNA.

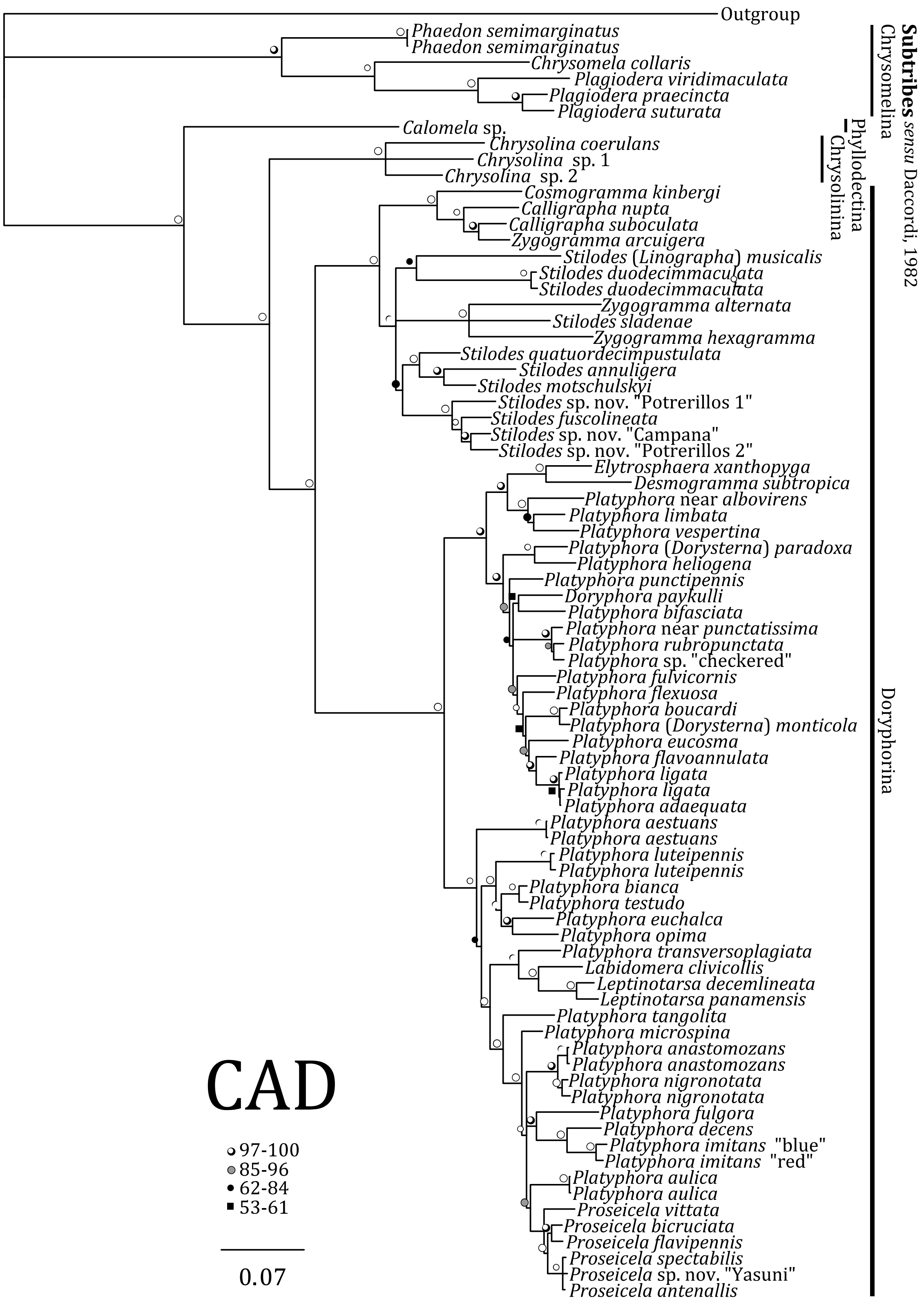

Figure S4.3: Topology and posterior probabilities values of the inferred phylogeny of Neotropical Chrysomelinae through Bayesian analysis of the nuclear protein-coding *CAD* (*Carbamoyl-phosphate synthetase 2, Aspartate transcarbamylase and Dihydroorotase*) segments.

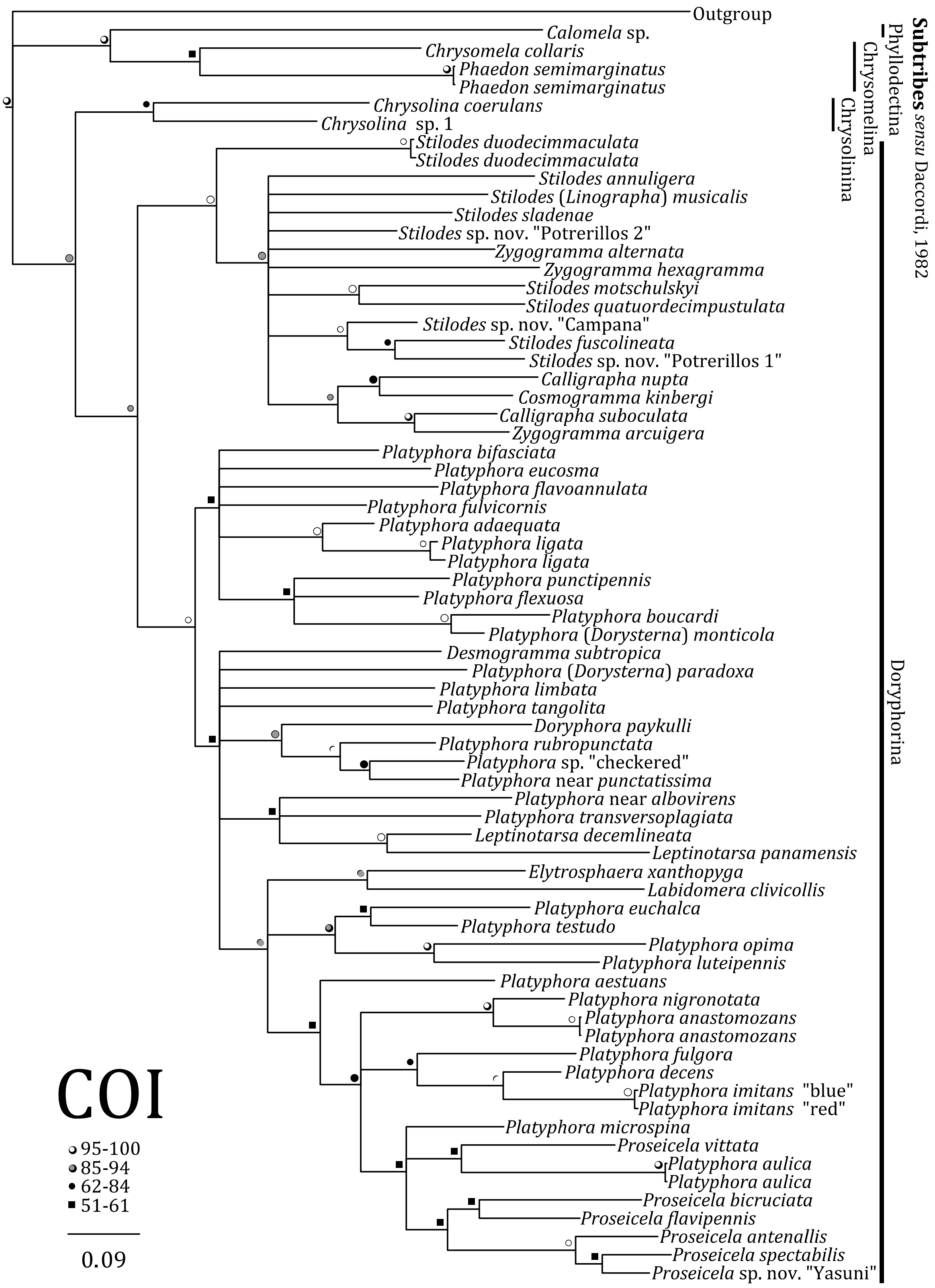

Figure S4.4: Topology and posterior probabilities values of the inferred phylogeny of Neotropical Chrysomelinae through Bayesian analysis of mitochondrial protein-coding *cytochrome oxidase I* (*COI*).

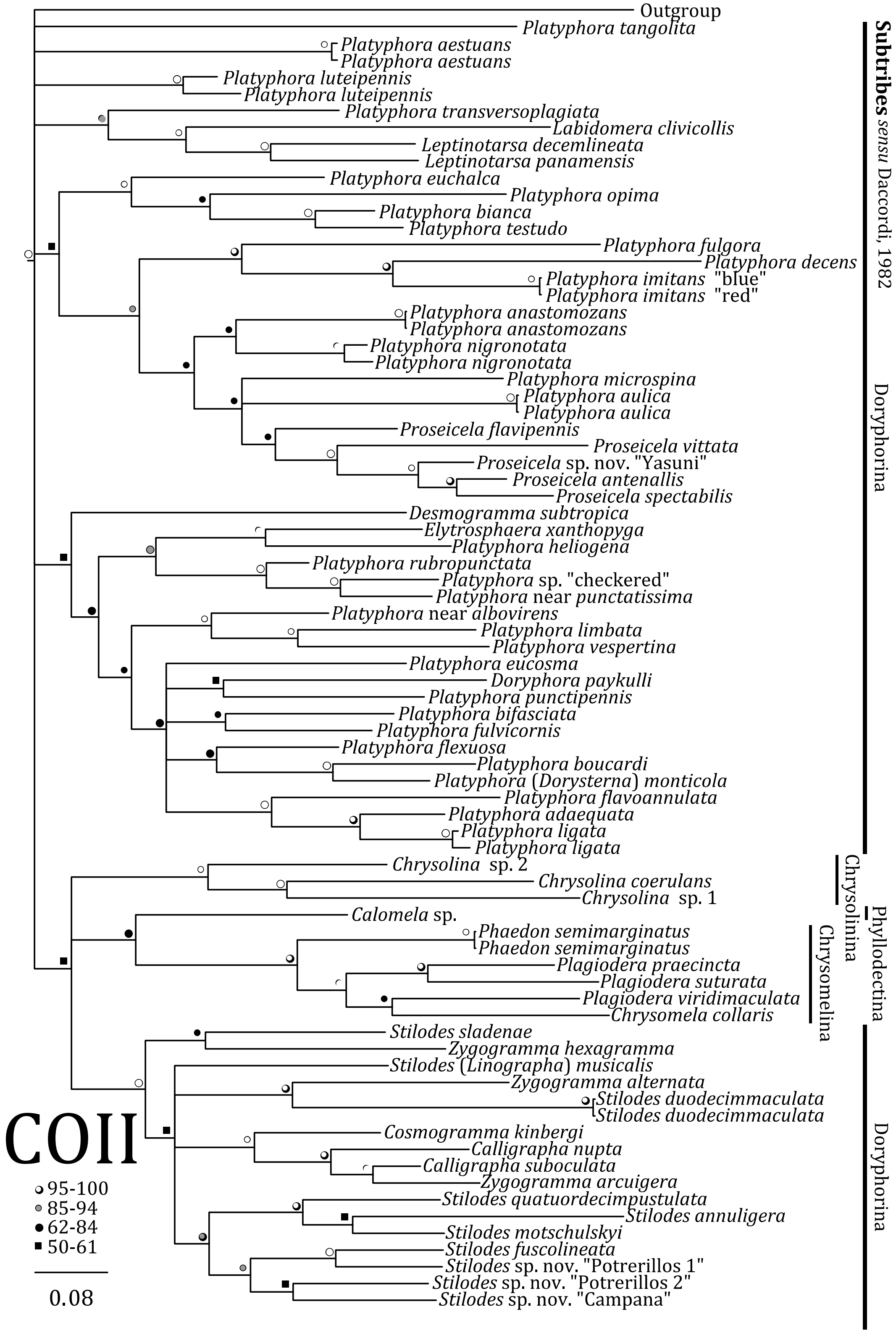

Figure S4.5: Topology and posterior probabilities values of the inferred phylogeny of Neotropical Chrysomelinae through Bayesian analysis of mitochondrial protein-coding *cytochrome oxidase II* (*COII*).

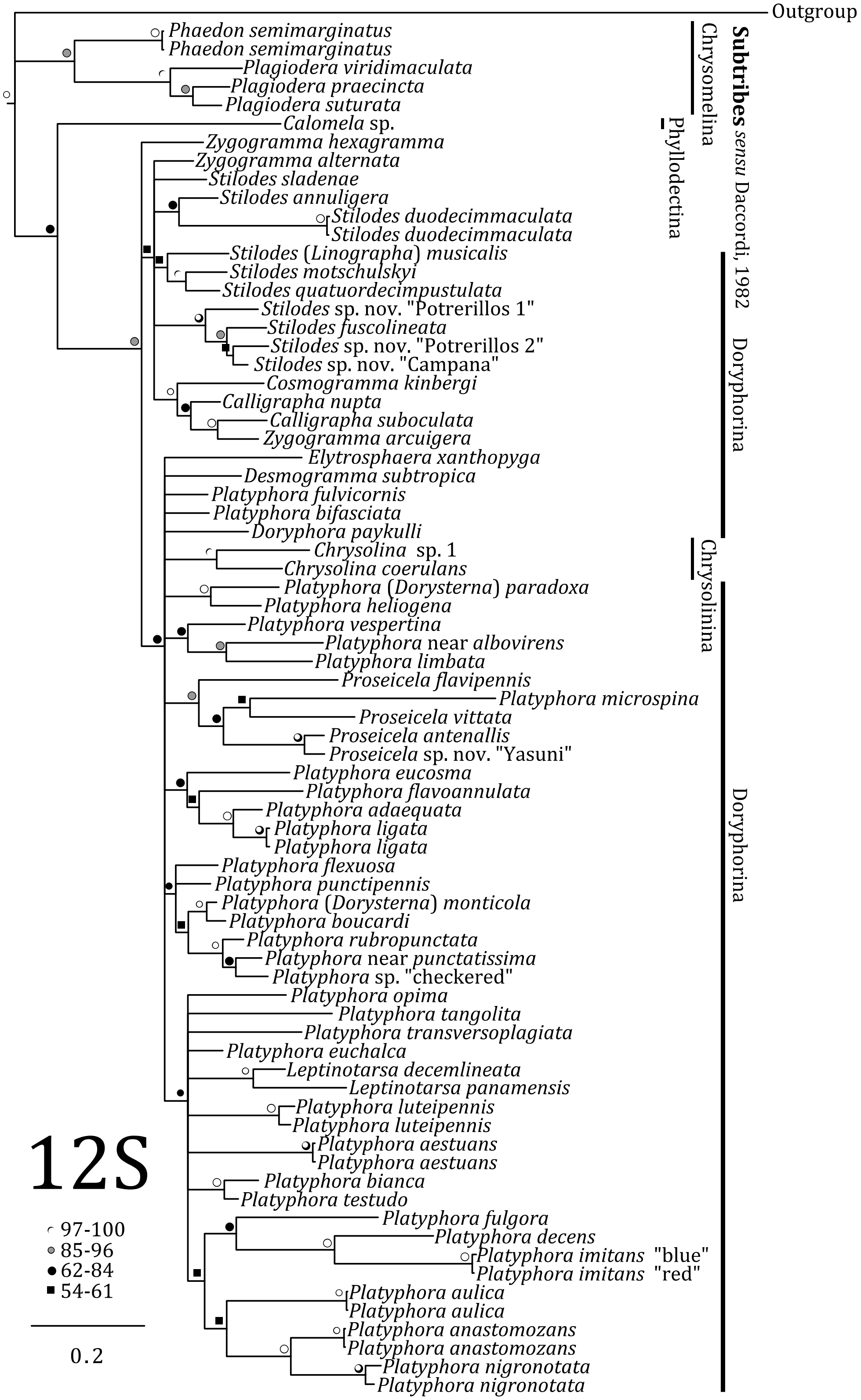

Figure S4.6: Topology and posterior probabilities values of the inferred phylogeny of Neotropical Chrysomelinae through Bayesian analysis of mitochondrial *12S* ribosomal DNA.

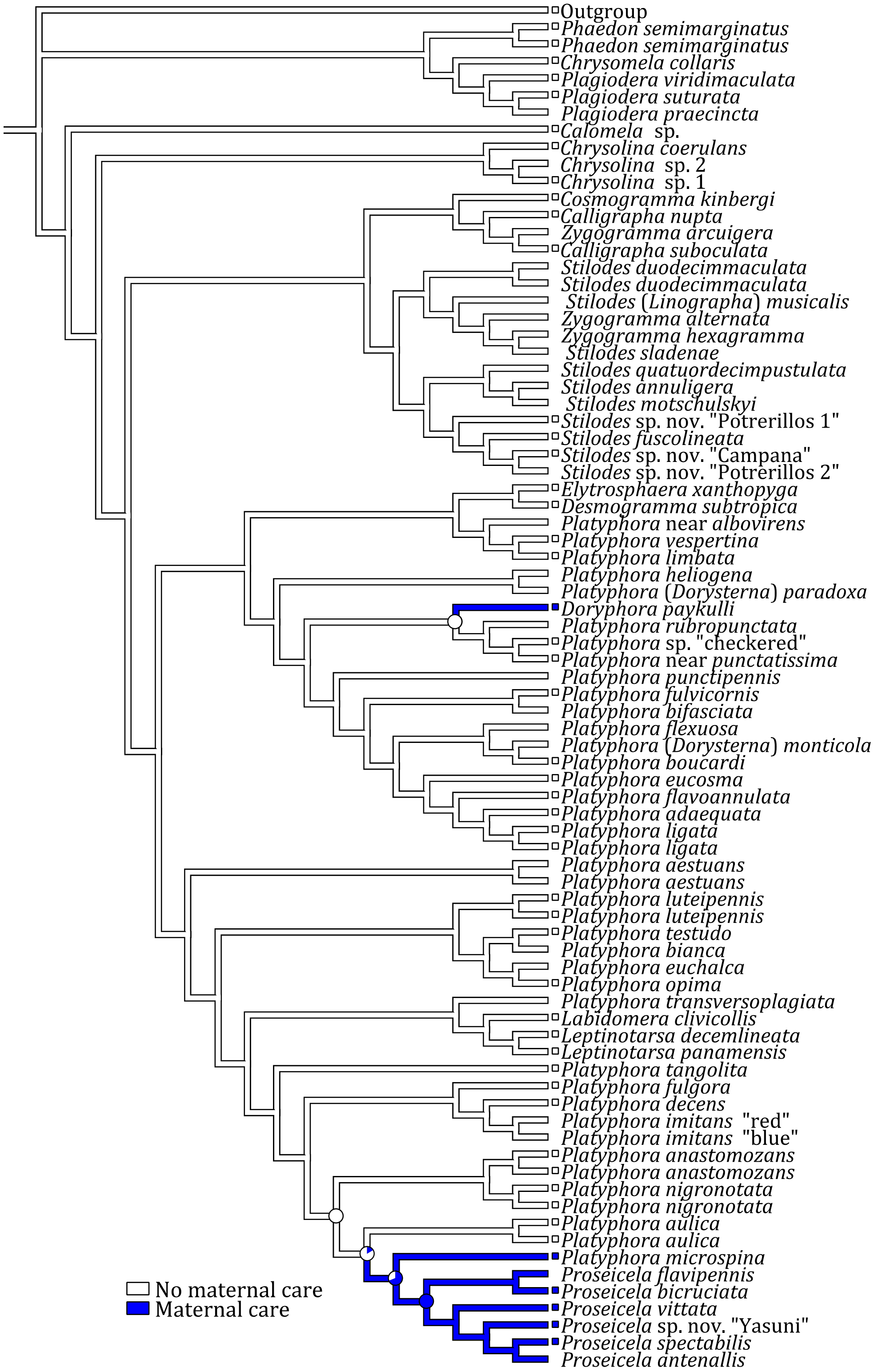

Figure S4.7: Cladogram representing the reconstruction of ancestral maternal care on maximum a posteriori probability (MAP) Bayesian tree from analysis of the concatenated gene dataset.

Colours on branches are from Maximum Parsimony reconstruction, pie charts at nodes represent Maximum Likelihood with Mk1 model of either state in the ancestor.

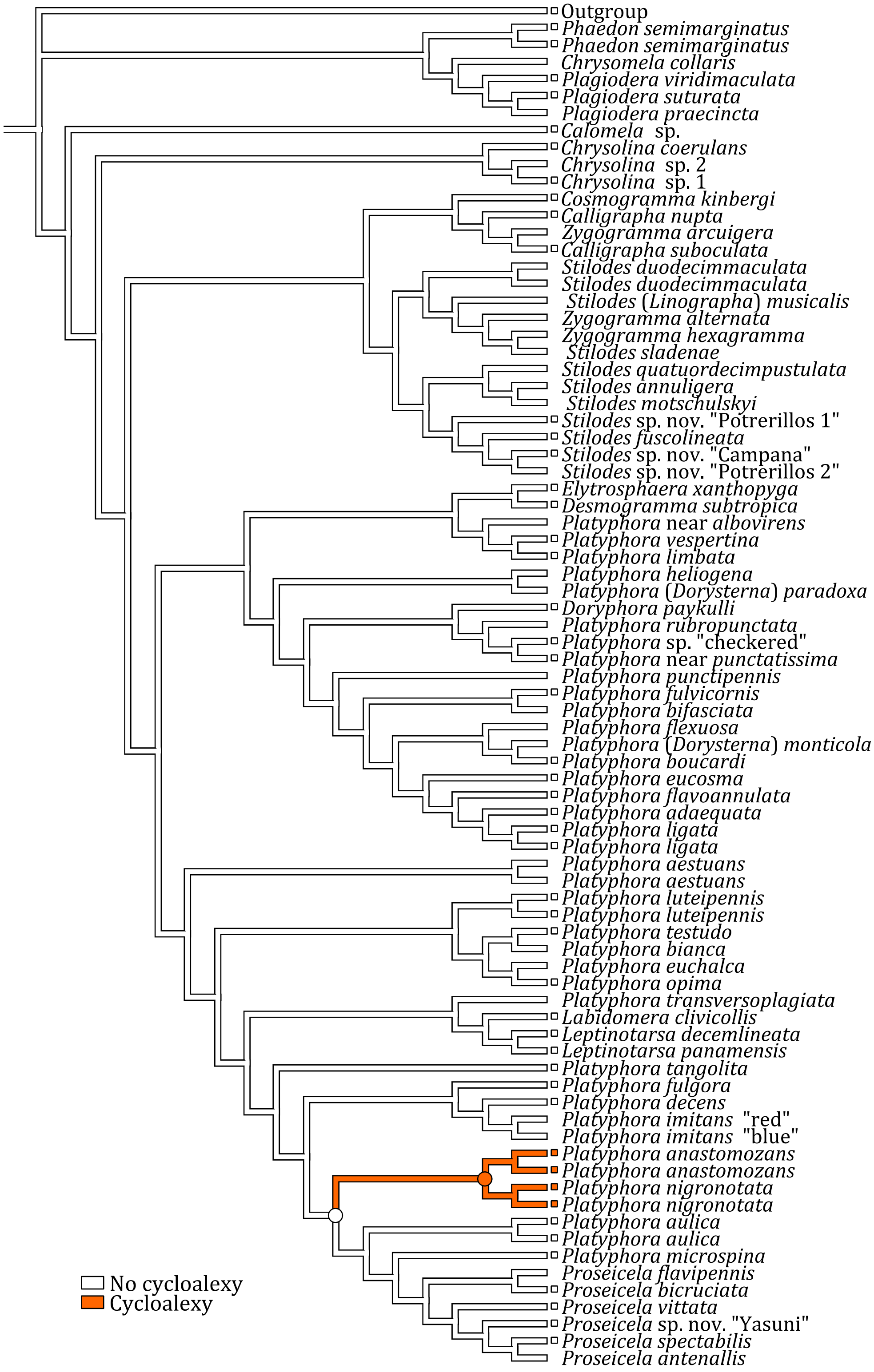

Figure S4.8: Cladogram representing the reconstruction of ancestral cycloalexy on maximum a posteriori probability (MAP) Bayesian tree from analysis of the concatenated gene dataset.

Colours on branches are from Maximum Parsimony reconstruction, pie charts at nodes represent Maximum Likelihood with Mk1 model of either state in the ancestor.

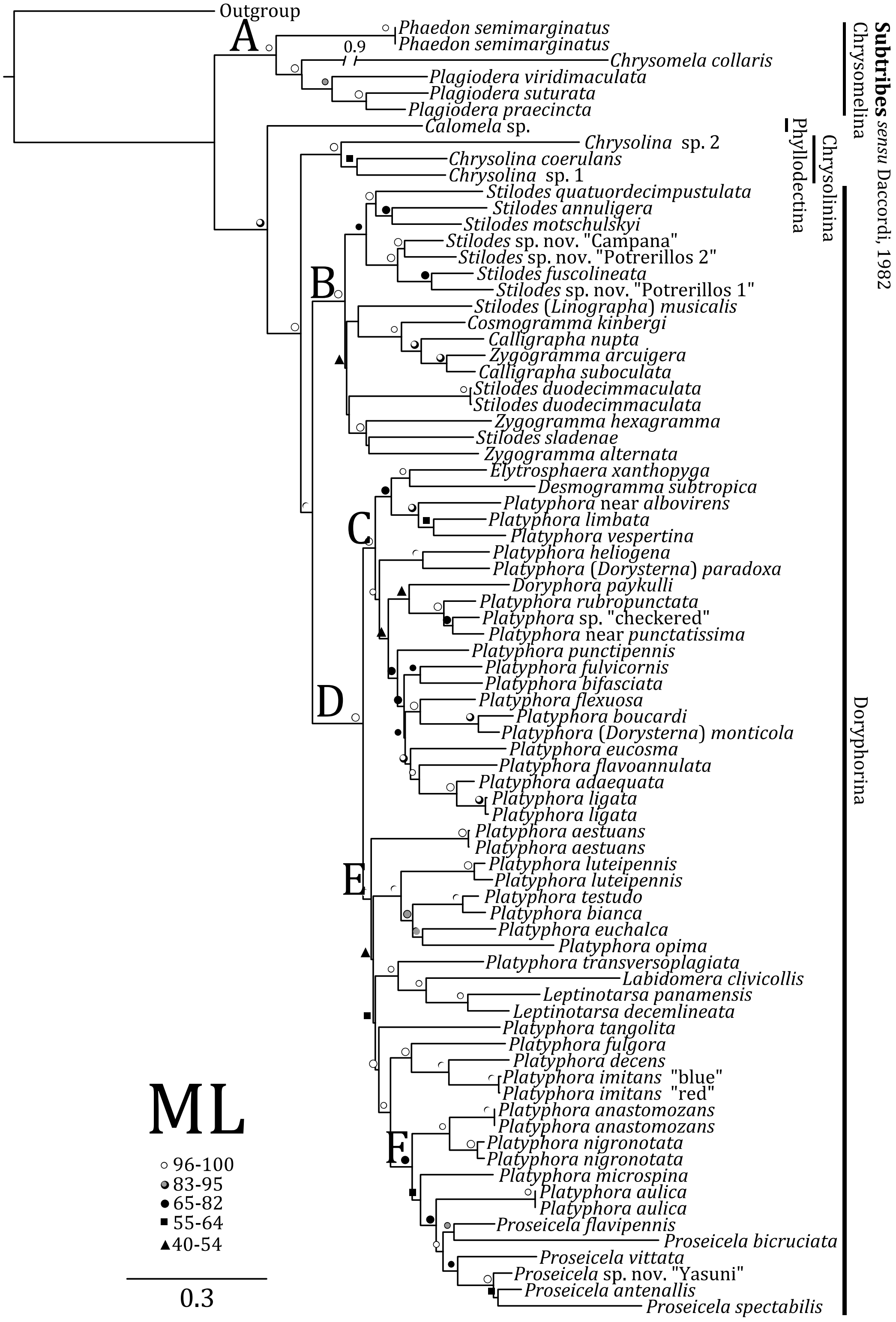

Figure S4.9: Topology and support values of the inferred phylogeny of Neotropical Chrysomelinae through Maximum Likelihood analysis of all genes concatenated (with regions of ambiguity from *28S* removed).

Bootstraps values are represented by the shapes on top of the nodes.

5.0. GENERAL DISCUSSION AND CONCLUSIONS

Our aim in this study was to build upon the knowledge of cycloalexy and larval gregariousness. First, we reviewed the literature for reports of cycloalexy and determined if these reports met the criteria for cycloalexy as originally defined by Jolivet et al. (1990). Then, using a molecular phylogeny, we reconstructed the historical relationships of Neotropical Chrysomelinae to gain a better understanding of the evolutionary history of larval gregariousness and cycloalexy.

"[Cycloalexy] takes time to be recognized" (Jolivet and Verma, 2011) and even more time to be fully documented. The original definition of the concept has several criteria: it is restricted to insect larvae, it is a tight circular group formation, taken at rest, with heads or abdomens at the periphery, sometimes with larvae in the middle of the circle. It is defensive and accompanied by coordinated movements to repel predator or parasitoid threats. The behaviour was first described in leaf beetles (Chrysolemidae), and is now known from four subfamilies: Cassidinae, Galerucinae, Criocerinae and Chrysomelinae. Cycloalexy is also found in two families of sawflies (Tenthredinoidea), the Pergidae and Argidae. Some caterpillars are cycloalexic with heads at the periphery of their circle. This is the case of *Lonomia* species (Saturniidae: Hemileucinae) and tentatively in *Papilio laglaizei* (Papilionidae: Papilioninae). Cycloalexy is rare in Curculionidae, where *Phelypera distigma* is the only known cycloalexic weevil, and the midge *Forcipomyia fuliginosa* (Ceratopogonidae) is the only known cycloalexic Diptera, although there exists no documentation of coordinated movements to repel threats in this group. The term has been applied to groups of animals like penguins and elk that are neither defensive nor are insects. Sometimes the circular defence is reactive rather than preventive, and not taken at rest but in face of an imminent threat, as occasionally observed in muskoxen, elands, water buffalo, red deer and killer whales. Tight defensive groups of owlfly larvae (Neuroptera: Ascalaphidae: *Ascaloptynx furciger*) are unidirectional rather than circular, with abdomens at one end and heads at the other, and their formation is used while feeding. Adult Hymenoptera are not cycloalexic, for example, stingless bees (Apidae: Meliponinae), ants (Formicidae) and wasps (Vespidae) actively guard their nest. Similarly, the term has been applied to the circular assembly of an amphipod crustacean (*Phronima sedentaria*) which helps the mother herd larvae.

Similarly, the tight circular grouping of *Doryphora* and *Proseicela* larvae, with heads inwards pose a special challenge to the definition of cycloalexy. Can we accept as cycloalexy if the group defence is given by the guarding mother rather than the larvae themselves? Given that these larvae lack coordinated group movements to repel threats, like larvae of the amphipod *P. sedentaria*, we suggest that they are not cycloalexic. The feeding aggregations of hymenopteran parasitoids on their host, and nymphs of Hemiptera are not cycloalexic because they are not taken specifically at rest. This strict definition of cycloalexy takes full advantage of the usefulness of the term and restricts it to clearly convergent behaviours of insect larvae.

After clarifying the original meaning of the term cycloalexy and what it does not mean, we study the evolution of the behaviour, focusing on Neotropical Chrysomelinae. I collected beetle specimens and observed behaviour of larvae in Panama, Ecuador, Canada and Mongolia, Dr. Windsor did the same in Panama, Bolivia, French Guiana and France. Observations and specimens from Brazil were made and collected by colleagues. We selected and amplified DNA sequences from five genes; three mitochondrial, *COI*, *COII* and *12S*, and two nuclear, *CAD* and *28S*. A molecular phylogeny was inferred using Bayesian and ML methods and ancestral larval behaviour was reconstructed with ML and MP methods. This molecular phylogeny of Chrysomelinae provides several taxonomic findings. Chrysomelina (*sensu* Daccordi, 1982) is monophyletic, confirming previous research by Termonia et al. (2001). The Neotropical Doryphorina (*sensu* Daccordi, 1982) is also monophyletic, divided into two clades, one clade with *Stilodes* paraphyletic with *Zygogramma*, with sister *Calligraphra* and *Cosmogramma*. This corresponds to the cardenolide-secreting clade of Pasteels and collaborators (2004). Second clade contains the polyphyletic genus *Platyphora* (clade D, Fig. 4.1), is divided into two clades. Species in these clades secrete saponins metabolized from sequestered plant amyrins. The first clade (C, Fig. 4.1) inside these saponin-secreting Chrysomelinae is composed of *Desmogramma* and *Elytrosphaera* sister to each other, and together sister to 18 species of *Platyphora*, and *Doryphora paykulli* Stål nested within those *Platyphora* spp. Clade E contains a polytomy of *Platyphora, Labidomera* and *Leptinotarsa*; the third (clade E) contains the remaining *Platyphora* spp. with monophyletic *Proseicela* species nested within.

Our reconstructions of ancestral larval behaviour show five independent evolutionary origins of larval gregariousness, one of which is accompanied by clear cycloalexy, in a clade containing *Platyphora anastomozans* and *Platyphora nigronotata*. Two other origins are more complicated in that the group defence is provided by the guarding mother rather than larvae of the group, this is the case in *Doryphora paykulli*, and in species of the genus *Proseicela* and *Platyphora microspina*. Two more origins of larval gregariousness were found: one without cycloalexy in genera *Chrysomela*, *Phaedon* and *Plagiodera* of subtribe Chrysomelina (sensu Daccordi, 1982). *Platyphora eucosma* larvae are definitely gregarious, without maternal care, and aggregate loosely at rest, with heads generally inwards, and apparently represent an independent evolutionary origin of gregariousness, but not cycloalexy.

6.0. RECOMMENDATIONS FOR FUTURE RESEARCH

Our phylogeny of Neotropical Chrysomelinae will help future endeavours to create a comprehensive tree of the subfamily, and ultimately help create a comprehensive tree for all of Chrysomelidae. Within the Chrysomelinae, analysis of our five gene segments did not resolve a polytomy inside *Platyphora* + *Labidomera* + *Leptinotarsa*. Although, CAD alone suggests that *Labidomera* + *Leptinotarsa* are nested within *Platyphora*. Additional gene segments with a similar evolutionary rate will help resolve that uncertainty. The inclusion of more species and genera with maternal care or cycloalexy, like *Platyphora selva* and *Eugonycha* could identify more independent origins of the behaviours.

Together with Chrysomelinae, Cassidinae, Criocerinae in the leaf beetles (Chrysomelidae) and Pergidae and Argidae in the sawflies (Tenthredinoidea) have several cycloalexic species. The evolutionary history of cycloalexy has not been studied in those groups, nor has it been studied in groups with few known cycloalexic species: Saturniidae with genus *Lonomia*, Galerucinae with genus *Coelomera*, Ceratopogonidae with *Forcipomyia fuliginosa* and Curculionidae with *Phelypera distigma*. Comparing the ecology of these species with that of non-cyloalexic and solitary species will help identify the evolutionary pressures leading to preventive circular group defence. By definition, cycloalexy is defensive; as such pressures by predators and parasitoids are probably significant to its evolution. Cassidinae would be ideal to study the interplay between maternal care and cycloalexy, and the closely related species of Solanaceae-feeding *Proseicela* and *Platyphora* would be ideal to study the evolution of maternal care.

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