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The Meat-Farming Ants: Predatory Mutualism Between Melissotarsus Ants (Hymenoptera: Formicidae) and Armored Scale Insects (Hemiptera: Diaspididae)

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THE MEAT-FARMING ANTS: PREDATORY MUTUALISM BETWEEN *MELISSOTARSUS* ANTS (HYMENOPTERA: FORMICIDAE) AND ARMORED SCALE INSECTS (HEMIPTERA: DIASPIDIDAE)

A Dissertation Presented

by

SCOTT A. SCHNEIDER

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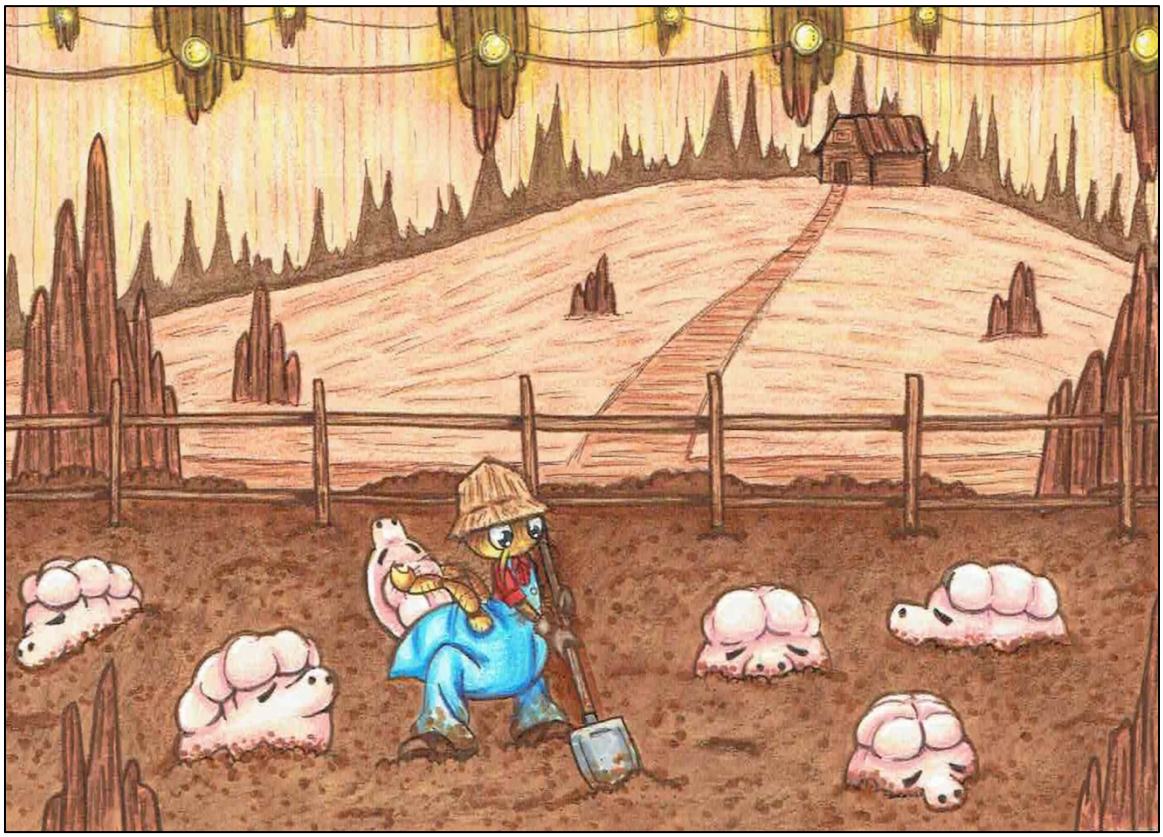
DEDICATION

I dedicate this work to my nephews and nieces
who continue to inspire and be inspired.

EPIGRAPH

J.R.R. Tolkien's writings have helped to inspire in me a love of nature and an appreciation for agriculture. We are often reminded of ourselves when we study behaviors of ants, particularly their aptitude for farming. Below I juxtapose Tolkien's favorite protagonists with my own subjects of study using the following illustration and a quotation from one of his classic works.



"It is clear that Hobbits had, in fact, lived quietly in Middle-earth for many long years before other folk became even aware of them. And the world being after all full of strange creatures beyond count, these little people seemed of very little importance."

− J. R. R. Tolkien, *The Fellowship of the Ring*

Illustration Credit:
Not Your Average Farmer
Tara Michelle Bradley
January 2016

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I would like to thank my advisor, Ben Normark, for always leading by example and demonstrating daily how to be a thoughtful scientist and admirable colleague. I would also like to thank my committee members, Lynn Adler, Laura Katz, and Aaron Ellison, for years of insightful guidance and stimulating conversation. I thank Akiko Okusu for teaching me about molecular systematics and serving as a secondary advisor on many occasions. It has been a joy and privilege to work alongside my fellow lab members, Daniel Peterson, Laura Ross, and Rodger Gwiazdowski.

ABSTRACT

THE MEAT-FARMING ANTS: PREDATORY MUTUALISM BETWEEN *MELISSOTARSUS* ANTS (HYMENOPTERA: FORMICIDAE) AND ARMORED SCALE INSECTS (HEMIPTERA: DIASPIDIDAE)

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Ant agricultural mutualisms are common, well studied, and receive attention from scientific and public spheres due to shared similarities with human agriculture (i.e. ant/fungus 'crop farming' and ant/insect 'dairy farming'). They also serve as important model systems for studying many facets of mutualism. This study reveals that the repertoire of ant agriculture may also include 'meat farming'. Predatory mutualisms occur between *Melissotarsus* ants and various species of armored scale insects. This dissertation employs a multi-disciplinary approach to investigate the evolutionary history and nature of ant/diaspidid mutualisms. Chapter 1 reviews the current state of knowledge regarding species composition of these associations and includes descriptions of three new diaspidid species. Also included is a discussion on new observations of foraging behaviors gathered from multiple colonies of *Melissotarsus emeryi* in South Africa. Chapter 2 reconstructs the phylogeny of the Aspidiotini tribe of armored scale insects from molecular data for 127 species from 31 genera. Nearly all known ant-associated diaspidids belong to the tribe Aspidiotini. The majority of aspidiotine genera are found to be paraphyletic as currently defined and recommendations to increase taxonomic stability for this tribe are provided. Myrmecophily among diaspidids has evolved no fewer than six times independently, four times within the Aspidiotini and two additional origins recorded from the Diaspidini. Relationships between ants/diaspidids are labile at the species level and partnerships can shift. However, several clades of ant-specialized diaspidids have evolved indicating that some relationships can be stable on an evolutionary timescale. Chapter 3 investigates the diet and relative trophic position of *Melissotarsus* ants by analyzing stable isotopic enrichment of $\delta^{15}N$ and $\delta^{13}C$, and by assaying ant gut contents for diaspidid COI mtDNA fragments. Diaspidid DNA is consistently amplified from gut contents of worker ants. Isotopic analyses indicate a strong positive relationship between $\delta^{15}N$ and $\delta^{13}C$ isotopes of worker ants and associated diaspidids; most variation in worker isotopes can be explained by variation in diaspidid isotopes. Worker ants are calculated to be approximately one trophic level above associated diaspidids. These dietary studies indicate that *Melissotarsus* ants are predators of mutualistically associated diaspidids. Predation plays a central role in the establishment and maintenance of ant/diaspidid mutualisms.

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CHAPTER 1

MUTUALISM BETWEEN ARMORED SCALE INSECTS AND ANTS: NEW SPECIES AND OBSERVATIONS ON A UNIQUE TROPHOBIOSIS (HEMIPTERA: DIASPIDIDAE; HYMENOPTERA: FORMICIDAE: *MELISSOTARSUS* EMERY)

1.1 Abstract

The association between African armored scale insects (Hemiptera: Coccoidea: Diaspididae) and ants belonging to *Melissotarsus* Emery (Hymenoptera: Formicidae: Myrmicinae) is the only trophobiosis known in which ants do not receive honeydew or nectar in exchange for protection and other services. Food reward for the ants in this mutualism remains unknown, despite repeated suggestions that diaspidids are consumed by the associated ants, thus serving as 'domestic cattle'. I describe new observations on interactions between *Melissotarsus emeryi* Santschi and the diaspidid *Morganella conspicua* (Brain) from South Africa. Worker ants exhibited previously undescribed tending behaviors, most notably a 'squeezing and licking' performed on an adult female diaspidid and 'culling', in which a worker removed an adult female armored scale from the host plant. These could represent the gathering of secretory products and the cultivation of an individual for consumption, respectively. An ant exclusion study over 12 days of isolation showed that adult female diaspidids and second-instar nymphs secreted no wax or exudates that attending ants would ordinarily collect. Workers of *M. emeryi* did not defend their nest against invading colonies of *Crematogaster* and other unidentified ants: I hypothesize that the primary mode of defense is maintenance of isolation within galleries. I describe three new ant-associated diaspidid species: *Affirmaspis cederbergensis* Schneider sp.n. from South Africa, *Diaspis doumtsopi*

Schneider sp.n. from Cameroon, and *Melissoaspis incola* Schneider sp.n. from Madagascar. *Melissoaspis formicaria* (Ben-Dov) comb.n. is transferred from *Morganella* (Brain). Diagnostic characteristics for *Melissoaspis* Ben-Dov are revised, and additional taxonomic information defining this genus allows ease of identification. An updated identification key to the species of ant-associated diaspidids is provided.

1.2 Introduction

The association of ants with honeydew-producing Hemiptera (aphids, scale insects, membracids, etc.), or with nectar-producing larvae of lycaenid butterflies, is a well-studied phenomenon termed trophobiosis. Trophobioses are complex, typically mutualistic, relationships in which ants provide protection and other benefits to a partner species and procure a reliable food reward from this partner in exchange for their attendance (reviewed by (Way 1963, Hölldobler and Wilson 1990, Gullan 1997, Gullan and Kosztarab 1997, Delabie 2001, Pierce et al. 2002). The only ant–hemipteran trophobiotic relationship in which honeydew appears not to be a 'currency' of exchange involves ants of the genus *Melissotarsus* Emery (Hymenoptera: Formicidae: Myrmicinae: Melissotarsini) and certain armored scale insects (Hemiptera: Diaspididae). The Diaspididae are one of a few families of scale insects that do not produce honeydew (Beardsley Jr and Gonzalez 1975, Foldi 1990, Foldi and Rosen 1990). Armored scale insects feed on the parenchyma tissues of host plants rather than on phloem or xylem fluids, which obviates the need to expel excess water and sugars as honeydew. It is uncertain what food source *Melissotarsus* ants procure from diaspidids and how stable mutualisms are maintained between such unlikely partners. However, *Melissotarsus*

workers actively tend diaspidid populations within their nests to the benefit of both parties and the association is obligate for the ants and potentially for the diaspidids as well (Mony et al. 2007, Ben-Dov and Fisher 2010).

A detailed review on the trophobiosis between *Melissotarsus* ants and diaspidids by Ben-Dov and Fisher (2010) is summarized briefly later on. Here, I describe new observations on associations between the ant *Melissotarsus emeryi* Santschi and the diaspidid *Morganella conspicua* (Brain) in South Africa. I describe three new species of ant-associated diaspidids and expand our understanding of the taxonomy and distribution of the trophobiosis.

1.2.1 *Melissotarsus* ants and Diaspididae: natural history and associations

The relationship between *Melissotarsus* ants and armored scale insects was first discovered in the 1970s in Côte d'Ivoire (Delage-Darchen 1972, Delage-Darchen et al. 1972) and shortly thereafter in South Africa (Prins et al. 1975, Ben-Dov 1978). The association occurs throughout continental Africa as well as Madagascar and Saudi Arabia (Ben-Dov and Matile-Ferrero 1984, Collingwood 1985, Dejean and Mony 1991, Mony et al. 2002). Little is known about *Melissotarsus* ants, due to their cryptic habits. These are gallery-forming ants with large polygynous colonies ranging from several thousand to over 1.5 million individuals (Mony et al. 2002). Worker ants birthed from multiple queens operate as a single unified colony within one host tree; there is little intercolony aggression (Mony et al. 2007). Workers excavate a network of tunnels in the bark of live trees and diaspidid populations are housed within the nest chambers along with ant brood. *Melissotarsus* workers enclose their galleries against the surrounding environment by forming a mortar from silk, sawdust and frass used to seal the entrances (Prins et al.

1975, Fisher and Robertson 1999). They are the only adult ants that produce silk. When a segment of the gallery roof is removed, workers immediately divert their attention to repairing and enclosing the nest chambers. It is difficult to observe normal behaviors and interactions between ants and diaspidids directly, due to this cloistered habit, but workers have been observed frequently to tend diaspidids (Delage-Darchen et al. 1972, Prins et al. 1975, Ben-Dov 1990, Mony et al. 2007). *Melissotarsus* workers do not forage outside of their galleries due to an unusual configuration of their middle pair of legs, which are tilted at an angle of nearly 180° to the front legs. This configuration allows workers to anchor themselves on the sides or roof of the tunnel, but renders them incapable of walking effectively on flat surfaces (Delage-Darchen 1972, Mony et al. 2007). Workers will stagger and flail about when placed on a flat surface.

Within the galleries of *Melissotarsus*, various insect inhabitants have been found, including putative predators and parasitoids (Encyrtidae, Reduviidae, and Bethylidae) and social parasites (Thysanura, Anthochoridae, and Aradidae) (Prins et al. 1975), but these are rare. Diaspidids are the only other abundant and consistent nest inhabitants, and it is likely that the nutritive demands of these massive ant colonies are derived in some form from diaspidids.

All life stages of diaspidids are found within *Melissotarsus* galleries, including first- and second-instar nymphs, adult females and adult males in biparental species (Ben-Dov and Matile-Ferrero 1984). Diaspidids are abundant within ant nests. One census found the diaspidid *Morganella pseudospinigera* Balachowsky to outnumber ants (*Melissotarsus beccarii* Emery) approximately three to one from randomly selected twigs (Ben-Dov and Matile-Ferrero 1984). Mony *et al.* (2002) estimated colonies of

Melissotarsus weissi Santschi and *M. beccarii* in mango (*Mangifera indica* L.) and safou (*Dacryodes edulis* Lam), respectively, to contain from 330 000 to 556 000 diaspidids. These staggering numbers suggest that diaspidids benefit from the association, and Ben-Dov & Matile-Ferrero (1984) proposed that the primary benefit received by diaspidids is protection within the enclosed galleries of ant nests. Aided dispersal is another potential benefit that may be received by diaspidid partners, but it remains unknown as to how ant colonies acquire diaspidids and if they are transported actively or recruited during colony foundation. Possibly ants participate actively in the introduction of diaspidids as crawlers (mobile first-instar nymphs) to the galleries (Ben-Dov and Fisher 2010).

All populations of ant-associated diaspidids are scale-less; they lack the characteristic scale covering for which the 'armored' scales have been named. The majority of ant-associated diaspidid species are known only from scale-less populations living with ants; however, *M. conspicua*, *M. pseudospinigera* and *Melanaspis madagascariensis* Mamet were originally described from free-living populations with normal scale covers (Brain 1919, Mamet 1951, Balachowsky 1956, Ben-Dov and Fisher 2010). In free-living populations the scale cover provides mechanical protection throughout most of the life stages (Foldi 1990) and provides effective protection even against predatory *Pseudomyrmex* ants associated with *Acacia* (Janzen 1966, Kosztarab 1987). For ant-associated populations, sequestration within galleries is apparently an effective substitute for this mode of protection.

It is unclear why ant-associated diaspidids lack scale covers. If diaspidids attempt to produce wax in galleries then ants either collect that wax or prevent its production, perhaps through chemical manipulation (Ben-Dov 1978). An exception apparently occurs

for male diaspidids; the prepupal and pupal instars of *M. conspicua* possess scale covers from which adult males emerge (Prins et al. 1975) S. A. Schneider, personal observation). If ants are responsible for the absence of scale covers, this suggests that *Melissotarsus* workers differentiate between the sexes and only interfere with females producing wax. Female diaspidids possibly cease wax production in the presence of *Melissotarsus* ants: wax production may be costly and it may be advantageous for diaspidids to invest in growth or reproduction instead. One symbiotic species of *Aspidiotus* completely lacks dorsal macroducts, the major secretory glands that are responsible for producing the scale cover (Delage-Darchen et al. 1972). Several other ant-associated diaspidids have few dorsal macroducts as well (Ben-Dov 2010). Currently we cannot know if the macroducts are functional in these species, because all known populations are ant-associated.

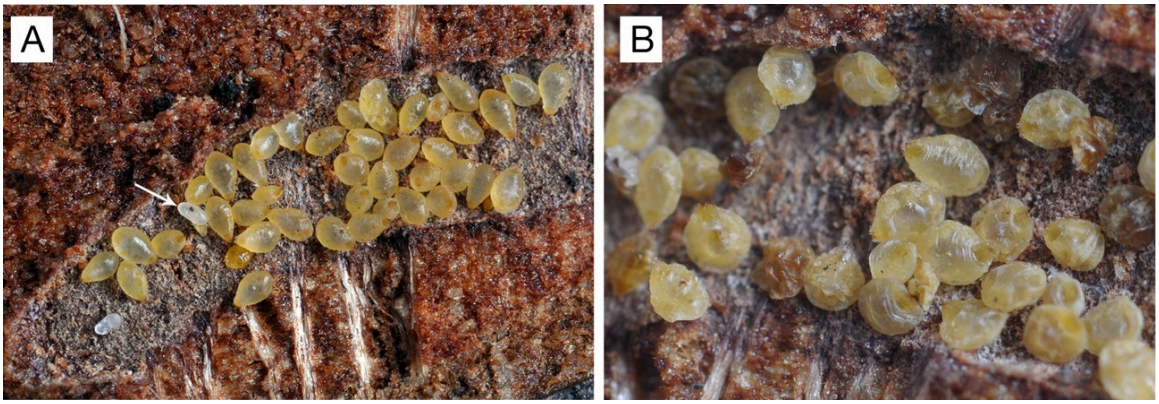
Authors have speculated about the potential food rewards that *Melissotarsus* could receive from diaspidids. Waxy glandular secretions from the macroducts have been suggested to serve as the primary food source for *Melissotarsus* (Ben-Dov and Matile-Ferrero 1984). Worker ants frequently probe the dorsum and pygidium of diaspidids with their mandibles; it is possible that they are collecting secretions in this way. This hypothesis is not supported by the observation that several ant-associated diaspidids either lack macroducts or possess only a few on the dorsal pygidium. However, both scale-covered and scale-less populations of *M. conspicua* have been found on the same tree in free-living and ant-associated populations, respectively (Prins et al. 1975), and so this possibility has not been ruled out. It has also been suggested that ants are maintaining armored scale insects as ‘domesticated cattle’ that are consumed as a source of ‘meat’ (Ben-Dov 1978). Trophobiotic ants occasionally consume mutualist partners in addition

to harvesting honeydew, but the degree to which this occurs is largely unknown (Stadler and Dixon 2008). This would be the first trophobiosis in which meat is the primary (and perhaps only) food reward for ant attendance. Meat farming would make for more than just an interesting case of natural history, as it would suggest that the relationships between *Melissotarsus* ants and diaspidids are simultaneously mutualistic and predatory. Further dietary studies of *Melissotarsus* ants are required to fully understand the nature and dynamics of this unusual form of mutualism.

1.3 Interactions between *M. emeryi* and *M. conspicua* from South Africa

1.3.1 Methods

I made several new observations on live populations of the ant species *M. emeryi* and the diaspidid *M. conspicua* in the Western Cape province of South Africa. In January 2012. S.A.S. and J.H.G. revisited Nardouwsberg, the locality from which Prins *et al.* (1975) first discovered this association within host trees of the species *Leucospermum praemorsum* (Meisn.) Phillips (Fabaceae). Ant colonies were discovered in 10 trees of *L. praemorsum* from several farms located between Nardouwsberg and Vanrhynsdorp in the Clanwilliam district (localities are listed under the heading for *M. conspicua* in 'Material examined' below). Infested trees were identified easily by vein-like markings on the smooth bark, indicating the presence of galleries under the surface. I haphazardly selected branches from infested trees, sawed them off and brought them back to Stellenbosch University for observation in the laboratory. I removed the bark to expose galleries and then observed the interactions between ants and diaspidids through a Leica Wild M8 dissecting microscope. Any evidence of ant-feeding behaviors was of particular interest.



1.1. Ant Exclusion − A segment of exposed galleries from the ant-exclusion study with adult females and second-instar nymphs of *Morganella conspicua*. (A) This photograph was taken at the beginning of the ant exclusion study on 7 January 2012. The white arrow indicates an ant larva that is also visible at the far left of the second pane. (B) This photograph was taken 20 days later on January 27, 2012. Daily observations were made for the period of January 7th through January 18th. During this time no armored scale insects were observed secreting wax from the macroducts or exudate from the anus. Individual diaspidids that died during this interval appear darkened and shriveled. (Photograph: Anton Jordaan, Stellenbosch Centre for Photographic Services.)

I exposed ant galleries on two segments of branch, each approximately 17 cm in length, cut from a tree at Duikerfontein farm in Nardouwsberg (locality: 32°1’55.56” S, 18°51’54.30” E). All worker ants were removed from these branch segments, such that the resident diaspidids (both adult females and second-instar nymphs) remained isolated from ant attendance for a total of 12 days. The goal of this ant exclusion was to determine whether or not armored scale insects would produce filaments of wax from their dorsal macroducts or droplets of exudate from the anus that ants would ordinarily collect. Armored scale insects were observed daily for any such evidence. One branch segment was left uncovered and exposed (**Fig. 1.1**); the other was wrapped in a piece of black plastic in an attempt to simulate an intact gallery roof. This latter attempt proved unsuccessful, due to accumulating condensation resulting in the growth of mold in the galleries. The mold grew over rapidly and killed most of the armored scale insects on this branch. I report observations for the uncovered branch only.

1.3.2 Ant behaviors observed

1.3.2.1 Tending

Consistent with previous reports, worker ants generally divert their attention to repairing exposed galleries with a combination of silk, sawdust and frass (Fisher and Robertson 1999). A few workers did focus attention on tending to brood and to armored scale insects. These workers were often busy using silk to coat the gallery walls and also sometimes placing strands of silk on larvae, pupae and diaspidids. The silk may be effective at reducing the build-up of moisture within galleries, as the tunnels appear to be considerably drier than the surrounding wood. Workers also used silk to bundle larvae together for transport to new locations.

I observed two curious tending behaviors that have not been reported previously in the literature. On one occasion I saw a worker ant tending an adult female armored scale that had a first-instar nymph partially breaching from the vulva. The worker ant repeatedly grabbed the adult female diaspidid around the thoracic/anterior abdominal margin with its mandibles and gently squeezed the body. The worker then grazed its mouthparts along the mid-dorsum of the diaspidid in what appeared to be a 'licking' behavior. This sequence of behaviors, 'squeezing and licking', was repeated multiple times for the duration of only a few minutes. The worker then ceased this behavior and walked away. The first-instar nymph took several hours to fully emerge from the female. It is possible that the 'squeezing and licking' was a form of foraging behavior, but without further information this remains purely speculative. If fluid or wax was secreted during this process, the amounts were too minute to see under the microscope at full magnification. On a separate occasion, a worker ant antennated an adult female diaspidid

and then seized the scale insect with its mandibles and pulled it away from the gallery wall. The worker drew the armored scale out until its mouthparts were mostly removed from the wood and then placed it down and walked away. The armored scale was marked to see if workers would come back to claim it later; however, all workers within this branch were killed soon after by an invading colony of unidentified ants that were present in the laboratory. The adult female diaspidid died within 24 h after being removed from the tree. It remains unclear whether this diaspidid was being harvested for consumption or if there is an alternative explanation for this behavior. There were no direct observations of ants consuming armored scales.

1.3.2.2 Defense

The invasion of *Crematogaster* workers and another unidentified ant species into the galleries of one branch presented an unexpected opportunity to observe the defensive behaviors of *M. emeryi* workers. *Melissotarsus* workers did not aggressively defend their galleries; when faced with an intruder, workers would pause or tuck themselves into small crevices. Invading workers of the unidentified ant species stung and killed *Melissotarsus* workers with little to no resistance. Invading *Crematogaster* workers were also observed pinching workers of *M. emeryi* with their mandibles, eliciting the same retreat response from *Melissotarsus*. This suggests that *Melissotarsus* colonies primarily defend themselves by maintaining enclosed galleries and avoiding interaction with competitors and/or predators. Invading workers took *Melissotarsus* larvae and pupae but did not pay any attention to the diaspidids.

1.3.3 Diaspidid products

1.3.3.1 Free-living *M. conspicua*

One sampled tree (at coordinates 31°59'33.30" S, 18°49'14.97" E) had a free-living population of *M. conspicua* with white scale covers on the exterior bark next to what appeared to be a tunnel leading into the branch. All diaspidids within the ant galleries lacked scale covers with the exception of males. This corresponds to a similar observation of free-living individuals of *M. conspicua* on trees containing ants and diaspidids made by Prins *et al.* (1975). All individuals from this population were dead upon discovery. The bodies of adult females from the free-living population were generally larger than those from within the galleries, but otherwise were similar in appearance.

1.3.3.2 Ant-exclusion study

For the duration of the ant exclusion, no diaspidids produced wax filaments from their macroducts or exuded any waste products from the anal opening. A more rigorous ant-exclusion experiment would certainly be necessary in order to draw any significant conclusions, but this observation at least indicates that, under the given conditions, adult female diaspidids and second-instar nymphs were not regularly producing secretory or excretory products (**Fig. 1.1**).

1.4 Taxonomy and distribution of species

Delimitation of new species was based upon discrete gaps in morphological character sets of adult females, primarily involving features of the pygidium. Such gaps are inferred to indicate long-standing reproductive isolation and divergence among

lineages under the biological species concept. Diaspidid specimens were slide-mounted following the techniques of the Systematic Entomology Laboratory (ARS, USDA, Beltsville, MD; http://www.ars.usda.gov/Main/docs.htm?docid=9832). Morphological terminology follows that of Miller and Davidson (2005). The abbreviations for type depositories are as follows: BMNH, The Natural History Museum, London, U.K.; CASC, The California Academy of Sciences Collection, San Francisco, CA, U.S.A.; UMEC, University of Massachusetts, Amherst Entomology Collection, Amherst, MA, U.S.A.; USNM, United States National Entomological Collection, U.S. National Museum of Natural History, Washington, D.C., U.S.A., housed at the U.S. Department of Agriculture, Beltsville, MD, U.S.A.

1.4.1 *Affirmaspis cederbergensis* Schneider sp.n.

(**Fig. 1.2**)

http://zoobank.org/urn:lsid:zoobank.org:act:785258DB-7A20-445B-8F12-5B17B63741E3

1.4.1.1 Description of adult female. Features of scale covering unknown, all specimens of type series scale-less. Mounted on a microscope slide, body turbinate, 0.62–0.71mm long, widest at metathorax, 0.5–0.54mm wide. Pygidium with pair of well-developed median lobes; second and third lobes represented by membranous points; third lobes sometimes absent. Median lobes each with one medial and one lateral notch, large paraphysis-like sclerotizations along medial margins of lobes, smaller sclerotizations at the base of lateral margins; second and third lobes triangular, poorly developed and about one-third the length of median lobes. Segmental setae of pygidium stout flagellate, those delineating abdominal segment VIII shortest, 13–14 μm; all others similar in length, 18–21 μm long. Plates present between median lobes and in first and second spaces, highly variable in type and shape; those between median lobes in pairs, either simple or

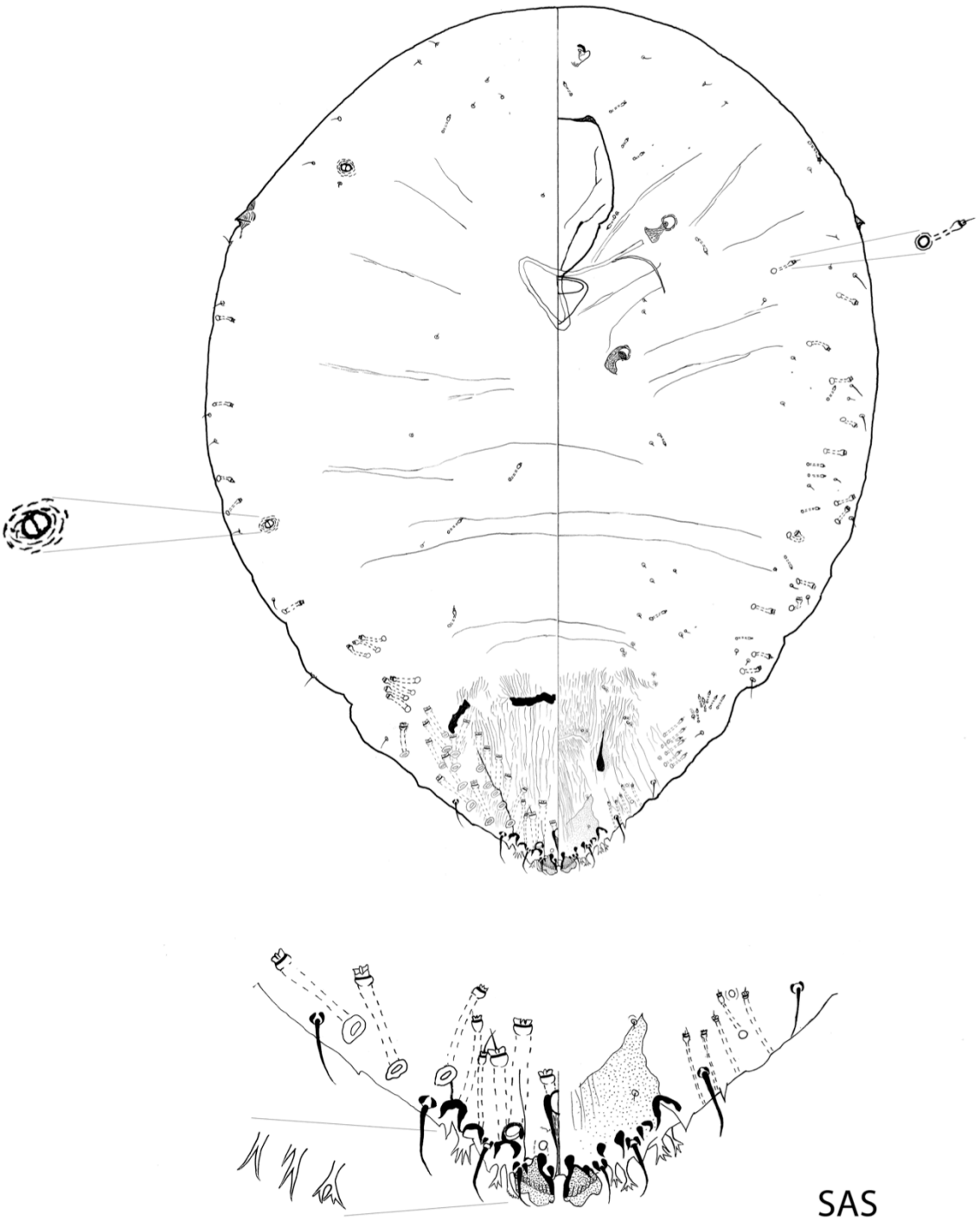


Fig. 1.2. *Affirmaspis cederbergensis* **sp.n.** adult female. Variable plates are illustrated below.

trifurcating; two fringed plates present in first space, variable in shape; second space with

two to three plates in various combinations of type and shape; simple, bifurcating, and fringed plates all represented in second space. Pair of large clavate paraphyses on VIII, lateral to median lobes. Dorsal pygidial macroducts of one-barred type, positioned along margin and with two submarginal rows running anteroposteriorly, length greater than five times the width of the opening, longest 36 μm. Shorter macroducts, 14–21 μm, present in groups of four to five on submargin of abdominal segment IV, three to four on submargin of abdominal segment III, and in pairs or triplets at margins of mesothorax through abdominal segment III. Long, thin ventral microducts in group of six on submargin of abdominal segment IV, 16 μm long; singular or in pairs along submargin of metathorax through abdominal segment III, 14 μm long; a few microducts also distributed medially on the head and thorax, 12 μm long. Intersegmental line between abdominal segments IV and III with sclerotized bands, one medial and two lateral. Anal opening 8 μm wide at longest axis, located about two times the width of anal opening from pygidial apex. Vulva located about four times further from the pygidial apex than the anal opening. Cicatrices present on dorsal submargin of prothorax and abdominal segment I, diameter 5 μm. Eye represented by small spur or dome near margin of mesothorax and level with middle of clypeolabral shield. Without perivulvar pores. Antennal tubercles each with one stout seta.

1.4.1.2 Material examined. **Holotype**: Adult ♀, SOUTH AFRICA, Cederberg Mts, 8 km NE Clanwilliam, Western Cape, 32°7’59.8794” S, 18°58’0.1194” E, found in nest galleries of *M. emeryi* from a branch of *Maytenus oleoides* Loes., ID# D1876D, 19.v.2002 (D.O. Burge) (CASC). **Paratypes**: SOUTH AFRICA, same data as holotype, one adult ♀ (D1876F) (USNM), one adult ♀ and one second-instar nymph (D1876A,

D1876E) (UMEC), one adult ♀ (D1876C) (BMNH).

1.4.1.3 Etymology. The epithet is a Latin adjective, formed from Cederberg (referring to the Cederberg Mountains of the Western Cape, South Africa) +-*ensis*, meaning 'of or from a place'. The Cederberg Mountains are currently the only locality from which this species has been collected.

1.4.1.4 Comments. Using Balachowsky's (1958) key to the genera of African Aspidiotina, this species keys out as *Diclavaspis*, which Balachowsky (1956) erected for three species: *Diclavaspis ehretiae* (Brain), *Diclavaspis socotrana* (Lindinger), and *Diclavaspis mashonae* (Hall). Two of these species, *D. ehretiae* and *D. socotrana*, had previously constituted the genus *Affirmaspis* MacGillivray (MacGillivray 1921). *D. socotrana* is the type species of *Affirmaspis* and *D. ehretiae* is the type species of *Diclavaspis*. Thus, under either MacGillivray's or Balachowsky's generic concepts, *Affirmaspis* and *Diclavaspis* are synonyms and *Affirmaspis* is the senior synonym and thus the valid name (Ben-Dov et al. 2013).

Adult females of *A. cederbergensis* are most similar to *Affirmaspis ehretiae* but may be distinguished by the following suite of characteristics. The second lobes are triangular and are not notched as in *A. ehretiae*. There are no plates anterior to the third lobes. Pairs of cicatrices are present on the dorsal submargins of the prothorax and abdominal segment I. The dorsal pygidial macroducts have wider openings and the ducts are not as long and thin as those in *A. ehretiae*. The dorsal macroducts are also more numerous on the pygidium and have a distinctive patterning, with clusters of four to five on the dorsal submargin of abdominal segment IV and three to four on the submargin of III.

This is the first species of *Affirmaspis* found associated with *Melissotarsus* ants. *Affirmaspis cederbergensis* was discovered within the galleries of *M. emeryi* from a host tree of *M. oleoides*. Whether free-living populations of *A. cederbergensis* exist, and whether these populations would produce a scale cover in the absence of ant attendance remains unknown.

1.4.2 *Andaspis formicarum* Ben-Dov, 1978: 316 – 319

This species was discovered originally in 1976 from East London in Eastern Cape Province, South Africa. It has now been discovered for the second time in association with *M. emeryi* from East London. Adult females lacked scale covers.

1.4.2.1 Material examined. SOUTH AFRICA, Eastern Cape, East London (*Ficus* sp.) 26.iii.2012 (K. Cole), one adult ♀ (D3660A) (UMEC).

1.4.3 *Diaspis doumtsopi* Schneider sp. n.

(**Fig. 1.3**)

http://zoobank.org/urn:lsid:zoobank.org:act:0A98FE3D-0E53-4CC1-9FC4-BEE05EB1E80B

1.4.3.1 Description of adult female. Features of scale covering unknown; all specimens of type series lacking scale. Mounted on a microscope slide, body oval, 0.63–0.7mm long, widest at metathorax, 0.53–0.56mm wide. Median lobes appear serrate with one medial notch and two lateral notches, well developed and sclerotized with large paraphysis-like sclerotizations along the medial edge and smaller sclerotizations at the lateral base, medial edges parallel or only slightly divergent, with one short pair of simple setae between median lobes; second and third lobes poorly developed and membranous, each with one notch near the center; position of fourth lobes occupied by a sclerotized spur on margin of abdominal segment V, triangular with blunted apex, more conspicuous than second and third lobes; with a sclerotized spur on margin of abdominal segment IV,

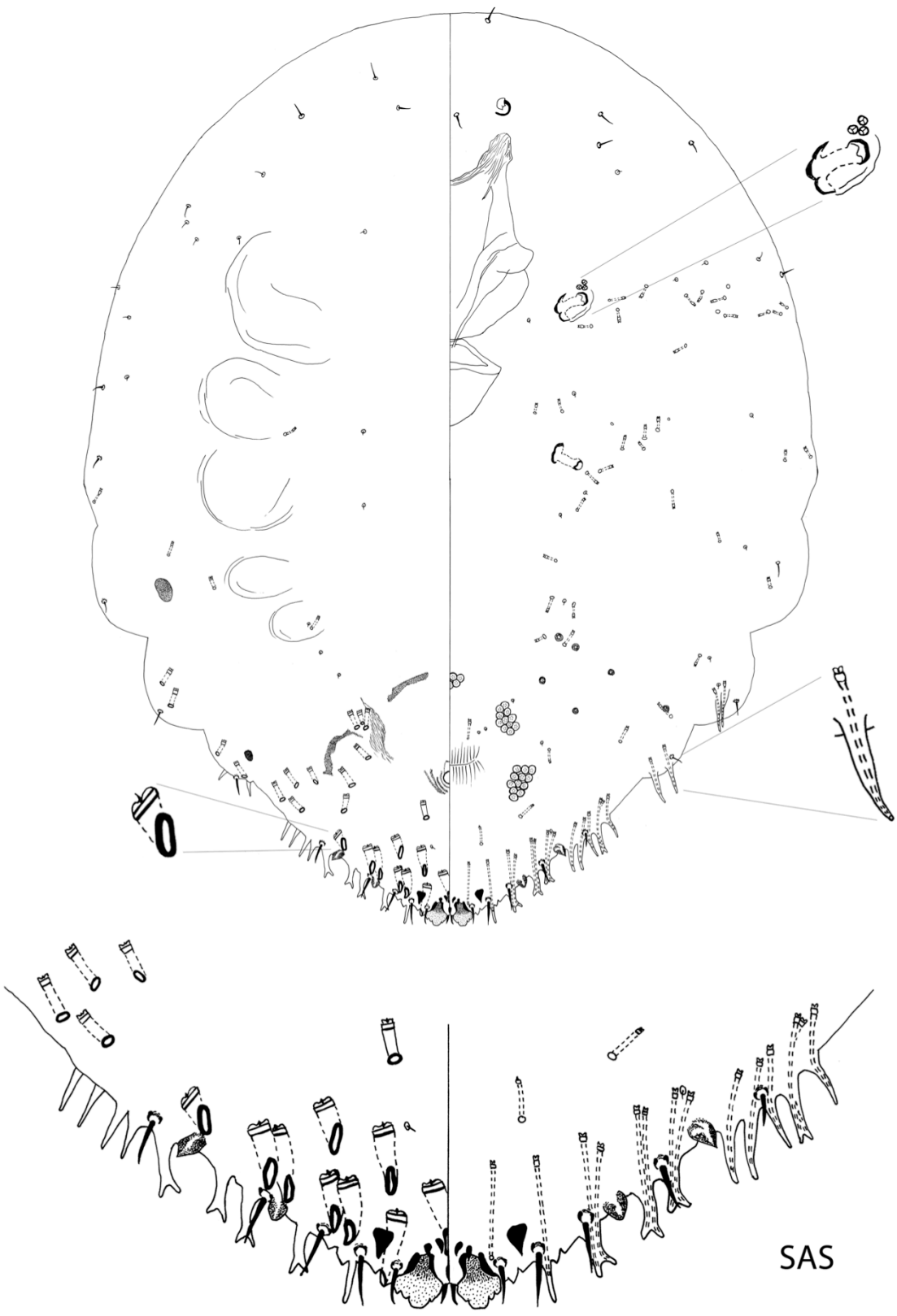


Fig. 1.3. *Diaspis doumtsopi* **sp.n.** adult female.

resembling a fifth pair of lobes. One pair of large turbinate paraphyses close to the notch of second lobes and intersegmental setae of segment VII, one pair of elongated comma-shaped paraphyses near medial base of fourth lobes. Segmental setae stout, flagellate, those of abdominal segment VIII projecting about as far as median lobes, 12–13 μm long; remaining pygidial segmental setae 15–20 μm long. Gland spines following formula (0, 1, 1, 3, 5–8, 2); gland spines of third and fourth spaces bifurcate, each apex subtended by a long, thin microduct, 40–45 μm long; remaining gland spines usually simple, with only one microduct. Dorsal pygidial macroducts of two-barred type present in two forms: large barrel-shaped macroducts with oval slit-like openings 9–10 μm wide at opening, 13–17 μm long, one present between proximal base of median lobes, two pairs present on submargin of abdominal segments VI and V, one pair at margin of VII, two pairs on margin of VI, two pairs on margin of V, one pair at margin of IV; thin, elongate macroducts with circular openings 4–5 μm wide at opening, 12–16 μm long, one pair present on submedian of abdominal segment VI with one pair of short setae always located posterior to them, 10–12 present on submedian/submargin of abdominal segments IV and III, in pairs on margins of III. Few ventral microducts present on submedian and submargin of pygidial segments, 9–14 μm long; present in bands running anteroposteriorly along the submargin of abdominal segment IV–mesothorax, 6–13 μm long. Perivulvar pores present in five clusters surrounding vulva, anterior-most group with six to eight pores, middle groups with 10–14 pores, posterior-most group with 12–15 pores, distribution of pores often asymmetrical but posteriormost clusters always containing more pores than others. Two sets of intersegmental sclerotizations present at median and lateral positions between abdominal segments IV and III. Anal opening

round, 7–10 μm wide, separated from pygidial apex about nine times the width of anal opening. Vulva approximately 50 μm wide, level with position of anal opening. Cicatrices sometimes present on dorsal submargins of abdominal segment III and prothorax, 6–12 μm in diameter, often absent or inconspicuous. Two to four pores present next to anterior spiracles. Dorsum of each segment from prothorax through abdominal segment III with large submarginal and submedial dorsal protuberances on each side, largest at prothorax and growing progressively smaller posteriorly, giving dorsum a coarsely hedgehog-like appearance. Eyes present and indicated by small dome-like projections at submargin of head. Antennal tubercles each with one stout seta.

1.4.3.2 Material examined. **Holotype**: Adult ♀, CAMEROON, Nkolbisson, 1°42'9.83" N, 11°42'9.83" E, elevation 602 m, found in nest galleries of *M. weissi* from *Mangifera* sp., ID# D3670A, 22.iv.2012 (A. Doumtsop) (UMEC). **Paratypes**: Same data as holotype, one adult ♀ (D3670E) (USNM); CAMEROON, Evodoula, found in nest galleries of *M. weissi* from *Dacryodes* sp., 28.iv.2012 (A. Doumtsop), one adult ♀ (D3669A) (UMEC), one adult ♀ (D3669C) (USNM), one adult ♀(D3669E) (BMNH); CAMEROON, Nkolbisson, 1°9'44.57" N, 11°42'9.83" E, elevation 602 m, found in nest galleries of *M. emeryi* from *Dacryodes* sp., 7.v.2012 (A. Doumtsop), one adult ♀ (D3674A) (USNM).

1.4.3.3 Etymology. This species is named in honor of a colleague, Armand Rodrigue Pascal Doumtsop Fotio, of the University of Maroua, Cameroon, who collected all known specimens, and who graciously provided samples of ants and armored scale insects from several infested mango and safou trees.

1.4.3.4 Comments. In Hall's (1946) key to African Diaspidini (Diaspidinae sensu

(Takagi 2002)), adult females of *D. doumtsopi* key to genus *Epidiaspis*. They resemble adult females of the only African species of *Epidiaspis*, *Epidiaspis ficifoliae* Hall, of Zimbabwe, but differ from *E. ficifoliae* in having perivulvar pores and furcate gland spines with multiple microducts.

Characters that distinguish *Diaspis* from *Epidiaspis* relate to the pygidial lobes. *Epidiaspis* has prominent median lobes, whereas those of *Diaspis* are sunken into the apex of the pygidium. The second and third lobes are well developed in *Diaspis* and reduced or obsolete in *Epidiaspis*. These are the same characters that distinguish the bark versus leaf phenotypes in polyphenic species of *Chionaspis* and *Diasipidiotus*, in which bark phenotypes have prominent median lobes and reduced second and third lobes, while the leaf phenotypes have recessed median lobes and more prominent second and third lobes (Liu et al. 1989, Miller and Davidson 2005). They are thus somewhat suspect as genus-defining characters. I place the species in *Diaspis* on the basis of DNA evidence indicating that *D. doumtsopi* is more closely related to the type species of *Diaspis* than to the type species of *Epidiaspis* (B.B. Normark *et al.*, unpublished data).

Dejean and Mony (1991) collected an unidentified *Diaspis* sp. in Cameroon inside galleries of *M. beccarii* from *D. edulis*. It is possible (yet remains to be confirmed) that these were also collections of *D. doumtsopi*. It is unknown whether free-living populations of *D. doumtsopi* exist and if these populations would produce a scale cover in the absence of ants.

1.4.4 *Melissoaspis* Ben-Dov 2010: 50 (type species: *Melissoaspis reticulata* Ben-Dov)

Melissoaspis fisheri Ben-Dov, 2010: 51

Melissoaspis formicaria (Ben-Dov, 2010: 54) **comb.n.**

Melissoaspis incola **sp.n.**

Melissoaspis reticulata Ben-Dov, 2010: 52

1.4.4.1 Diagnosis. Body of adult female circular to oval in shape with pygidium heavily constricted near abdominal segment V; pygidial segments often compressed and forming roughly triangular projection at posterior end. Pygidium comprising segments V, VI, VII, and VIII, with two to four pairs of lobes. Median lobes simple and poorly developed, appearing continuous with abdominal segment VIII, without paraphysis-like sclerotizations or other features defining basal boundaries. Setae of abdominal segment VIII short, lanceolate. Dorsal macroducts and ventral microducts long and thin, present in small numbers on pygidium and other body segments. Paraphyses present or absent; when present, only occurring in pairs between abdominal segments VIII and VII, VI and V. Two pairs of cicatrices present on dorsum of prothorax and abdominal segment I or II. Antennal tubercle submarginal with one seta. Spiracles without perispiracular pores. Plates absent, sometimes possessing pygidial marginal microducts with protruding orifices that resemble simple plates. Without perivulvar pores.

1.4.4.2 Comments. I gathered new information regarding *Melissoaspis* through inspection of additional specimens. Two diagnostic characteristics are modified herein from the original generic description, regarding the absence of paraphyses and the presence of distinctive patterning on the pygidium. I have noted that paraphyses are sometimes present on adult female specimens of *M. fisheri* and are always present on *M. formicaria* and *M. incola*. The presence of distinctive light and dark patterning on the dorsal pygidium of *M. fisheri* and *M. reticulata* may be a synapomorphy linking these two as sister species; however, it is not characteristic of the genus as a whole. I describe

multiple traits that help to link species and further characterize *Melissoaspis*, especially regarding the constriction and shape of the pygidium, the development of the median lobes, the presence and distribution of cicatrices, and the description of the posterior-most pair of segmental setae. Characterizing these traits has allowed me to reassign *M. formicaria* and to place the new species, *M. incola*. Phylogenetic analyses of DNA sequence data recover *Melissoaspis* as a monophyletic clade (Chapter 2).

1.4.5 *Melissoaspis fisheri* Ben-Dov, 2010: 51–52

Additional collections of this species have been made from the nests of *Melissotarsus insularis* Santschi in Madagascar (nine specimens, eight from two new localities). Adult females lack scale covers. Identity of specimens confirmed by Y. Ben-Dov.

1.4.5.1 Material examined. MADAGASCAR, Toliara, Berenty, Forêt de Bealoka, 14.6 km 329° NNW Amboasary, 24°57'24.84" S, 46°16'17.4" E, elevation 35m, 3–8.ii.2002 (B.L. Fisher), six adult ♀ (D1885B,C,D,E,F, D2733C) (UMEC); MADAGASCAR, Toliara, Forêt de Mîte, 20.7 km 29° WNW Tongobory, 23°31'27.12" S, 44°07'16.6794" E, elevation 75m, 27.ii.2002–2003.iii.2002 (B.L. Fisher), one adult ♀ (D1895A) (CASC); MADAGASCAR, Toliara, Andohahela National Park, Manantalinjo Forest, 33.6 km 63° ENE Amboasary, 7.6 km 99° E Hazofotsy, 24°49'0.84" S, 46°36'35.9994" E, elevation 150 m, 12.i.2002 (B.L. Fisher), two adult ♀ (D1897A,C) (CASC).

1.4.6 *Melissoaspis formicaria* (Ben-Dov) comb. n.

Morganella formicaria Ben-Dov, 2010: 54–56

Prior to Takagi's (2007) revision of *Morganella*, five African species were placed in the genus: *M. acaciae* Munting, *M. conspicua* (Brain), *M. pseudospinigera*

Balachowsky, *M. spinigera* (Lindinger), and *M. vuilleti* (Marchal). Takagi excluded all five of these species from *Morganella*, and remarked that they were not particularly closely related to *Morganella*, but did not indicate to what genus they ought to belong. When Ben-Dov (2010) described *M. formicaria* and placed it in *Morganella*, he did not cite Takagi (2007) or propose an alternative to Takagi's restricted definition of *Morganella*.

The transfer of this species to *Melissoaspis* was based upon the poor development of median lobes that appear continuous with abdominal segment VIII, distinctive lanceolate setae on abdominal segment VIII, and a pygidium that is compressed and triangular in shape. These combined characteristics are unique to *Melissoaspis*.

Additional collections of this species have been made from the nests of *M. insularis* in Madagascar (six specimens, two from a new locality). Adult females lack scale covers.

1.4.6.1 Material examined. MADAGASCAR, Toliara, Forêt de Beroboka, 5.9 km 131° SE Ankidranoka, 22°13'59.16" S, 43°21'58.68" E, elevation 80m, 12–16.iii.2002 (B.L. Fisher), three adult ♀ (D1880A, D1883B,C) (UMEC); MADAGASCAR, Toliara, Berenty, Forêt de Bealoka, 14.6 km 329° NNW Amboasary, 24°57'24.84" S, 46°16'17.4" E, elevation 35m, 3–8.ii.2002 (B.L. Fisher), one adult ♀ (D1882A) (CASC); MADAGASCAR, Toliara, Forêt de Mîte, 20.7 km 29° WNW Tongobory, 23°31'27.12" S, 44°07'16.6794" E, elevation 75m, 27.ii.2002 (B.L. Fisher), two adult ♀ (D1890A,B) (CASC).

1.4.7 *Melissoaspis incola* Schneider sp. n.

(**Fig. 1.4**)

http://zoobank.org/urn:lsid:zoobank.org:act:7866FBCDE4A9-47AC-AB6C-A5C3FD709BD1

1.4.7.1 Description of adult female. Scale cover unknown; all specimens of type series lack scales. Mounted on microscope slide, body circular to ovoid, 0.44–0.6mm long, widest at metathorax, 0.37–0.46mm wide. Median and second lobes present, simple and poorly developed. Median lobes with lateral notch, apex of each lobe rounded, medial edges either parallel or slightly converging. Second lobes about one-half length of median lobes and narrowly triangular coming to sharp pointed apex without notch, pressed closely to lateral edge of median lobes. Pygidium strongly constricted between abdominal segments V and VI, segments VI, VII, and VIII forming roughly triangular projection at posterior apex. Intersegmental space between segments IV and III with sclerotized bands, one medial and two lateral. Comma-shaped paraphyses in pairs between median and second lobes and at pygidial constriction between abdominal segments V and VI, medial paraphysis larger than lateral. Pair of segmental setae delineating abdominal segment VIII distinctively stout, lanceolate, extending slightly beyond apex of median lobes, 6–8 μm long; remaining pygidial segmental setae stout, flagellate, 20–28 μm long. Plates absent. Few long, thin dorsal macroducts of one-barred type present primarily at pygidial margin, one or two sometimes present at submargin, one situated between median lobes; shortest at anterior 16–28 μm, longest at posterior apex 32–44 μm, diameter of each macroduct opening approximately 1 μm. Pygidium with pair of microducts with protruding orifices that extend beyond body margin, resembling simple plates; position variable but falling between abdominal segments IV–VI. Few submarginal and submedial ventral microducts present on head, thorax, and abdominal segments I–III, 9–18 μm long. Anal opening10–14 μm wide at longest axis, located 0.5–1.5 times the width of anal opening from the pygidial apex. Opening of vulva

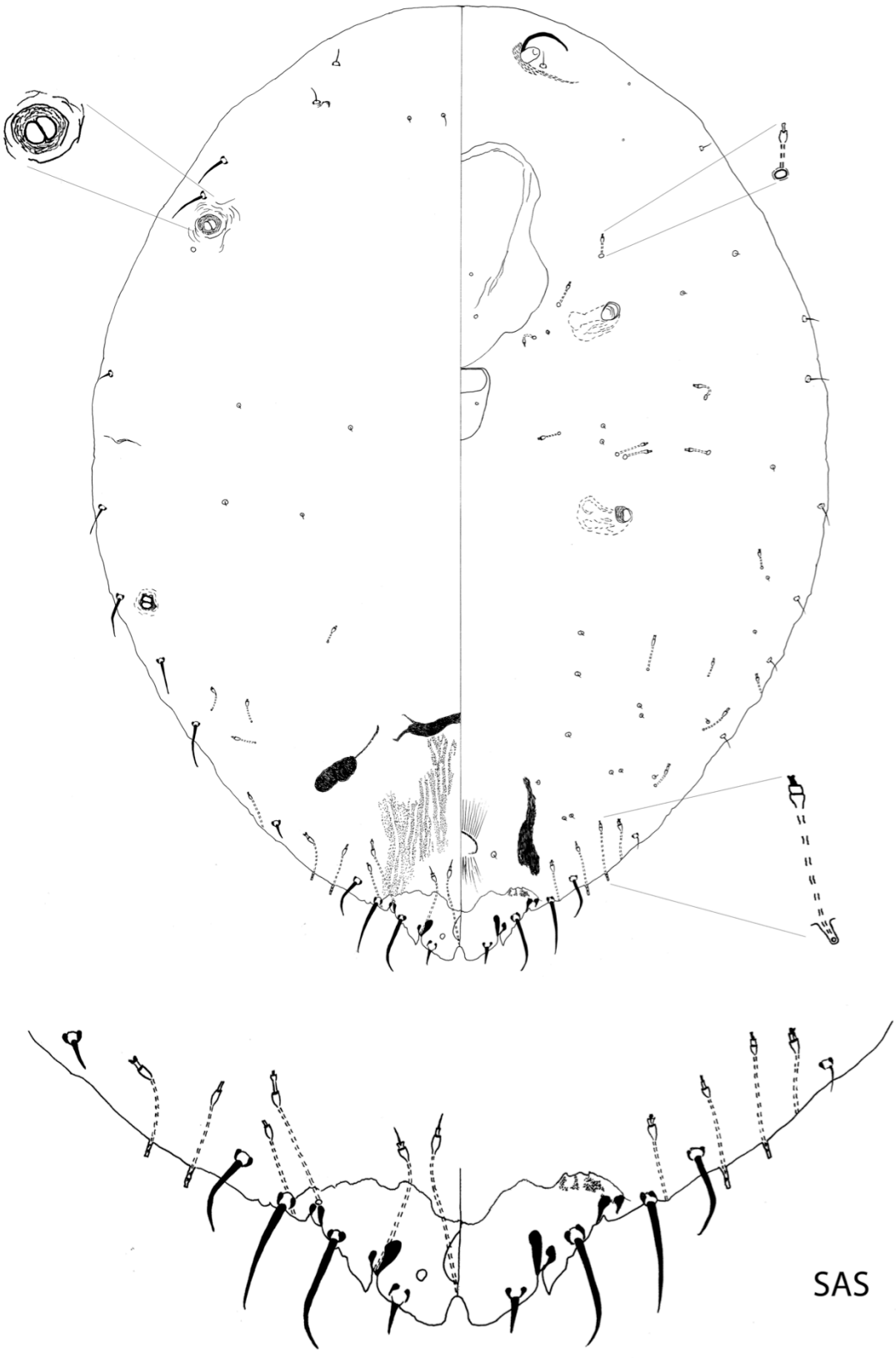


Fig. 1.4. *Melissoaspis incola* **sp.n.** adult female.

approximately 16–22 μm wide, situated 2.5–5 times further from the pygidial apex than the anal opening. Pairs of cicatrices present on prothorax and abdominal segment I, diameter 8–10 μm. Eyes present and indicated by small dome-like projections at submargin of head. Without perivulvar pores. Antennal tubercles each with one stout seta.

1.4.7.2 Material examined. **Holotype**: Adult ♀, MADAGASCAR, Toliara 6 km 146° SSE Belo sur Mer, 20°46'18.1194" S, 44°02'48.12" E, elevation 15m, found in nest galleries of *M. insularis* from *Euphorbia* sp., ID# D1875D, 10.xii.2001 (B.L. Fisher) (CASC). **Paratypes**: Same data as holotype, one adult ♀(D1875A) (UMEC); MADAGASCAR, Toliara, 6 km 131° SE Lavanono, Soamanitra, elevation 150 m, 25°26'44.1594" S, 44°59'44.88" E, found in nest galleries of *Melissotarsus insularis* from Euphorbiaceae sp. undet., 17.ii.2002 (B.L. Fisher), one adult ♀ (D1877A) (USNM); MADAGASCAR, Toliara airport, 23°22'59.8794" S, 43°43'0.12" E, elevation 40m, found in nest galleries of *M. insularis* from Euphorbiaceae sp. undet., 7.i.2001 (B.L. Fisher), one adult ♀ (D1896A) (BMNH), one adult ♀(D1896B) (UMEC), one adult ♀ (D1896C) (USNM).

1.4.7.3 Etymology. The Latin noun '*incola*' means 'resident' and is used here in reference to the symbiotic relationship that exists between this species and *Melissotarsus* ants. Like its congeners, *M. incola* is unknown outside the nest galleries of *M. insularis*.

1.4.7.4 Comments. Adult females of *M. incola* are most similar in appearance to *M. formicaria*, particularly in that both species lack the distinctive reticulated light and dark patterning that is found on the dorsal pygidium of *M. fisheri* and *M. reticulata*. The following suite of characteristics distinguishes *M. incola* from its congeners. The median

lobes in adult females of this species possess a lateral notch and the second lobes are without notches. By contrast, none of the lobes are notched in *M. fisheri* and *M. reticulata*, and in *M. formicaria* this trait is reversed, i.e., the second lobes possess a notch rather than the median lobes. The adult female of *M. incola* is further distinguished from *M. formicaria* by the absence of ventral microducts on the pygidial submargin and the presence along the pygidial margin of protruding microduct orifices resembling simple plates or gland spines.

1.4.8 *Morganella conspicua* (Brain)

Diaspis (Epidiaspis) conspicua Brain, 1919: 228.

Morganella conspicua (Brain); Balachowsky, 1956: 124.

Additional collections of this species were made from Madagascar in association with *M. insularis*, from South Africa in association with *M. emeryi*, and from Cameroon in association with *M. weissi* (35 new specimens, 32 from 12 new localities). Male prepupal and pupal instars from ant-associated populations form normal scale covers, but adult females and second-instar nymphs lack scale coverings. Collections from South Africa also represented free-living diaspidids with scale covers found on the exterior bark of *Leucospermum praemorsum* containing ant and armored scale insect populations lacking scale covers (identified as D3610B,C,D, D3611B). *M. conspicua* is apparently the most geographically widespread of the ant-associated diaspidids.

Following Takagi (2007), this species clearly does not belong in *Morganella*, but lacking allocation to another genus, for the present it remains in *Morganella*.

1.4.8.1 Material examined. MADAGASCAR, Toliara, Libanona, Tolganaro, 25°2’13.9194” S, 46°59’53.88” E, elevation 35 m, 10.i.2001 (D.O. Burge), one adult ♀

(D1881A) (CASC); MADAGASCAR, Toliara, Réserve Privé Berenty, Forest Bealoka, Mandraré River, 14.6 km 329° NNW Amboasary, 24°57’24.84” S, 46°16’17.4” E, elevation 35m, 3–8.ii.2002 (B.L. Fisher), one adult ♀ (D1892B) (CASC); MADAGASCAR, Toliara, Andohahela National Park, Manantalinjo, 33.6 km 63° ENE Amboasary, 7.6 km 99° E Hazofotsy, 24°49’0.84” S, 46°36’35.9994” E, elevation 150 m, 12–16.i.2002 (B.L. Fisher), two adult ♀ (D1898A,B) (UMEC); SOUTH AFRICA, Western Cape, Clanwilliam, Nardouw farm, 32°0’4.26” S, 18°50’20.40” E, elevation 358 m, 5.i.2012 (S.A. Schneider), four adult ♀ (D3559A,C, D3579A, D3582A) (UMEC); SOUTH AFRICA, Western Cape, Clanwilliam, Duikerfontein farm, 32°1’55.56” S, 18°51’54.30” E, elevation 439 m, 4.i.2012 (S.A. Schneider), one adult ♀ (D3567A) (UMEC); SOUTH AFRICA, Western Cape, Clanwilliam, Duikerfontein farm, 32°2’3.78” S, 18°52’2.82” E, elevation 443 m, 5.i.2012 (S.A. Schneider), two adult ♀ (D3570A, D3572A) (UMEC); SOUTH AFRICA, Western Cape, Clanwilliam, Duikerfontein farm, 32°1’55.80” S, 18°51’52.86” E, elevation 437 m, 5.i.2012 (S.A. Schneider), four adult ♀ (D3573A, D3575A, D3576A, D3577A) (UMEC); SOUTH AFRICA, Western Cape, Clanwilliam, Brakfontein farm, 31°54’58.14” S, 18°46’15.66” E, 5.i.2012 (S.A. Schneider), two adult ♀ (D3588A, D3589A) (UMEC); SOUTH AFRICA, Western Cape, Gifberg near Vanrhynsdorp, 31°48’36.48” S, 18°46’24.78” E, elevation 400 m, 5.i.2012 (S.A. Schneider), two adult ♀ (D3599A, D3600A) (UMEC); SOUTH AFRICA, Western Cape, Clanwilliam, Brakfontein farm, 31°54’32.34” S, 18°45’49.14” E, elevation 349 m, 5.i.2012 (S.A. Schneider), three adult ♀ (D3606A, D3607A, D3608A) (UMEC); SOUTH AFRICA, Western Cape, Clanwilliam, Nardouw farm, 31°59’33.30” S, 18°49’14.76” E, elevation 386 m, 6.i.2012 (S.A. Schneider), eight

adult ♀ (D3610B,C,D, D3611B, D3613A, D3614A, D3615A, D3616A) (UMEC); SOUTH AFRICA, Western Cape, Clanwilliam, Nardouw farm, 31°59'33.00" S, 18°49'16.86" E, elevation 389 m, 6.i.2012 (S.A. Schneider), two adult ♀ (D3620A, D3621A) (UMEC); SOUTH AFRICA, Western Cape, Clanwilliam, Nardouw farm, 31°59'32.04" S, 18°49'15.60" E, elevation 389 m, 6.i.2012 (S.A. Schneider), one adult ♀ (D3631A) (UMEC); CAMEROON, Nkolbisson, 1°9'44.57" N, 11°42'9.83" E, elevation 602 m, 22.iv.2012 (A. Doumtsop), two adult ♀ (D3668A, D3672A) (UMEC).

1.5 Key to the species of ant-associated armored scale insects (adapted from Ben-Dov, 2010)

1. Pores present near anterior and/or posterior spiracles; macroducts and microducts of two-barred type 2

– Pores absent from areas adjacent to spiracles; macroducts and microducts of one-barred type 3

2. Antennal tubercle with six setae; pygidium with two pairs of lobes . . . . . *Andaspis formicarum* Ben-Dov

– Antennal tubercle with single seta; pygidium with five pairs of lobes . . . . . *Diaspis doumtsopi* Schneider **sp.n.**

3. Fringed plates present in first and second spaces; macroducts with large round openings occurring in pairs or triplets along margin of thorax through abdominal segment III . . . . . *Affirmaspis cederbergensis* Schneider **sp.n.**

– Plates present or absent; if present, never fringed; macroducts of this type absent from margin of metathorax through abdominal segment III 4

4. Body strongly constricted near proximal base of pygidium at abdominal segment V; pygidium compressed and roughly triangular; stout spine-like setae on abdominal segment VIII; plates absent; median lobes simple and poorly developed . . (*Melissoaspis*) 5

– Body gently tapering toward posterior end without a strong constriction near proximal base of pygidium; pygidium may be flat, rounded, or triangular in shape; stout flagellate setae on abdominal segment VIII; plates present or absent; median lobes well developed and sclerotized 8

5. Pygidial dorsum with a reticulated pattern of bright and dark lines disposed perpendicular to margin; paraphyses typically absent 6

– Pygidial dorsum without such a reticulated pattern; paraphyses always present in pairs 7

6. Median lobes distinctly projecting from margin . . . . . *Melissoaspis fisheri* Ben-Dov

– Median lobes not projecting from margin . . . . . *Melissoaspis reticulata* Ben-Dov

7. Pygidium with submarginal microducts present on venter; median lobes without notch, second lobes with one lateral notch . . . . . *Melissoaspis formicaria* (Ben-Dov) **comb.n.**

– Pygidium lacking submarginal microducts on venter, only present along margin; median lobes with one lateral notch, second lobes without notch . . . . . *Melissoaspis incola* Schneider **sp.n.**

8. With ten pairs of paraphyses on pygidium . . . . . *Melanaspis madagascariensis* Mamet

– With two pairs of paraphyses on pygidium 9

9. All pygidial plates simple with pointed apices . . . . . *Morganella conspicua* (Brain)

– Some pygidial plates with bi- or trifurcating apices . . . . . *Morganella pseudospinigera* Balachowsky

1.6 Summary

The unique trophobiosis between *Melissotarsus* ants and Diaspididae remains poorly understood: any observations of foraging behaviors and interactions between species provide useful information regarding the nature of the association and merit attention. Future studies should focus on determining the diet of *Melissotarsus* ants and the nature of interactions between these unlikely partners. There are now ten described species of ant-associated armored scale insects (Ben-Dov and Fisher 2010 and the new species described herein) and it is likely that more new species await discovery within the galleries of *Melissotarsus* ants.

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synonym of *Diclavaspis*, and to Douglas Williams for his concurring opinion. I thank Stellenbosch University for providing access to laboratory space during my visit to South Africa. Special thanks go to Bennie and Carina Bezuidenhout for their gracious hospitality on Nardouw Farm and for their enthusiasm regarding this project. Thanks to John Martins (Mount Holyoke College) for providing assistance with figure editing.

CHAPTER 2

MOLECULAR PHYLOGENETICS OF ASPIDIOTINI ARMORED SCALE INSECTS (HEMIPTERA: DIASPIDIDAE) REVEALS RAMPANT PARAPHYLY AND MULTIPLE ORIGINS OF ASSOCIATION WITH *MELISSOTARSUS* ANTS (HYMENOPTERA: FORMICIDAE)

2.1 Abstract

Ant agricultural interactions are important model systems for studying mutualism. Our ability to study the evolution and ecology of mutualisms between ants and commonly associating scale insects is restricted by a limited understanding of scale insect systematics. The armored scale insects (Hemiptera: Diaspididae), a relatively well-studied family in this regard, provide a rare opportunity to study the evolution of myrmecophily across an entire scale insect family. Relationships between armored scales and *Melissotarsus* Emery ants are unique and could provide valuable insights regarding the interplay between predation and mutualism. In this article I reconstruct a molecular phylogeny for the Aspidiotini tribe of armored scale insects that expands greatly upon taxonomic and character representation from previous studies. Our dataset includes 127 species (356 terminal taxa) and four gene regions: 28S, EF-1α, COI-COII, and the newly included protein-coding gene CAD. Phylogenetic data were analyzed in a Bayesian framework using the MC^3 algorithm as implemented in MrBayes 3.2.6. I find that the majority of aspidiotine genera are paraphyletic as currently defined and provide recommendations that would increase taxonomic stability for this tribe. Myrmecophily among diaspidids has evolved no fewer than six times independently, four times within the Aspidiotini and two additional origins recorded from the Diaspidini. Relationships are labile at the species level and partnerships can shift. However, several clades of ant-

specialized armored scales have evolved, indicating that these relationships can be stable on an evolutionary timescale.

2.2 Introduction

Relationships between ants and partner species are important model systems for studying facets of mutualism, including interaction stability, symbiont fidelity, multi-trophic interactions, diversification rates and patterns, and lability vs. constraint (see Currie 2001, Pierce et al. 2002, Mueller et al. 2005, Stadler and Dixon 2005, Ivens 2014 for reviews). Most research efforts have focused on ant associations with fungal cultivars, lycaenid caterpillars, or aphids (Mueller et al. 2001, Stadler and Dixon 2008). Ants also frequently engage in relationships with scale insects (Hemiptera: Coccoidea), an important group of myrmecophiles that have largely been overlooked in studies of mutualism (Delabie 2001).

Scale insects are among the most commonly ant-tended taxa yet we know very little about the evolutionary history of myrmecophily for this clade. The families Pseudococcidae (mealybugs), Rhizoecidae (root mealybugs), Coccidae (soft-scales), and Stictococcidae contain the majority of ant-associated species. To date, few studies have used phylogeny of scale insects to examine the evolution of ant/scale associations (Ueda et al. 2008, Ueda et al. 2010, Schneider and LaPolla 2011).

Deficiencies in our understanding of scale insect systematics are partially responsible for our knowledge gap regarding the history of ant/scale symbioses. Coccoidea have great potential to serve as important evolutionary and ecological model systems but are seldom used due to an incomplete systematic framework (Hardy 2013).

Scale insects often are poorly sampled, have cryptic life histories, and pose various challenges for molecular study (Gullan and Cook 2007). The largest scale insect families comprise the majority of myrmecophilic species. Addressing these challenges requires conducting in-depth studies of scale insect phylogeny in concert with taxonomic revision. These efforts would enable a more comprehensive understanding of how myrmecophily has evolved within particular groups of interest and, more broadly, across families of Coccoidea.

Relationships between armored scale insects (Hemiptera: Coccoidea: Diaspididae) and *Melissotarsus* ants (Hymenoptera: Formicidae) provide an opportunity to investigate origins of myrmecophily across a relatively well-studied family of scale insects. The phylogeny of Diaspididae has been more closely studied using molecular data than most scale insect families (Hardy 2013). However, Andersen et al.'s (2010) recent phylogeny of Diaspididae emphasized the need for more thorough taxonomic and character sampling, particularly for the tribe Aspidiotini. Many nodes within this group remain unresolved and monophyly for most aspidiotine genera is questionable. Most of the myrmecophilous armored scale species fall within Aspidiotini and thus the challenge of systematic uncertainty applies in this situation, making it difficult to confidently ascertain the identities and relationships of ant-associated lineages. Increasing our taxonomic representation and improving phylogenetic estimations among aspidiotine species is necessary before drawing any meaningful conclusions about the origins of myrmecophily in this clade.

Aspidiotini contains a large number of pest species and is an especially important group of scale insects, both in terms of economic importance and ecological significance

(Miller and Davidson 2005, Normark et al. 2014). Nearly 30% of the aspidiotine species included in this study are identified as extremely polyphagous pests, known to feed on at least 20 different families of host plants (Miller and Davidson 2005, Normark and Johnson 2011).

This study addresses the challenges in scale insect systematics in order to evaluate the evolutionary history of diaspidid symbiosis with *Melissotarsus* ants. This study is the first of its kind in evaluating the evolution of myrmecophily across a family of scale insects. Furthermore, *Melissotarsus*/diaspidid mutualisms are particularly interesting because they represent unique ant symbioses in which neither honeydew nor secretory byproducts seem to play a role in the establishment and maintenance of interactions (Schneider et al. 2013). Thus this system may prove valuable in future studies on the intersection between predation and mutualism. I significantly expand upon Andersen et al.'s dataset (2010) by increasing both taxonomic representations across Aspidiotini, including newly sampled myrmecophilous species, and incorporating an additional nuclear protein-coding locus to the character set. I include a discussion of the current status of aspidiotine genera and suggestions for improving taxonomic stability in this clade. Finally, I map myrmecophily onto the resulting phylogeny and discuss the history of ant-association among the Diaspididae.

2.3 Methods

2.3.1 Taxonomic Sampling

For the purposes of this study I define the Aspidiotini as the monophyletic core aspidiotines identified as "Clade F" in Andersen et al. (2010). Characters defining this

clade within Aspidiotinae include early paternal genome elimination and a lack of pores near the anterior spiracles. Several genera that were traditionally recognized as belonging to Aspidiotini have been found to lie outside of it and are not considered here. One hundred twenty-eight species are represented from 31 out of a total of 89 aspidiotine genera, a nearly 4-fold increase in representation of aspidiotine species from the Andersen et al. study. *Aonidia*, *Parlatoria*, and *Prodigiaspis* serve as outgroups for core aspidiotines; eleven species from these genera comprise outgroups for our analyses. Amplification was attempted for all aspidiotine species that were available to the Normark lab group for molecular work as of January 2015; I aimed at sequencing three individuals per species, representing geographic variation when possible. Some species are represented by fewer than three specimens because of a lack of material or failure to amplify or sequence the target gene fragments. Unsuccessfully amplified taxa were attempted twice before being excluded from the dataset. For all ant-associated species, I attempted to sequence four or more specimens. The ant-associated species represented in our analyses include: *Affirmaspis cederbergensis*, *Melanaspis madagascariensis*, *Melanaspis* undescribed sp., *Melissoaspis fisheri*, *M. formicaria*, *M. incola*, *M.* undescribed sp., and *Morganella conspicua*. I also sequenced more than three specimens for particular taxa if there was reason to suspect patterns of cryptic species diversity, peripatric speciation, or other potentially interesting patterns of species diversity. The final full dataset comprised 356 specimens. Specimens are stored at the University of Massachusetts Amherst; dry material is frozen at -80 °C; preserved specimens are in 100% ethanol and stored at -20 °C. Sequence data were also downloaded from GenBank for six of the taxa included in our analyses.

2.3.2 Character Sampling

DNA extractions were completed using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Valencia, California) following the standard methods with modifications as outlined in Andersen et al. (2010), except that the two elution steps were each completed using 100µl AE buffer. The cuticle from each specimen was slide-mounted following the protocol of the Systematic Entomology Laboratory (SEL, ARS, USDA) at Beltsville, Maryland (http://www.ars.usda.gov/Main/docs.htm?docid=9832). Vouchers are kept at the University of Massachusetts Insect Collection.

Four gene fragments were used for molecular phylogenetic analysis: the D2 expansion segment of the large subunit ribosomal RNA gene (28S), 606 bp; a segment of the nuclear protein-coding gene Elongation Factor-1α (EF-1α), 952 bp; a segment of the nuclear protein-coding gene Carbamoyl-phosphate synthetase (CAD), 464 bp; and a region of mitochondrial DNA encompassing the 30 portion of cytochrome oxidase I (COI) and the 50 portion of cytochrome oxidase II (COII), 787 bp. The four gene regions add up to 2809 bp combined. Primer sets and standard PCR protocols are listed in **Table 2.1**. The forward amplification and sequencing primers for CAD (CAD_s2100dryr and CAD_s2103rdr respectively) were developed by SAS by first using the 787F primer from Moulton and Wiegmann (2004) and developing a new internal forward primer that amplified more successfully for aspidiotines. Sequencing of CAD amplicons was more successful when using the internal sequencing primer (**Table 2.1**). I used either GoTaq® Green or GoTaq® G2 hot-start polymerase (Promega Corporation, Madison, Wisconsin) for standard PCR amplification. PCR products were visualized using 1.5% agarose gel electrophoresis with SYBR® Safe (Life Technologies, Carlsbad, California) ultraviolet

Gene Region	Forward Primer	Reverse Primer	Annealing temperature profile
28S	28s_s3660 5' GAG AGT TMA ASA GTA CGT GAA AC -3'	28s_a335 5' TCG GAR GGA ACC AGC TAC TA -3'	58-48°C, -1°C/3 cycles + 11 cycles @ 48°C
EF-1α	EF-1α(a) 5'- GAT GCT CCG GGA CAY AGA G -3'	EF2rod 5'- ATG TGA GCG GTG TGG CAA TCC AA -3'	58-42°C, -2°C/3 cycles + 11 cycles @ 42°C
CAD	CAD_s2100dryr *(amplification)* 5' - CDA RAG TYA GCA CRA AGG T -3' CAD s2103rdr *(internal/sequencing)* 5' - GTT AGC ACR AAG GTD RG -3'	CAD R2564 5'- CAA TTT GCT TAT CCG AAA AAC -3'	50-40°C, -1°C/3 cycles + 5 cycles @ 40°C
COI-COII	C1-j-2753ywr 5' GTA AAC CTA ACA TTT TTY CCW CAR CA -3'	C2-n-3362 5' CCA CAA ATT TCT GAA CAT TGA CC -3'	35 cycles of 47°C for 1 minute

Table 2.1: PCR protocols – This table outlines the primers and standard PCR protocols used to amplify/sequence each of the four gene regions. The forward primers for CAD are newly designed for use on aspidiotine taxa. Their utility for other scale insect taxa has not yet been determined.

stain. PCR products were purified by treating with Exonuclease I and Shrimp Alkaline Phosphatase (Exo-SAP) (Affymetrix, Santa Clara, California) at 37°C for 25 minutes, followed by 80°C for 15 minutes to denature the proteins. Purified products were then sent to the UMass Genomics Resource Lab (Amherst, Massachusetts) for Sanger sequencing using an ABI Model 3130XL sequencer (Life Technologies, Carlsbad, California).

I used Sequencher 4.2 (Gene Codes Corporation, Ann Arbor, Michigan) to edit DNA sequence data. Sequence alignments for each locus were made by importing sequences to Mesquite 2.75 (Maddison and Maddison 2015) and conducting a MUSCLE alignment (Edgar 2004). These alignments were further refined in PASTA 1.6.0 (Mirarab et al. 2014). I kept the default settings in PASTA using MAFFT as the aligner tool, OPAL as the merger, FASTTREE as the tree estimator, and specified the model as GTR+G20. These settings were applied to all four gene sets for three iterations of tree estimation and re-alignment with the maximum subproblem set to 50% and decomposition set to centroid. I visually inspected the resulting alignments and decided that no further adjustments were required.

2.3.3 Phylogenetic analysis

Two concatenated datasets were generated for phylogenetic analyses, the full dataset containing all 356 taxa (127 species) and a restricted dataset with 330 taxa (120 species). The restricted dataset includes a taxon if data are available for at least 28S or for any combination of two from EF-1α, CAD, or COI-COII; 26 taxa were excluded after failing to meet these criteria. Each gene region was also analyzed independently. I calculated the fit of available evolutionary models to each data partition (i.e. gene fragment) in jModelTest 2.1.7 (Darriba et al. 2012) and compared models using the Bayesian Information Criterion (BIC). For each data partition the model of best fit was determined as follows: for 28S, the generalized time reversible model (Tavaré 1986) with invariant sites and gamma-distributed rates (GTR+I+G); for EF-1α, the three-parameter model (Kimura 1981) with unequal frequencies, invariant sites and gamma-distributed rates (TPM3uf+I+G); for CAD, the Hasegawa-Kishino-Yano model (Hasegawa et al. 1985) with invariant sites and gamma-distributed rates (HKY+I+G); and for COI-COII, the transversional model (Posada 2003) with invariant sites and gamma-distributed rates (TVM+I+G). The best fitting models were implemented in all subsequent analyses; the nexus-formatted data file is provided as supplementary material, including the concatenated taxon-by-character nucleotide matrix and MrBayes block detailing the parameter settings.

Bayesian inference using Metropolis-coupled Markov chain Monte Carlo (MC^3) methods were employed in MrBayes 3.2.6 (Ronquist and Huelsenbeck 2003) to reconstruct a phylogeny of the Aspidiotini based upon DNA sequence data. These analyses were completed with support from the Cyberinfrastructure for Phylogenetic

Research (CIPRES) Science Gateway 3.3 and Extreme Science and Engineering Discovery Environment (XSEDE) computational resources (Miller et al. 2010, Towns et al. 2014). For each concatenated dataset, 2 independent analyses were conducted concurrently with 4 chains each (3 hot, 1 cold); each analysis was allowed to run for 20 million generations, sampling parameters every 1000 generations. Stationarity was reached by 1 million generations as determined by visualizing the likelihood-by-generation plot, the potential scale reduction factor (PSRF 1.0), and the standard deviation of split frequencies (≤ 0.02). The first 5 million generations were discarded as the burn-in, leaving a total of 15,001 trees from each run available for reconstruction of a majority-rule consensus tree. A consensus tree was generated using the *sumt* command in MrBayes, providing branch lengths as substitutions per site and branch support values as posterior probabilities. FigTree 1.4.2 was used to format the majority-rule consensus trees (http://tree.bio.ed.ac.uk/software/figtree/). For independent genealogical analyses, the same methods as above were followed except that analyses were allowed to run for 10 million generations, the burn-in was set to 5 million, leaving 5000 trees per gene region available for constructing each consensus tree. Genealogies were used to assess congruence of nodes on the concatenated majority-rule tree for the restricted dataset via visual inspection (included as **Supplementary Fig. 1—4**; Appendix).

2.4 Results

2.4.1 Phylogenetic Results

The majority-rule consensus tree resulting from analysis of our restricted dataset reveals four independent origins of ant association among the Aspidiotini and confirms

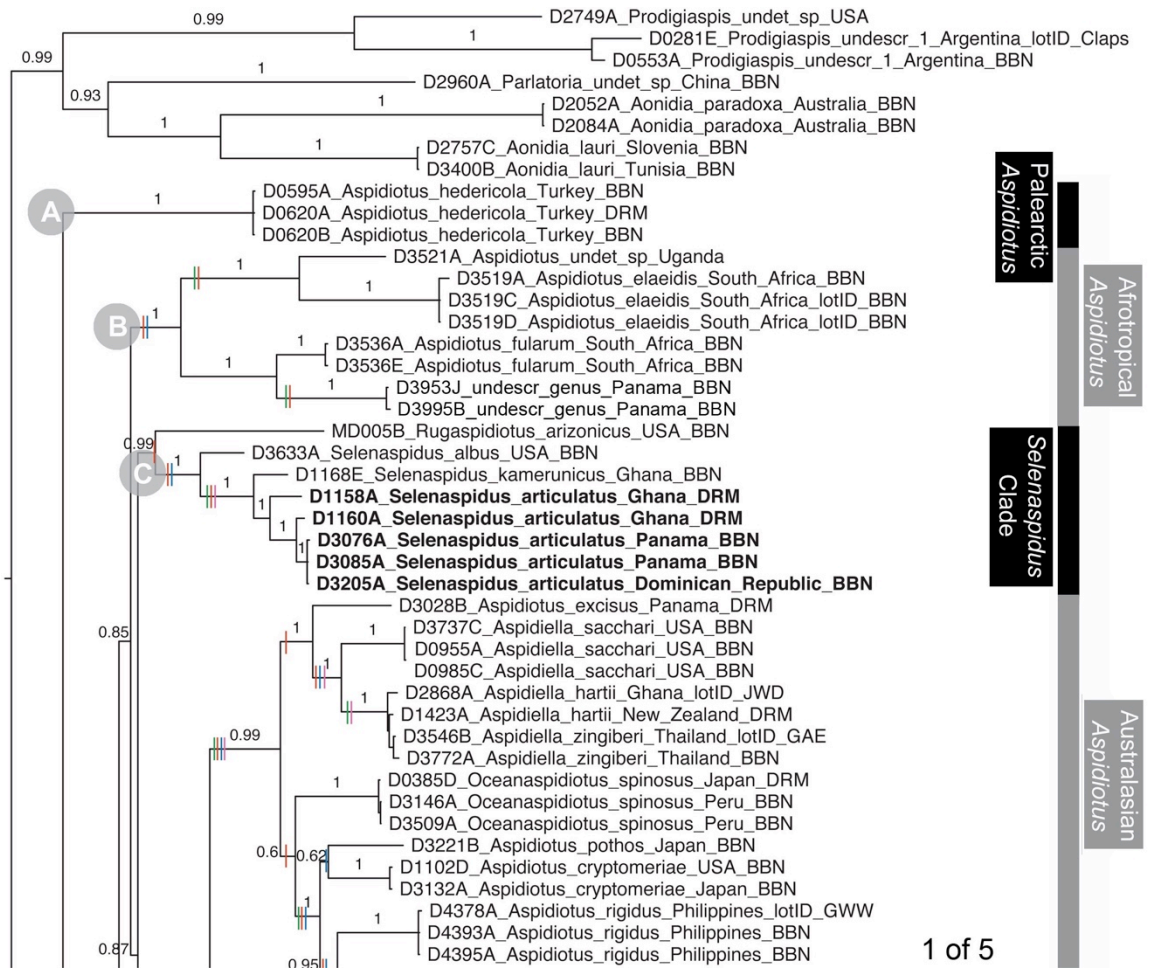
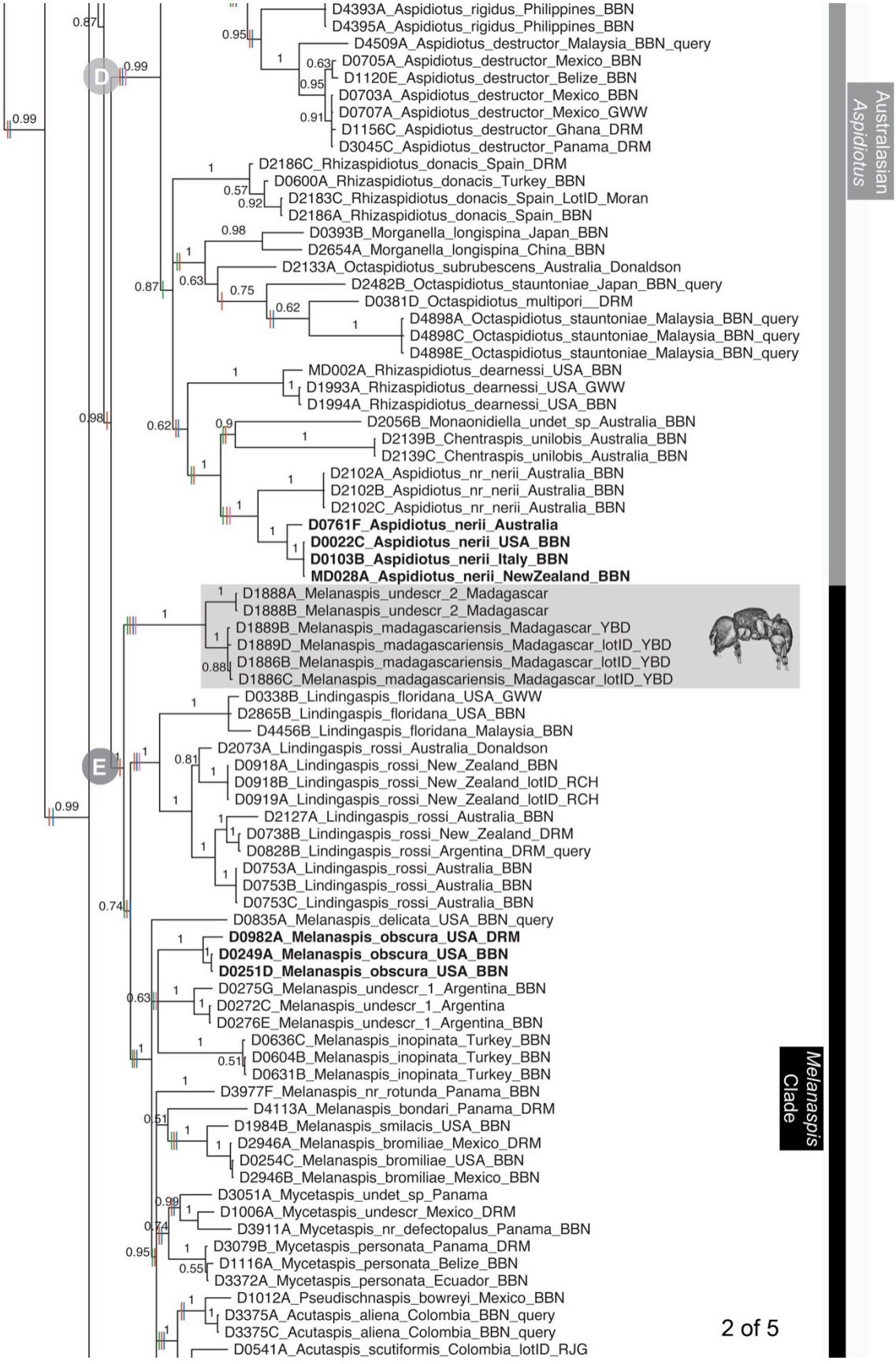


Figure 2.1: Phylogeny of the tribe Aspidiotini highlighting myrmecophilous clades — restricted taxon set – The majority-rule consensus tree resulting from Bayesian analysis of the concatenated dataset for four gene regions (28S, EF-1α, CAD, COI-COII) and a restricted taxon set of 120 species, 330 total taxa. Taxa are listed as: Identification # (e.g. D0001C)_Species name_Locality_Identifier. Specimens designated as "query" indicates uncertainty regarding their species designation. Those designated as "undescr" are undescribed new species. "Undet" specimens are unidentified. Congruency of nodes for each individual genealogy is indicated by colored bars: 28S = green, EF-1α = red, CAD = blue, COI-COII = pink). Congruency is only reported up to the level of species and "no bars" indicates that none of the individual genealogies recovered that particular grouping of species. Branch support is indicated as posterior probabilities. Clades A—J highlight various monophyletic groupings discussed in the text: Clade **A** depicts the Palearctic *Aspidiotus*, Clade **B** the Afrotropical *Aspidiotus*, Clade **C** the genus *Selenaspidus*, Clade **D** the Australasian *Aspidiotus,* Clade **E** the *Melanaspis* Clade, Clade **F** the *Diaspidiotus* Clade, and then the monophyletic genera *Davidsonaspis* (**G**), *Affirmaspis* (**H**), *Melissoaspis* (**I**), *Clavaspis* (**J**) and *Hemiberlesia sensu stricto* (**K**). Type species for genera (when available) are indicated in bold text. Alternating black and gray bars running along the right-hand side of the figure designate the major monophyletic divisions of the phylogeny. Myrmecophilous clades are outlined in a light gray box with a *Melissotarsus* worker depicted next to them.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D0761F_Aspidiotus_nerii_Australia
D0022C_Aspidiotus_nerii_USA_BBN
D0103B_Aspidiotus_nerii_Italy_BBN
MD028A_Aspidiotus_nerii_NewZealand_BBN
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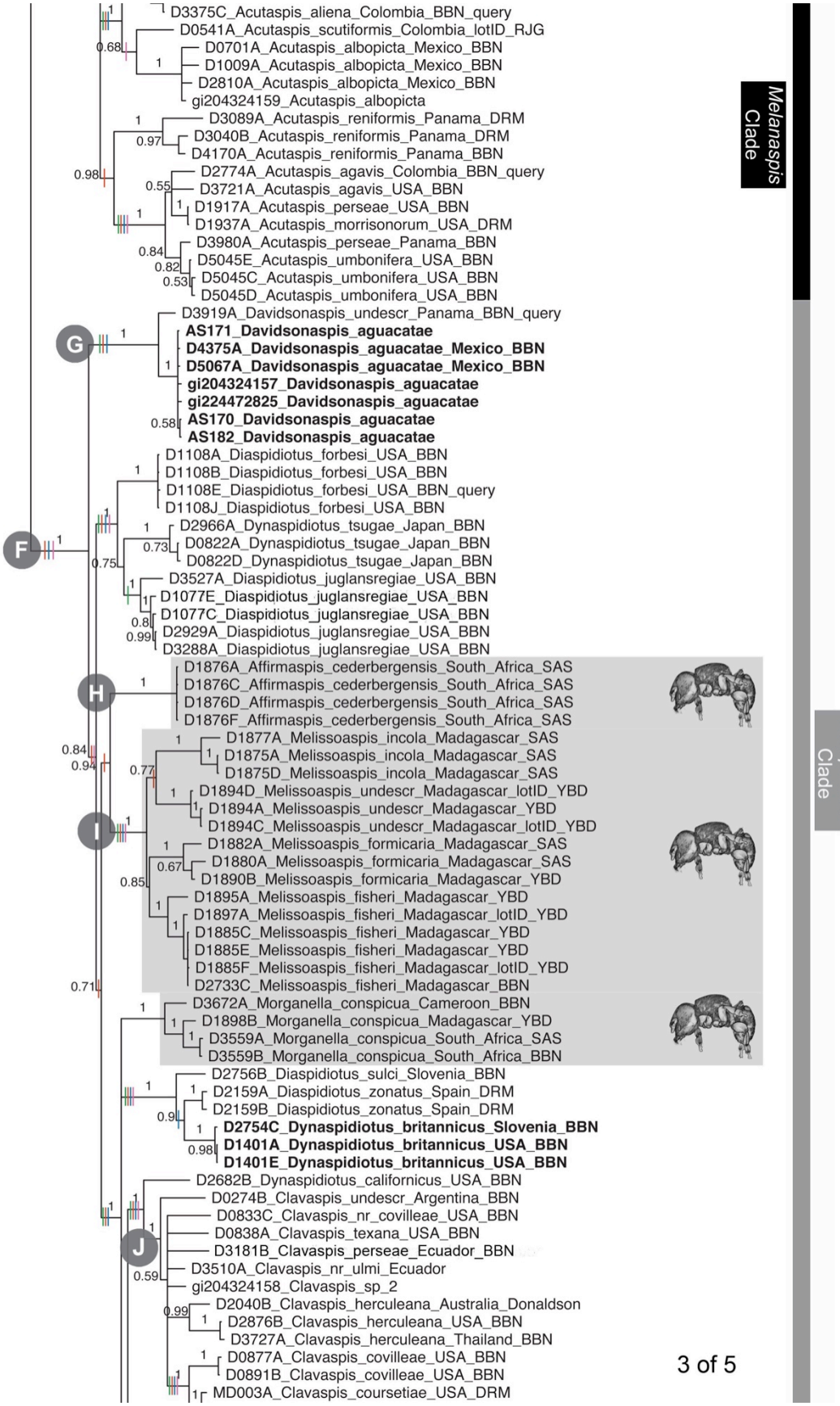
D
E
Australasian Aspidiotus
Melanaspis Clade
2 of 5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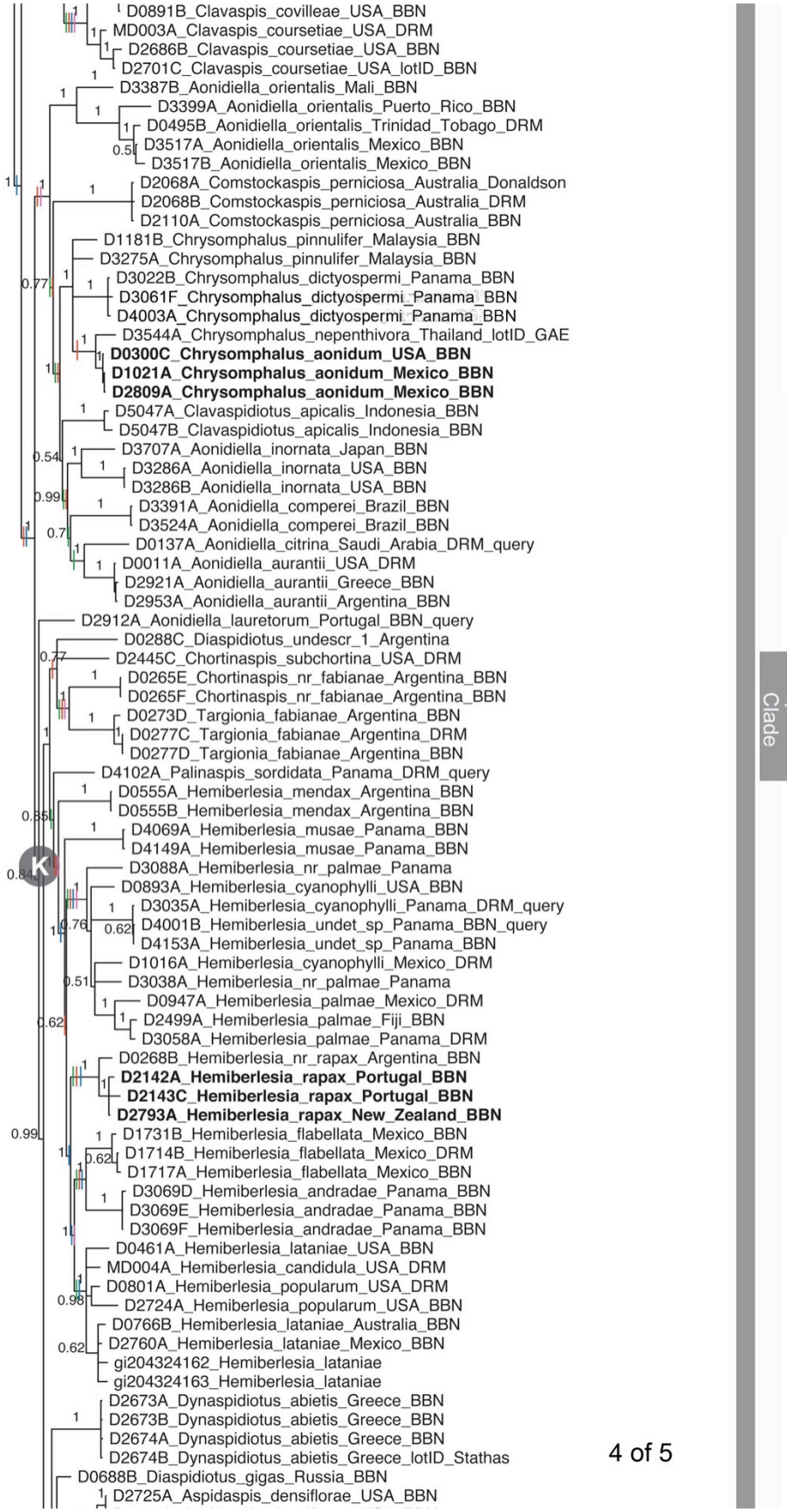
Melanaspis Clade
Diaspidiotus Clade
3 of 5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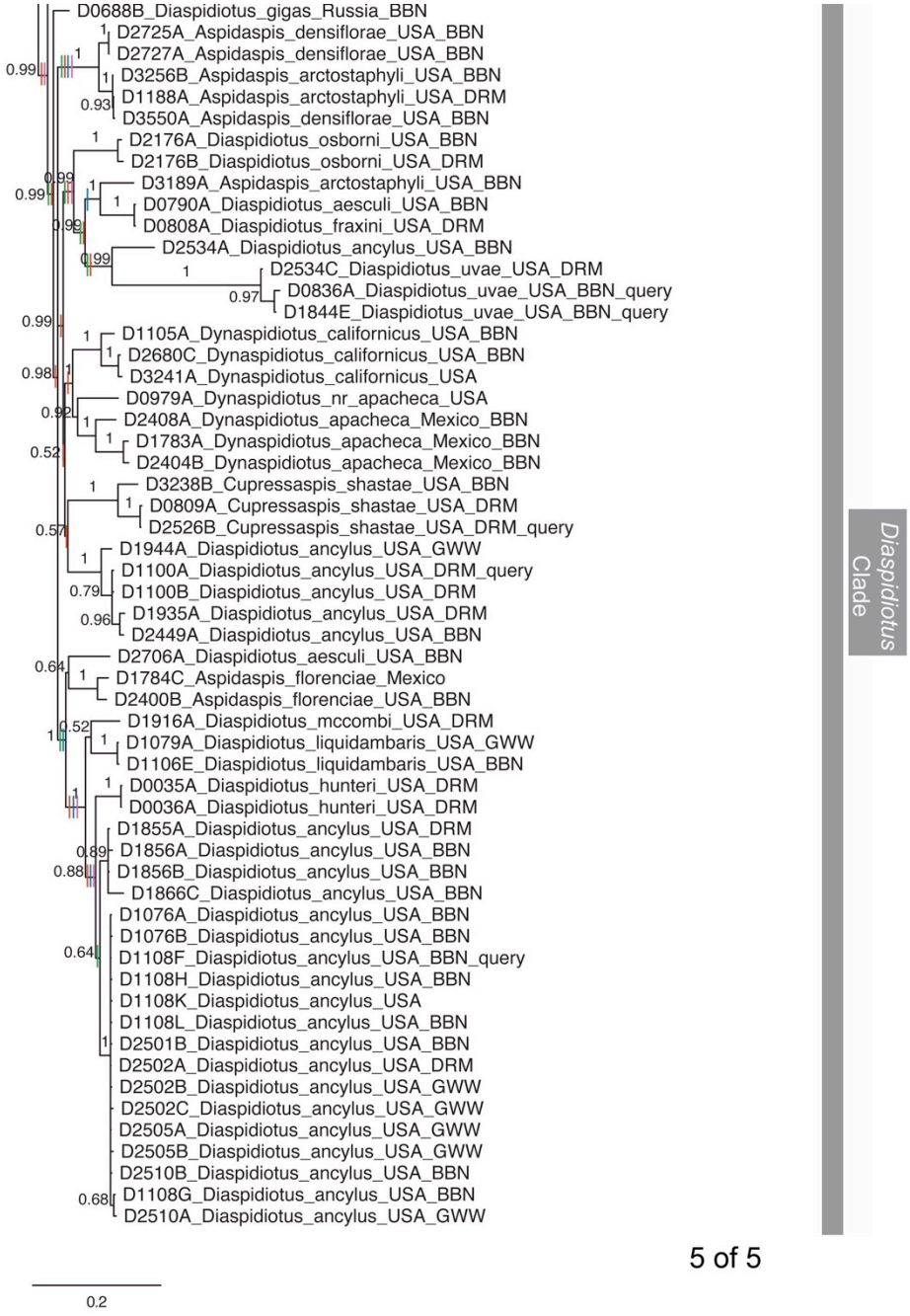
Diaspidiotus Clade
4 of 5



that most of the aspidiotine genera included are paraphyletic (**Fig. 2.1**). Results from the full dataset consensus tree (**Fig. S5**) are generally consistent with those from the restricted set. The greatest difference is in the placement of *Rugaspidiotus arizonicus* as either sister to the *Diaspidiotus* clade (full dataset) with weak support (posterior probability = 0.62) or as sister to the *Selenaspidus* clade (restricted dataset) with strong

support (pp = 0.99). The nodes separating *R. arizonicus* from *Selenaspidus* in the full dataset phylogeny are all weakly supported. The predominantly Afrotropical *Aspidiotus* clade (B in **Fig. 2.1**) also differs in position between analyses; however, the nodes responsible for this distinction are weakly supported in both consensus trees. The sister to the Afrotropical *Aspidiotus* clade remains indeterminate.

As expected, the majority of aspidiotine genera are non-monophyletic. For ease of discussion I identify six clades below, named for the genus that dominates each clade and describe both the phylogenetic patterns and status of genera within each clade (gray shading **Fig 2.1**). I also describe revisionary changes based upon our findings that would remedy various non-monophyletic designations and increase taxonomic stability within Aspidiotini. Following consideration of aspidiotine systematics, I report on the evolutionary origins of association between armored scale insects and *Melissotarsus* ants.

2.4.1.1 The Palearctic, Afrotropical, and Australasian *Aspidiotus* Clades

Aspidiotus is a polyphyletic genus with three distinct origins recovered in this analysis. *Aspidiotus hedericola* (Palearctic, Clade A) was recovered as the sister to the rest of Aspidiotini. This is interesting because many species across the Aspidiotini have at one point been described as belonging to *Aspidiotus*. The Afrotropical *Aspidiotus* clade (B) is sister to clades C, D, and E as indicated on the phylogeny (**Fig. 2.1**). The remaining *Aspidiotus* species are found within the strongly supported Australasian *Aspidiotus* clade (D), which encompasses several other genera: *Aspidiella*, *Chentraspis*, *Chortinaspis*, *Monaonidiella*, *Morganella*, *Oceanaspidiotus*, *Octaspidiotus*, and *Rhizaspidiotus*. The geographic divisions of these clades refer to the species' regions of origin, not necessarily to their current distributions. Many of these species have expanded distributions due to

anthropogenic interference, primarily transportation on fruit and vegetable exports (Miller and Davidson 2005).

The majority of "*Aspidiotus"* clades require revision to establish monophyletic genera that are well supported by molecular data (**Fig. 2.1**). Clade D contains the type species *Aspidiotus nerii*, indicated in bold text (**Fig. 2.1**), and thus should remain *Aspidiotus*. This clade is strongly supported (pp = 0.99) and is recovered by 3 of 4 genes; 28S generally provides limited resolution to the interior nodes (**S1**). The results suggest that the genera listed above are synonymous with *Aspidiotus* (Clade D). Two new genera may need to be established to describe Clades A and B. Both clades are strongly supported (pp = 1) and recovered by EF-1α and CAD.

2.4.1.2 The *Selenaspidus* Clade

Selenaspidus (Clade C) is one of the few genera confirmed to be monophyletic with strong support (pp = 1; **Fig. 2.1**). This topology is recovered by 3 of 4 genealogies; 28S cannot resolve the relationship between *S. albus* and the remaining species. I consider *Rugaspidiotus* to belong within the *Selenaspidus* Clade rather than assigning it's own separate clade. The monophyly of *Rugaspidiotus* is questionable because the dataset only includes one specimen that is recovered as the sister to *Selenaspidus*. It is possible the inclusion of additional *Rugaspidiotus* and/or *Selenaspidus* species would indicate the two genera are synonymous.

2.4.1.3 The *Melanaspis* Clade

The *Melanaspis* Clade (E) is strongly supported (pp = 1) but revision of several genera would be necessary to achieve monophyly (**Fig. 2.1**). Our results indicate that *Acutaspis*, *Mycetaspis*, and *Pseudischnaspis* are junior synonyms of *Melanaspis*. Whether

or not *Lindingaspis* is also a junior synonym depends upon the positioning of the two ant-associated *Melanaspis* species, which are currently positioned as sister to the rest of Clade E but with weak support (pp = 0.74) deriving mainly from EF-1α. Both 28S and CAD cannot resolve the relationship and COI-COII recovers the two ant-associated species as nested within the rest of *Melanaspis*. *Lindingaspis* is monophyletic and should remain valid until these relationships are better resolved. The *Melanaspis* Clade is equivalent to the Melanaspidina described by Deitz and Davidson (1986).

2.4.1.4 The *Diaspidiotus* Clade

The large *Diaspidiotus* Clade (Clade F) is also strongly supported (pp = 1) and is recovered by all genes except 28S (**Fig. S1**). Within this clade there are four well-supported, monophyletic genera that require no taxonomic revision: *Davidsonaspis* (Clade G), *Affirmaspis* (Clade H), and *Melissoaspis* (Clade I), and *Clavaspis* (Clade J). The primary *Hemiberlesia* clade (Clade K) would also fall under this category were it not for *Hemiberlesia oxycoccus* being excluded, as indicated in the full dataset consensus tree (**Fig. S5**). The remaining genera require extensive revisionary work. The genera *Diaspidiotus* and *Dynaspidiotus* are polyphyletic with respect to Clade F. *Aspidaspis*, *Chortinaspis*, *Cupressaspis*, *Morganella*, *Palinaspis*, and *Targionia* are among the list of genera that require further taxonomic consideration. The available molecular data do a poor job at resolving relationships among this clade. Short branches, like the one uniting *Hemiberlesia* (Clade K), are commonplace and likely reflect a history of rapid diversification in this clade.

Our analyses do identify particular clades within the *Diaspidiotus* Clade that would form monophyletic genera following revision. The genera *Aonidiella*,

Chrysomphalus, *Clavaspidiotus*, and *Comstockaspis* form a strongly supported monophyletic clade (pp = 1), as recovered by EF-1α and COI-COII, which could be synonymized as *Chrysomphalus*. However, this clade excludes *Aonidiella lauretorum* so some further revision of this species would be necessary. The true *Dynaspidiotus* can be identified as the clade containing the type species, *D. britannicus* (in bold **Fig. S5**), as well as *Diaspidiotus sulci* and *Diaspidiotus zonatus*. The clade is strongly supported (pp = 1) and recovered by all four genes. The remaining *Dynaspidiotus* species are distributed in three different locales across Clade F. The true *Diaspidiotus* remains a mystery because the type species, *Diaspidiotus ostreaeformis* (Curtis), is not included. Unfortunately this leaves several demonstrably paraphyletic genera with equivocal taxonomic designations for the present: *Cupressaspis* (*Aonidia*), *Aspidaspis*, *Chortinaspis*, the remaining *Dynaspidiotus*, *Morganella*, and *Targionia*.

2.4.2 Origins of ant association

My analyses recover four independent origins of ant association in Aspidiotini: *Affirmaspis cederbergensis*, the *Melanaspis* clade (2 species), the *Melissoaspis* clade (5 species), and *Morganella conspicua*. Associated species or clades are outlined on the phylogeny with a gray shaded box and an image of a *Melissotarsus* worker next to them (**Fig. 2.1**). *Affirmaspis cederbergensis* was found to be sister to *Melissoaspis* but it may be appropriate to treat each as representing an independent origin because the node linking them is weakly supported (pp = 0.94) and only recovered by EF-1α. Our taxonomic sampling may bias the result as well; there are several species of *Affirmaspis* not represented in our dataset and their inclusion could definitively separate *A. cederbergensis* from *Melissoaspis*. The origins of association for the *Melanaspis* clade

and *M. conspicua* are unequivocally independent of the others. Two additional origins of ant association are known from the tribe Diaspidini (unpublished data) involving *Andaspis formicarum* from South Africa (Ben-Dov 1978) and *Diaspis doumtsopi* from Cameroon (Schneider et al. 2013). A newly discovered species of *Diaspis* from Uganda, determined by SAS to be sister to *D. doumtsopi* based upon morphological traits of adult females (unpublished data), has also been discovered in association with *Melissotarsus weissi*.

Our analyses reveal that there are two or three clades of strictly myrmecophilous armored scale insect species. The best example of this is the *Melissoaspis* clade from Madagascar, with five species all known exclusively from populations associated with *Melissotarsus insularis*. One of the five members of *Melissoaspis* is a new species that has not yet been described but is genetically distinct from the others in our analyses. The *Diaspis* clade, consisting of two species from Diaspidini, is another example of species known only from *Melissotarsus* galleries. Northern African colonies have been sparsely sampled and it is possible that more *Diaspis* species may be discovered from *M. weissi* galleries. *Melanaspis madagascariensis* and a new sister species revealed in this study are both associated with *Melissotarsus insularis*. *Melanaspis madagascariensis* was originally described from a free-living population with normal scale covers (Mamet 1951). Yair Ben-Dov identified specimens as *Melanaspis madagascariensis* but molecular data from both free-living and ant-associated populations should be used to confirm that they are the same species. It is possible that ant-associated populations resemble *M. madagascariensis* but are actually a separate, obligately associated species. *Morganella conspicua* associates with all four *Melissotarsus* species but the relationship

is apparently facultative. This species was originally described from free-living populations and has also been found living on the exterior bark of trees colonized by *Melissotarsus* (Prins et al. 1975, Schneider et al. 2013).

2.5 Discussion

The phylogeny of the Aspidiotini confirms that this tribe requires extensive taxonomic revision and helps lay the groundwork for future endeavors (**Fig. 2.1**). Only 7 of the 31 genera included in our analyses (~23%) require no revisionary changes to be considered monophyletic: *Affirmaspis*, *Clavaspis*, *Davidsonaspis*, *Lindingaspis*, *Melissoaspis*, *Rugaspidiotus*, and *Selenaspidus* (**Figs. 2.1, S5**). Providing the means for reliable and stable taxonomic identification of damaging pest species is of paramount importance to agricultural research. The taxonomic revisions suggested herein would establish monophyly for 25 of the 31 genera included in these analyses (80%) leaving six genera in need of further consideration.

A few aspidiotine species may also require taxonomic revision (**Fig. S5**). One example is the extremely polyphagous pest *Diaspidiotus ancylus*, which shows up in three separate locations on our phylogeny. In this case, specimens might be misclassified, there might be multiple cryptic species described as *Diaspidiotus ancylus*, or the species is extremely diverse and has given rise to several specialists. Interestingly, several nodes on the tree might illustrate peripatric speciation events in which a more recently diverged species appears nested within the parental species. This could explain why *Hemiberlesia candidula* and *H. popularum* are nested within a more widespread polyphagous pest

species, *H. lataniae*. Alternatively, these nodes may comprise multiple synonymous species that require revision or they may represent host morphs.

Our analyses were unable to fully resolve relationships within the *Diaspidiotus* Clade likely due to a history of rapid radiation. Morphological traits from immature instars and adult males might prove more useful in reconciling these relationships, where traits from adult females and molecular data have failed to do so (Hodgson and Hardy 2013).

2.5.1 Patterns of Myrmecophily Across Diaspididae

Armored scale insects have evolved to engage in mutualisms with *Melissotarsus* ants at least six times independently. This observed pattern of association conforms to expectations drawn from other ant mutualisms. Some agricultural ant taxa will readily associate with various myrmecophilic species rather than demonstrate patterns of strict partner fidelity (Maschwitz and Hänel 1985, Blüthgen et al. 2006, Schneider and LaPolla 2011). Often an ant taxon will associate with a particular clade or clades of specialized mutualists in which relationships are labile at the species level and frequently shift, as demonstrated among the attine ants (Schultz et al. 2015). Species-level relationships shift among *Melissotarsus* colonies; each of the four ant species has been found to associate with different diaspidid species across colonies. Likewise, some diaspidid species associate with more than one ant species. These patterns stand in contrast to typical host-endosymbiont or host-parasite patterns of association that often demonstrate a higher degree of partner fidelity, and sometimes result in cophylogenesis (Hafner and Page 1995, Page 2003, Vienne et al. 2013).

The aspidiotine phylogeny (**Fig. 2.1**) shows that several diaspidid clades have associated with *Melissotarsus* ants for extended periods of time; long enough for speciation and diversification to have occurred after their ancestors first engaged in myrmecophily. These long-lasting relationships demonstrate stable association between lineages on an evolutionary timescale. *Melissoaspis* is a prime example for specialization on myrmecophily; all five species are known exclusively from *Melissotarsus* galleries. Both *Diaspis* (not shown) and *Melanaspis* have also been ant-associated long enough for speciation events to occur.

One distinct characteristic of ant/diaspidid mutualisms is that it is difficult to identify a specific clade of primary associates among the Diaspididae. Agricultural ants often have a long history of association with one particular "primary" group of mutualists, with fewer numbers of colonies acquiring novel "secondary" associates from outside of this group. For example, *Acropyga* ants primarily associate with root mealybugs from the subfamily Xenococcinae but a subset of colonies associate with scale insects from outside of this clade (LaPolla 2004, Smith et al. 2007, LaPolla et al. 2008, Schneider and LaPolla 2011). The Xenococcinae have a long history of association with *Acropyga* (Johnson et al. 2001) and it is clear that associations evolved between the common ancestors of both clades ~40 MYA. It is not apparent which diaspidids first evolved associations with the ancestor of extant *Melissotarsus* species. It is possible that none of the myrmecophilous species represented herein are descendants of the original ant-associated armored scales. This suggests that there is a certain degree of fluidity in ant/diaspidid relationships. Ant colonies are apparently opportunistic in their choice of partners and species have acquired novel diaspidid associates repeatedly throughout their

history. Perhaps the potential for myrmecophily is commonplace among the Diaspididae, requiring only the opportunity for the association to arise.

Myrmecophily is not restricted to any particular group of armored scales, although it does occur most frequently among aspidiotine species. It might be the case that any armored scale species that can successfully invade *Melissotarsus* galleries and tolerate association with ants could benefit from these interactions. This remains to be confirmed as no researchers have yet attempted to introduce new partners to *Melissotarsus* galleries. It would be interesting to see how readily colonies may adopt novel mutualists. It is worth noting that none of the known myrmecophilous armored scales belong to common pest species. One might think that commonly occurring species have a high likelihood of encountering *Melissotarsus* colonies and engaging in interactions with them. Apparently ant/diaspidid relationships face selective barriers in the early phases of establishment, but once a diaspidid species has adapted to myrmecophily it can readily associate with multiple *Melissotarsus* species.

Some diaspidid populations also appear to shift back and forth between free-living and myrmecophilous life histories. While association is always obligatory for *Melissotarsus* ants, there are examples both of facultative and obligate associates among the armored scales. Most myrmecophilic diaspidids are putatively obligate associates, known only from populations living in ant galleries. However, the most commonly encountered and widely distributed myrmecophile is a facultative associate, described from both free-living and ant-associated populations (Schneider et al. 2013). *Morganella conspicua* is an especially successful nest associate found in galleries from all four species of ants across the African continent and Madagascar. Why has a facultative

myrmecophile experienced greater success relative to other, obligately associating species? Facultative relationships might exist as a precursor to more derived obligate associations. It is also possible that sampling error has misled us and all armored scales are actually facultative associates. Facultative association could lend a competitive edge to the mutualism if the maintenance of free-living populations on the exterior of trees increases the likelihood for offspring to disperse to neighboring ant colonies.

2.6 Summary

Relationships between ants and scale insects are an important class of ant agricultural interactions that can provide key insights into the evolution of mutualisms. They are a key feature in the success of arboreal ants (Blüthgen et al. 2006) and can indirectly benefit host plants in ant/plant mutualisms (Pringle et al. 2011). Future research efforts should focus on continuing to improve the systematic framework for diverse scale insect families that frequently engage in myrmecophily, making them more accessible for studies of this kind.

2.7 Acknowledgements

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CHAPTER 3

FARMING AT SMALL SCALES: *MELISSOTARSUS* ANTS ARE PREDATORY MUTUALISTS OF ARMORED SCALE INSECTS

3.1 Abstract

Ants are commonly suspected of preying upon mutualist hemipterans, but very few studies have investigated the extent to which this actually occurs. Research suggests that ants prey on associates in a context-dependent manner, when associates are abundant and honeydew is readily available. Atypical associations between *Melissotarsus* ants and armored scale insects present the opportunity to study ant/hemipteran mutualisms in the absence of honeydew. A dietary study of *Melissotarsus emeryi* worker ants was conducted to determine if ants are predators of mutualist diaspidids. Stable isotope analyses were used to evaluate the relative trophic position of worker ants and determine if diaspidids serve as a significant source of dietary nitrogen and carbon for colonies. In addition, a molecular assay of ant gut contents was conducted to determine the presence of diaspidid DNA inside the gut of ants. The relative trophic position of worker ants was recovered as 1.1 ± 04 trophic levels above diaspidids. Elemental analyses indicate that diaspidids are a major contributing source of nitrogen and carbon for ant colonies. Diaspidid DNA is consistently amplified from the gut contents of associated ants. The combined results from these dietary studies strongly indicate that *Melissotarsus* ants are predators of their mutualist partners, diaspidids.

3.2 Introduction

Ants are generally considered to be opportunistic predators and yet a remarkable number of ant species engage in stable mutualisms with populations that are potential prey. The most common associations occur between ants and species of Hemiptera (Delabie 2001). Many ant-attended hemipteran populations are largely sessile or otherwise vulnerable to attack. Attendant ants are often suspected of exploiting relationships by consuming mutualist hemipterans, but the actual frequency of attack and impact of exploitative predation in ant/hemipteran mutualisms remains poorly understood (Bronstein 2001, Stadler and Dixon 2008). Relationships are stable despite the expectation that mild to moderate predation on mutualists occurs regularly.

Predation can play a multitude of roles in mutualistic interactions and substantially impact their dynamics. Although predation is typically considered to be antagonistic (Berryman 1992, Abrams 2000), under certain circumstances it can serve to stabilize mutualisms. This has been documented in three-species models involving two mutualist populations and a third-party predator; moderate levels of predation on one mutualist population can prevent the growth of both populations from escalating toward instability (Heithaus et al. 1980). In two-species models, the influence of predation on mutualisms is not as straightforward. Some research suggests that predation is tolerable between mutualist partners to a point but that exploited species might evolve mechanisms to exclude their exploiters (Bronstein 2001). For instance, some yucca plants selectively abort fruits that are infested with too many seed-eating pollinator larvae (Pellmyr and Huth 1994). Others have argued that ant/aphid interactions (and presumably other ant/insect associations) alternate between mutualism and predation (Cushman and

Addicott 1991). This implies that predation substantively disrupts mutualism dynamics in these systems. Stable mutualisms are more likely to evolve in symbioses where costs of association remain relatively low and benefits high; mortality is an inherently high cost to associates (Bronstein 2001, Leigh 2010).

In ant/hemipteran mutualisms, predation is thought to occur between partners in a context-dependent manner. The general expectation is that ants are most likely to consume mutualist partners when population densities of mutualists are high and honeydew is readily available (Sakata 1995). The need for honeydew as a reliable food source is a critical component of interactions; it may prevent attendant ants from feeding too heavily on mutualist hemipteran populations, which could result in negative feedback on their own success (Bull and Rice 1991). Under these circumstances, it is in the ants' best interest to limit predation on their insect partners and seek out other protein sources when honeydew is limiting. Empirical studies investigating these dynamics have been sparse (Pontin 1958, Sakata 1995, Ivens et al. 2012).

Predation might play a central role in symbioses between *Melissotarsus* ants and an unusual group of mutualists, the armored scale insects. *Melissotarsus* is an African ant genus that associates exclusively with insects belonging to the family Diaspididae (Ben-Dov and Fisher 2010, Schneider et al. 2013). Their relationships are unconventional compared to other ant/insect mutualisms. Diaspidids are unique among ant-associates because they are incapable of producing honeydew and do not exude any other known by-products as a food reward for ant attendance (Beardsley Jr and Gonzalez 1975). Researchers have speculated for decades as to how ants benefit from these obligatory relationships (Prins et al. 1975, Ben-Dov 1990, Mony et al. 2002, Ben-Dov and Fisher

2010). The prevailing hypothesis is that *Melissotarsus* ants farm armored scale insects as a source of 'meat' (Ben-Dov 1978, Schneider et al. 2013). If this is true then ant/diaspidid relationships are simultaneously predatory and mutualistic, implying that diaspidids experience a net benefit from ant association in spite of incurring high costs from mortality. Several armored scale clades have a long history of association with ants (Chapter 2) but the stability of associations cannot be attributed to any obvious third-party predators/competitors outside of the mutualism (Chapter 1) or to selective preferences for typical food rewards (i.e. honeydew). Predation of ants on diaspidids could stabilize the associations (Heithaus et al. 1980). Indeed, predation is likely to have an appreciable impact on the stability of ant/diaspidid mutualisms if the fundamental basis of their association is predatory in nature.

The reclusive nature of *Melissotarsus* colonies makes it difficult to study their foraging behaviors and diet. Colonies reside within a network of galleries excavated by workers in the bark of live trees. *Melissotarsus* workers enclose their galleries against the surrounding environment by forming a mortar from silk, sawdust and frass used to seal the entrances (Prins et al. 1975, Fisher and Robertson 1999). When a segment of the gallery roof is removed, most workers immediately divert their attention to repairing their enclosure, which serves as the primary defense for colonies (Schneider et al. 2013). Direct observations of foraging behavior are hindered by this cloistered habit, requiring more innovative approaches to study *Melissotarsus* diet.

One such approach takes advantage of the utility of naturally occurring stable isotopes to reconstruct relative trophic relationships. Stable isotope techniques are commonly used to untangle complex trophic webs (Post 2002, Layman et al. 2012). With

one trophic exchange (e.g. between a primary producer and primary consumer) the heavier nitrogen isotope, ^{15}N, is preferentially incorporated into the tissues of the consumer, becoming enriched relative to the concentration of the lighter, more abundant isotope, ^{14}N. The expected mean differences in the ratios of these isotopes ($\delta^{15}N$) have been estimated from a wealth of studies on trophic webs from terrestrial, aquatic, and marine systems (see Post 2002, Blüthgen et al. 2003, McCutchan et al. 2003, Fiedler et al. 2007 for representative examples). This allows for the estimation of the relative trophic position of *Melissotarsus* ants by calculating $\delta^{15}N$ among host plants, diaspidids, and ants in this study system. The same principle applies to carbon enrichment except that $\delta^{13}C$ is more useful in tracking carbohydrate sources than distinguishing trophic level (Post 2002). In this study, I used stable isotopic ratios for nitrogen and carbon from *Melissotarsus* ant ecosystems (involving host plants, associated diaspidids, and worker ants) to assess ant trophic level. I focused on associations between populations of the ant, *Melissotarsus emeryi*, and diaspidids, *Morganella conspicua*, from South Africa. Data for the trophic enrichment of ^{15}N and ^{13}C isotopes were tested for consistency with the hypothesis that armored scales are a contributing source of dietary nitrogen and carbon for ants.

I studied worker ant diet more directly by employing a molecular analysis of ant gut contents targeting armored scale mtDNA from preparations of worker ants. Sets of diaspidid-specific primers were used to test worker ants for the presence of armored scale DNA from gut contents in contrast to amplification results from their legs.

3.3 Methods

3.3.1 Trophic position and resource tracking – Study design

In January 2012 I collected specimens of host plants (*Leucospermum praemorsum*), resident ants (*Melissotarsus emeryi*), and associated diaspidids (*Morganella conspicua*) from 9 sites located in the Clanwilliam district of Western Cape, South Africa. Locality data for sampled colonies are recorded in **Table 3.1**. Specimens were stored in 100% ethanol prior to analysis. Samples of host plants, adult worker ants, and diaspidids were prepared for stable isotopic analysis first by drying specimens at 55°C for a minimum of 72 hours. All dried samples for each tissue type were finely ground with a mortar and pestle. Ant samples consisted only of the head and alitrunk; the gaster of worker ants was removed so that gut contents did not skew the calculation of $\delta^{15}N$ (Tillberg et al. 2006). Plant samples consisted of wood collected from areas adjacent to galleries where diaspidids feed. Samples of diaspidids consisted of second instar nymphs and adult females. Wood samples, ants and diaspidids were drawn from various segments of the galleries to better represent isotopic signatures across the colony. For each of 9 sites, $\delta^{13}C$ and $\delta^{15}N$ were calculated for 7 samples of pooled ground tissue per tissue type to provide accurate estimation of means and standard deviations for $\delta^{15}N$ and $\delta^{13}C$ at each site (with the exception of diaspidids for Sites 2 and 3 for which there were only enough specimens to make 3 and 6 pooled samples, respectively). Elemental analyses of nitrogen and carbon were conducted at the Department of Geosciences Stable Isotope Laboratory, University of Massachusetts, Amherst using a Finnigan Delta XL+ ratio mass spectrometer (Thermo Fisher Scientific, Waltham, MA) and a Costech Elemental Analyzer (Costech Analytical Technologies, Inc., Valencia, CA).

Site	Locality		Elevation (m)	Included?	
	Latitude	Longitude		Trophic study	Gut content study
1	32° 01.926 S	18° 51.905 E	439	✓	✓
2	32° 02.063 S	18° 52.047 E	443	✓	✓
3	32° 00.071 S	18° 50.340 E	358	✓	✓
4	31° 54.969 S	18° 46.261 E	357	✓	✓
5	31° 48.608 S	18° 46.413 E	400	✓	✓
6	31° 54.539 S	18° 45.819 E	349	✓	✓
7	31° 59.555 S	18° 49.246 E	386	✓	✓
8	31° 59.550 S	18° 49.281 E	389	✓	✓
9	31° 59.534 S	18° 49.260 E	389	✓	✓
10	32° 01.930 S	18° 51.881 E	437		✓

Table 3.1: Locality Data – This table summarizes locality data for the ten sites in Western Cape, South Africa from which *Melissotarsus emeryi* colonies were collected.

Relative trophic position can be calculated for a consumer of interest from a particular food web when data are available for both: (a) $\delta^{15}N$ (expressed as ‰) for the source (i.e. primary producer/consumer) and the consumer of interest, and (b) expected $\delta^{15}N$ enrichment per trophic exchange. Post (2002) developed this simple model for calculating relative trophic position: trophic level = $\lambda + (\delta^{15}N_{\text{secondary consumer}} - \delta^{15}N_{\text{base}}) / \Delta_n$, where λ is the trophic position of the organism used to calculate $\delta^{15}N_{\text{base}}$ and Δ_n is the expected $\delta^{15}N$ enrichment per trophic level. Studies of terrestrial consumers raised on invertebrate diets have established that the average $\delta^{15}N$ enrichment is 1.4 ± 0.2 ‰ per trophic exchange (Scrimgeour et al. 1995, Ostrom et al. 1996, Pinnegar et al. 2001, Oelbermann and Scheu 2002, reviewed in McCutchan et al. 2003).

Isotope data can also be used to track resources from a source to a potential consumer. Baseline isotopic values vary widely from place to place; even plants within a few meters apart can have very different isotopic values. Natural variations in nitrogen and carbon isotope ratios across landscapes can be useful in determining which sources contribute to the diet of a consumer. If *Melissotarsus* ants are dependent upon diaspidids

as a primary food source both nitrogen and carbon isotopic enrichment should indicate a strong positive relationship between ants and diaspidids. If ants consume host plants (as suggested by Mony et al. 2013) ant isotopic signatures for carbon and nitrogen should track most closely to the plant. If the hypothesis that *Melissotarsus* ants are dependent upon either the host plant or diaspidids as a food source were wrong (e.g. if they regularly forage outside the galleries for other food sources) there should be at most a weak to moderate relationship between worker ant and diaspidid or worker ant and host plant isotopic values. Strong relationships between worker ant isotopes and host plant or diaspidid isotopes should only exist if the ants are dependent upon them as a primary food source.

3.3.2 Trophic position and resource tracking – Statistical analysis

Relative trophic position of worker ants was calculated using the model described above with data for $\delta^{15}N$ enrichment from ants and diaspidids at each of 9 sites. Relative trophic position was calculated for each site using corresponding diaspidid isotopic values as $\delta^{15}N_{base}$ and worker ant values as $\delta^{15}N_{consumer}$. The trophic position of diaspidids is 1 ($\lambda = 1$) and the expected $\delta^{15}N$ enrichment per trophic level is 1.4‰ ($\Delta_n = 1.4$). The mean relative trophic position for worker ants from all sampled colonies was then calculated by averaging across results from all 9 sites.

Stable isotopic data for $\delta^{15}N$ and $\delta^{13}C$ were analyzed using linear regression to determine if either plant or diaspidid isotopic values are significant predictors of a relationship with worker ant isotopic enrichment, indicative of resource tracking. Model selection, testing of assumptions, and linear regression analyses were conducted in R statistical software. The response variable for nitrogen enrichment models was $\delta^{15}N$ of

worker ants ($\delta^{15}N_{worker}$). Predictor variables considered for these models included $\delta^{15}N$ for diaspidids ($\delta^{15}N_{diaspidid}$) and plants ($\delta^{15}N_{plant}$), magnitude of change between mean diaspidid and worker isotopic values for each site (M_{DxW}, calculated as the Euclidean distance between points in a $\delta^{13}C/\delta^{15}N$ biplot, **Fig. 3.1**), magnitude of change between mean plant and worker isotopic coordinates for each site (M_{PxW}, plot not shown), the direction of change between mean diaspidid and worker isotopic coordinates for each site (S_{DxW}, calculated as the slope of the line connecting two points in a $\delta^{13}C/\delta^{15}N$ biplot, **Fig. 3.1**), and the direction of change between mean plant and worker isotopic coordinates for each site (S_{PxW}, calculated in the same manner). Melville and Connolly (2003) found correlation of magnitude and direction in $\delta^{13}C/\delta^{15}N$ biplots to be an important indicator of resource tracking between a producer and consumer. The direction of change indicates *how* isotopic values differ between source and consumer; the magnitude is a measure of *how much* they change. The response variable for carbon enrichment models was $\delta^{13}C$ of worker ants ($\delta^{13}C_{worker}$). Predictor variables considered included diaspidid and plant $\delta^{13}C$ ($\delta^{13}C_{diaspidid}$ and $\delta^{13}C_{plant}$), magnitude of change (M_{DxW} and M_{PxW}), and direction of change (S_{DxW} and S_{PxW}) as described above.

Model selection was conducted by calculating stepwise AIC for the full model using the "stepAIC" command from the *MASS* package in R, adding variables both forwards and backwards for comparison. This procedure was followed for both the nitrogen and carbon linear regression models. The model with the lowest AIC score was regarded as the model of best fit for each linear regression.

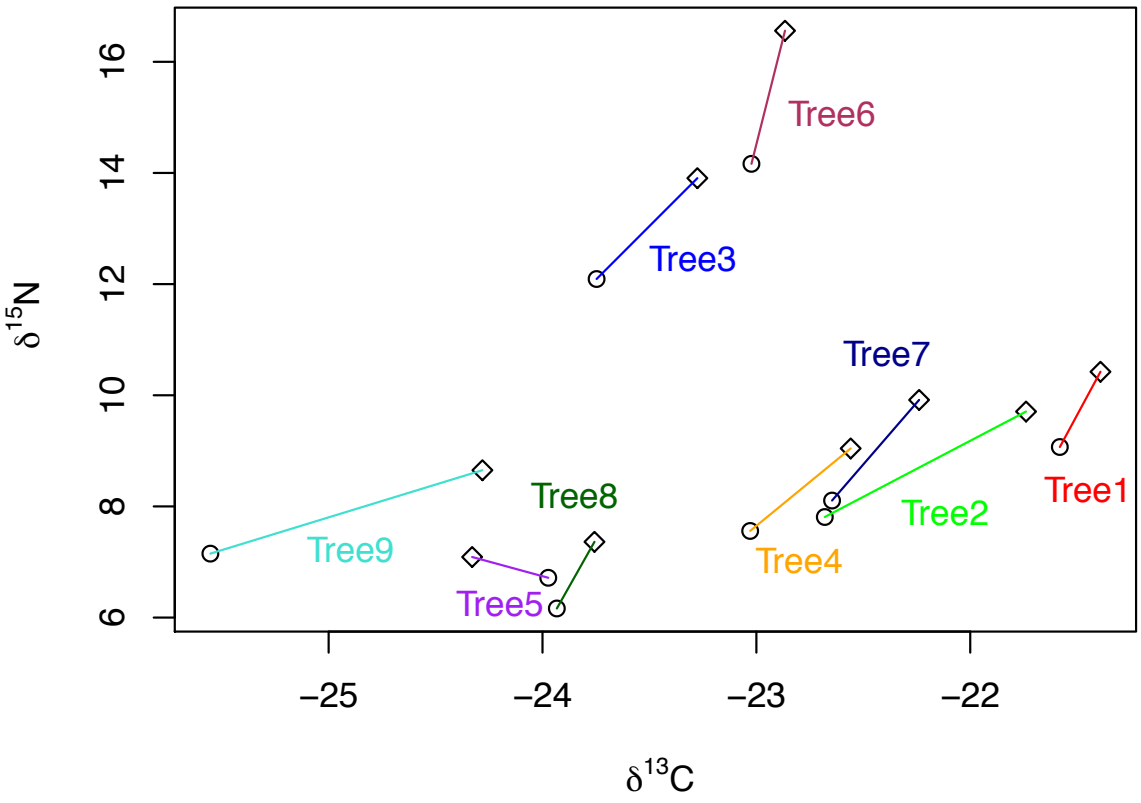


Fig. 3.1. $\delta^{13}C$/ $\delta^{15}N$ boxplot for diaspidids and workers – A biplot of $\delta^{13}C$ and $\delta^{15}N$ with lines connecting the mean value for diaspidids (circles) and worker ants (diamonds) from each site (Tree1 – Tree9). The magnitude of difference between diaspidid and worker values, calculated as the Euclidean distance between each diaspidid and worker per site, was included in the linear regression models for nitrogen and carbon. The slope of each connecting line was also included to indicate direction of change. Lines are colored by site.

Major assumptions of linear regression models were tested in R. Normality of residuals was assessed by visualizing QQ plots, histograms, and with Shapiro-Wilk tests normality when necessary. Potential outliers were evaluated using Cook's D statistic to determine if they had undue influence on the model; no significant outliers were detected. Non-constant Variance Score tests were used to evaluate residuals for homoscedasticity. Risk of autocorrelation among of predictor variables was assessed using a Durbin-Watson test for multicollinearity. All statistical tests were evaluated against a Type I error threshold of 0.05 ($\alpha = 0.05$).

3.3.3 Molecular assay of ant gut contents – Study design

Melissotarsus worker diet may also be assessed more directly by analyzing their gut contents. To accomplish this, I designed 3 diaspidid-specific primer pairs of varying product length for the COI barcoding region of the mitochondrial genome. First, the COI barcoding region was amplified for ants and diaspidids using primer pairs from Park et al. (2010). Polymerase chain reaction (PCR) protocols were as follows: an initial melting step at 95°C for 2 minutes; 5 cycles of melting at 95°C for 30 seconds, annealing at 50°C for 1 minute, and elongation at 72°C for 2 minutes; 30 cycles of melting at 95°C for 30 seconds, annealing at 55°C for 1 minute, and elongation at 72°C for 2 minutes; followed by a final elongation step at 72°C for 5 minutes. PCR products were purified by treating with Exonuclease I and Shrimp Alkaline Phosphatase (Exo-SAP) (Affymetrix, Santa Clara, California) at 37°C for 25 minutes, followed by 80°C for 15 minutes to denature proteins.

Purified products were then sent to the UMass Genomics Resource Lab (Amherst, Massachusetts) for Sanger sequencing using an ABI Model 3130XL sequencer (Life Technologies, Carlsbad, California). DNA sequences were aligned in Mesquite 2.75 (Maddison and Maddison 2015) using the MUSCLE alignment tool (Edgar 2004) and then used as a guide for primer design. To prevent annealing with DNA strands from ants, diaspidid-specific primer pairs coincide with regions where ants possess an insertion or deletion in the sequence. Three primer pairs were designed to represent relatively short-length (~150 bp), intermediate-length (~400 bp), and long (~600 bp) amplification products. The primer sets are as follows: *COIbcF150* - CATTACCTGTGCTAGCAAGAAG (150bp forward primer); *COIbcF400* -

ATACAGGATGAACATTATACCC (400bp forward primer); *COIbcF600*: ATAGAA CTATTACAATTCATGCTT (600bp forward primer); and *COIbcR1*: TAATTGGGTTA CCATTACCTCTTGG (reverse primer for all fragments). By chance, short fragments of DNA have a higher probability than longer fragments of remaining intact during digestion. If diaspidid DNA is present and being digested in the gut contents of ants, there should be an inverse relationship between amplicon length and amplification success.

Worker ant specimens from 10 sites (see **Table 3.1**) were prepared for gut content assay in the following way. Four worker ants were selected at random from each of the 10 sites. For each site, 3 ants were initially washed in 500 µL of DNA Away™ Surface Decontaminant (Thermo Fisher Scientific, Waltham, MA) for 10 minutes to degrade any contaminant diaspidid DNA on the exterior surface, and one ant specimen was washed in 500µL of sterile deionized water (ddH_2O). Specimens were randomly assigned to initial wash protocols. The two wash protocols were used to test whether any failure to amplify PCR products was due to the DNA Away™ wash working too effectively and destroying DNA in the gut contents. All specimens were then transferred to 500µL ddH_2O and washed for an additional 10 minutes. Specimens were dissected with clean forceps into two separate DNA extractions, one for the gaster segment and another for the legs. The gaster contains most of the digestive tract of ants, including the crop where partially digested food material is stored. Diaspidid DNA will only amplify from DNA extractions of ant legs if the initial wash steps fail to remove all contaminant DNA from the exterior surface. DNA extractions were completed using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Valencia, California) following standard protocols with two elution steps each

completed using 100µl AE buffer (as in Chapter 2). Two controls were also prepared, a diaspidid specimen collected from the 6th site served as the positive control (specimen ID# D3595A) and an unrelated ant (*Crematogaster* sp.; specimen ID# F0026A) that did not live in close contact with diaspidids was included to confirm that diaspidid-specific primers do not amplify ant DNA.

PCRs were performed on all DNA extractions (gaster preps, leg preps, diaspidid - positive control, *Crematogaster* ant - negative control) for each of the three COI barcoding fragments. PCR products were visualized using 1.5% agarose gel electrophoresis with SYBR® Safe (Life Technologies, Carlsbad, California) ultraviolet stain. PCR products were purified following the same protocols as stated above.

The concentration of amplified diaspidid dsDNA from worker ant tissues and controls was quantified using the Quant-iT™ PicoGreen® dsDNA assay (Life Technologies, Carlsbad, California) following the kit's standard protocols. A standard curve was produced by mixing a series of dilutions ranging from 0.0—50 ng/ml of purified Lambda DNA using TE (10 mM Tris, 1 mM EDTA, pH 7.5) as the solvent. Working PicoGreen reagent was prepared by diluting the provided PicoGreen stock solution 1:200 with TE according to the kit instructions. Equal volumes of DNA samples and PicoGreen reagent were combined and incubated for 5 minutes; aliquots were transferred to a 96-well microplate. Fluorescence was measured at the UMass Genomics Research Laboratory using an MX3000p real-time PCR machine (Stratagene, La Jolla, CA) with the plate reader set for excitation at 485nm and emission at 530nm. Fluorescence was compared against the standard curve to calculate dsDNA concentration (ng/ml) for unknown samples. Products with high dsDNA concentration were successful

in amplifying diaspidid DNA and those with low products failed to amplify. I interpret failure to amplify as a true negative, i.e. no intact diaspidid DNA present for a particular fragment (short, intermediate, or long).

3.3.4 Molecular assay of ant gut contents – Statistical analysis

Molecular assay data were analyzed using two-way ANOVA with fixed effects; fragment size (short, intermediate, long) and tissue type (gaster, leg, diaspidid, ant) are the dependent variables and DNA concentration is the independent response variable. PicoGreen assays of PCR products can be influenced by the presence of PCR reagents, which tend to depress fluorescence measurements. To correct for this effect and make all DNA concentration measurements positive numbers, the value of the lowest measurement was corrected to equal 1 ng/ml and all other DNA concentration measurements were corrected accordingly. The relative concentration of DNA from samples is of greater importance to these analyses than measurements of actual DNA concentration.

Analyses were conducted in R. For gaster and leg tissue types, each DNA fragment had 40 replicates. Fewer replicates were conducted for the positive and negative controls (3 each for diaspidids and ants) to conserve costs and because they are included mainly as a comparison to determine if DNA from gaster and leg tissues amplified as expected. Type III sums of squares were calculated to accommodate this unbalanced design. Type II sums of squares would be more powerful but only appropriate if the interaction were not significant. Controls were included for pairwise comparisons of gaster to diaspidid and ant to leg for each fragment since the gaster is assumed to contain diaspidid DNA and the legs are not. Pairwise comparisons were also conducted for the

short to intermediate and intermediate to long fragments for gaster tissues to determine if amplification is inversely related to amplicon length. All pairwise comparisons were achieved using Tukey's HSD.

Assumptions for ANOVA were evaluated in R as well. One potential outlier was identified but the Cook's D statistic indicated no undue influence of this data point on the model so it was kept in all subsequent analyses. Inspection of QQ plots and treatment-by-residual plots indicated that the assumptions of normality and homoscedasticity of residuals might be violated. A log transformation of DNA concentration data was used to correct this.

3.4 Results

3.4.1 Trophic position and resource tracking

The relative trophic position of *Melissotarsus* workers is 1.1 ± 0.4 trophic levels above associated diaspidids. This result is consistent with the hypothesis that ants are predators of diaspidids but is not sufficient to draw this conclusion.

The model of best fit for nitrogen linear regression was: $\delta^{15}N_{worker} = \delta^{15}N_{diaspidid} + M_{DxW} + S_{DxW}$. Linear regression analyses indicate that both $\delta^{15}N_{diaspidid}$ and M_{DxW} are significant predictors of a strong positive relationship with $\delta^{15}N_{worker}$ ($F_{3,5} = 1681$, Adjusted $R^2 = 0.9984$, $p = 6.286 \times 10^{-8}$, regression equation: $\delta^{15}N_{worker} = 1.01(\delta^{15}N_{diaspidid}) + 0.831(M_{DxW}) + 0.022(S_{DxW}) - 0.067$; $\delta^{15}N_{diaspidid}$ $p = 1.88 \times 10^{-7}$; M_{DxW} $p = 3.52 \times 10^{-4}$; **Fig. 3.2**). S_{DxW} was not a significant term in the model ($p = 0.157$). $\delta^{15}N_{plant}$ was not found to be a significant predictor of $\delta^{15}N_{worker}$ when included in the model ($p = 0.611$). Nearly all variation in worker ant nitrogen isotopic ratios can be explained by variation

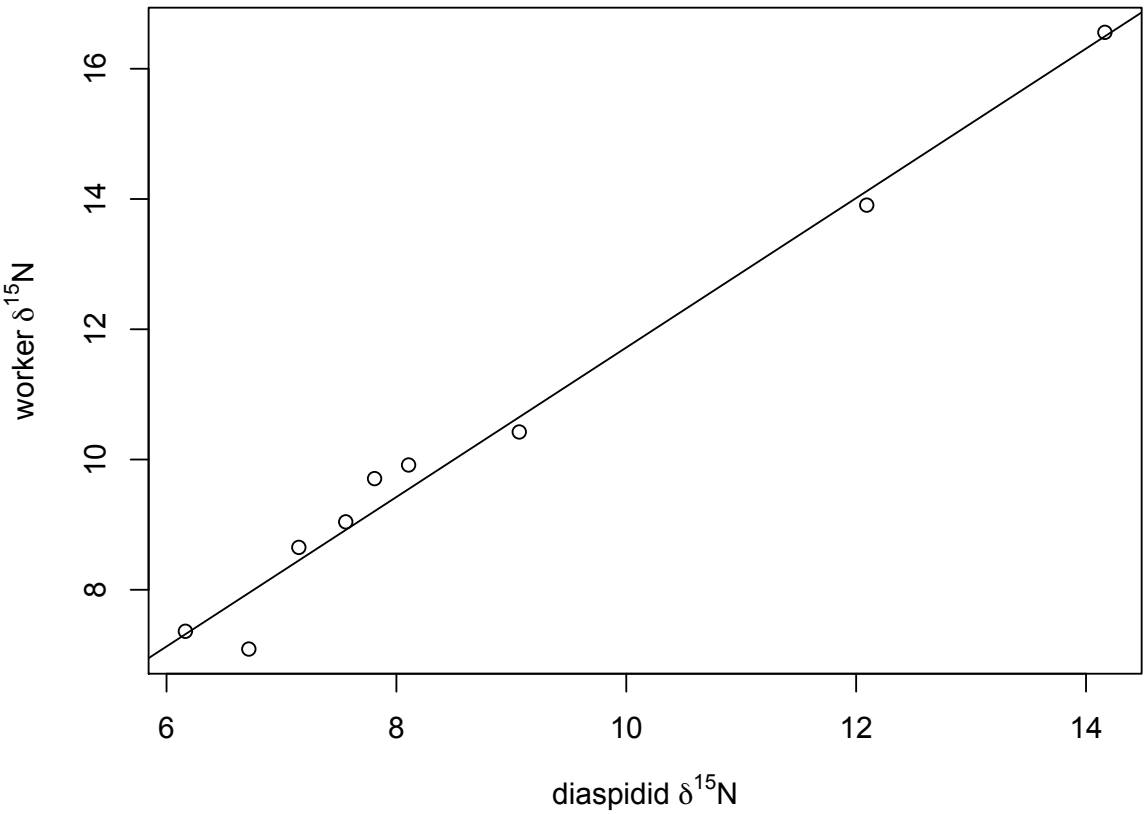


Fig. 3.2: Relationship between worker and diaspidid nitrogen isotopic values – A plot of the linear regression of $\delta^{15}N$ for diaspidids vs. workers showing the strong positive relationship between nitrogen enrichment in worker and diaspidid tissues. Much of the variation in worker nitrogen values across colonies can be explained by diaspidid nitrogen values, indicating that diaspidids are a significant source of nitrogen for ants.

from diaspidid nitrogen ratios. Ant nitrogen isotopes track strongly to diaspidids as the source. The host plant is not a significant source of nitrogen for ants.

For linear regression of carbon isotope data, the full model was determined to be the best fitting model: $\delta^{13}C_{worker} = \delta^{13}C_{plant} + \delta^{13}C_{diaspidid} + M_{DxW} + M_{PxW} + S_{DxW} + S_{PxW}$. Analyses indicate that $\delta^{13}C_{plant}$ ($p = 0.006$), M_{PxW} ($p = 0.008$), and S_{PxW} ($p = 0.017$) are all significant predictors of a strong positive relationship with $\delta^{13}C_{worker}$ ($F_{6,2} = 929.2$, adjusted $R^2 = 0.998$, $p = 0.0011$, regression equation: $\delta^{13}C_{worker} = 1.048(\delta^{13}C_{plant}) - 0.053(\delta^{13}C_{diaspidid}) - 0.278(M_{DxW}) + 1.05(M_{PxW}) + 0.011(S_{DxW}) - 1.99(S_{PxW}) + 0.453)$. $\delta^{13}C_{diaspidid}$ was not found to contribute significantly to the model ($p = 0.579$). A separate

linear regression analysis for carbon was conducted considering only $\delta^{13}C_{diaspidid}$ and $\delta^{13}C_{plant}$ as predictor variables; $\delta^{13}C_{diaspidid}$ was found to be a significant predictor of variation in $\delta^{13}C_{worker}$ ($p = 0.0018$) but $\delta^{13}C_{plant}$ was not ($p = 0.2423$). The ultimate source of carbon for both diaspidids and ants is likely to be the host plant; thus it is not surprising that ants share a significant relationship to plants as the carbon source. *Melissotarsus* can apparently digest various plant polysaccharides and the host plant may actually serve as an important source of dietary carbon (Mony et al. 2013). This might explain why regression analyses conflict on the contributions of diaspidids as a carbon source; both host plants and diaspidids are contributors of carbon.

3.4.2 Molecular assay of ant gut contents

Diaspidid DNA was successfully amplified from gaster preps of worker ants for the shortest and intermediate sized DNA fragments but not for the longest fragment (**Fig. 3.3**). For the 150bp fragment, 40/40 gaster preps (100%) contained intact diaspidid DNA. A lower proportion of samples amplified diaspidid DNA for the 400bp fragment, 30/40 gaster preps (75%). The 600bp fragment did not successfully amplify for any gaster preps, possibly indicating that intact DNA of that length was rare or absent. However, the 600bp fragment also failed to amplify for the majority of diaspidid samples suggesting that the primer set anneals poorly to the target region. For this reason, I only report on pairwise comparisons for the shorter two fragments. Low levels of contamination were present in leg preps, indicating that the wash protocols did not destroy all contaminant DNA on the exterior of worker ants. Still, significant differences were found between gaster and leg preps for the 150bp fragment (Tukey's HSD: $p < 0.001$) and the 400bp fragment (Tukey's HSD: $p < 0.001$). DNA concentration for gaster and diaspidid preps

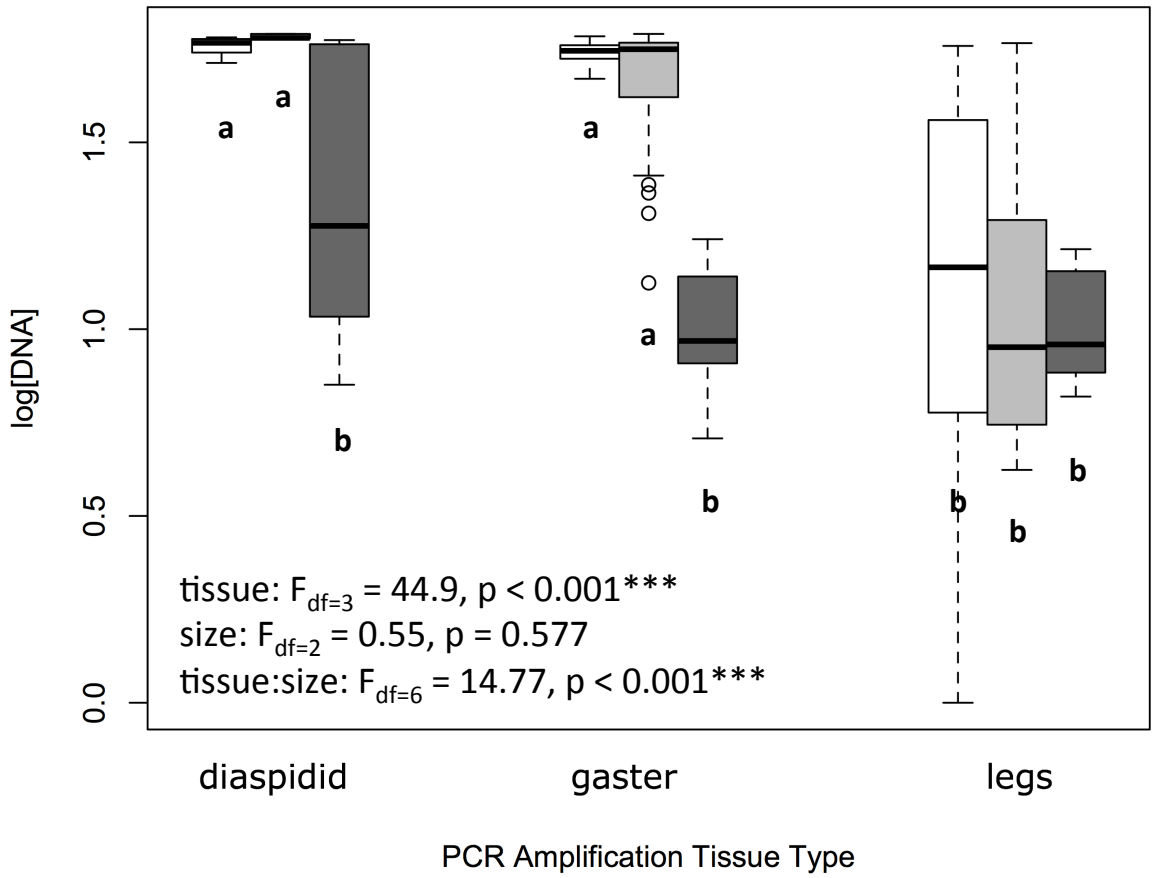


Fig. 3.3: Worker ant gut content assay – A boxplot illustrating comparisons made in the two-way ANOVA for log(DNA concentration) with the dependent variables: tissue type [diaspidid, gaster preps, leg preps, and ants (not shown)] and fragment length [150bp (white), 400bp (light gray), and 600bp (dark gray)]. Mean values of diaspidid and gaster preps for the 150bp and 400 bp fragments are significantly different from the remaining treatment combinations.

does not differ significantly for the 150bp and 400bp fragments (Tukey's HSD: p = 0.999, p = 0.998 respectively). The same is true for comparisons of legs and *Crematogaster* ants (Tukey's HSD: 150bp p = 0.912; 400bp p = 0.999). Diaspidid DNA is amplified equivalently from diaspidid preps and from gaster preps of worker ants for the short and intermediate length fragments. There is no statistical support for an inverse relationship between fragment length and amplification success (Tukey's HSD: gaster 150bp:400bp p = 0.977). No significant amount of diaspidid DNA was amplified from leg preps of worker ants or from unrelated ants that I assume have had no contact with

diaspidids. Results of two-way ANOVA indicate that a significant interaction exists between tissue type and fragment length. The effects of tissue type and fragment length are related and cannot be discriminated in this study.

3.5 Discussion

The combined analyses of relative trophic position, resource tracking, and molecular assay of gut contents for focal ant colonies all strongly support the prevailing hypothesis that *Melissotarsus* are predators of diaspidids. *Melissotarsus emeryi* cultivates populations of armored scale insects as a source of meat, an arrangement that apparently works to the mutual benefit of both parties. Based upon these results and a study on the evolutionary history of ant association among diaspidids (Chapter 2), I assert that ant/diaspidid relationships are simultaneously mutualistic and predatory, a condition that I refer to here as predatory mutualism. Myrmecophilous diaspidid populations do well in association with ants (Chapter 1) and several lineages have experienced a long legacy of association with *Melissotarsus* species (Chapter 2).

The rewards received by diaspidid populations from such an unusual mutualism remain up for debate. Protection from predation or parasitism is usually assumed to be a primary benefit to engaging in mutualism with ants. Although the ants are predators of armored scales the rate at which associated populations are preyed upon may be lower than that experienced by free-living populations. One important benefit that has not yet been explored, but could prove to be a significant reward for myrmecophilous populations, is the maintenance of microclimates within galleries that are suitable to the growth and reproduction of diaspidids (Ivens 2014). Being able to reinvest resources into

reproductive output rather than using those resources to develop defensive structures could also contribute to the success of myrmecophilous diaspidids. For "armored" scale insects, waxy scale coverings are the primary mode of defense and ant-associated populations do not produce them. *Melissotarsus* galleries serve as a proxy for waxy scale covers and the loss or reduction of wax producing structures in myrmecophilous populations might be selectively favored (Schneider et al. 2013).

Diaspidid cultivation by *Melissotarsus* ants is in some ways analogous to relationships between humans and livestock. Humans live in close association with many of their prey (livestock), and these prey species have experienced a substantial fitness benefit from associating with their predators (Diamond 2002). The main benefits received by domesticated animals are food, shelter, assisted dispersal, and protection from natural enemies (Rindos 1984, Budiansky 1992, Zeder 2006). Diaspidids benefit from access to food and shelter inside ant galleries and they might gain assistance in dispersal as well, but this remains to be discovered.

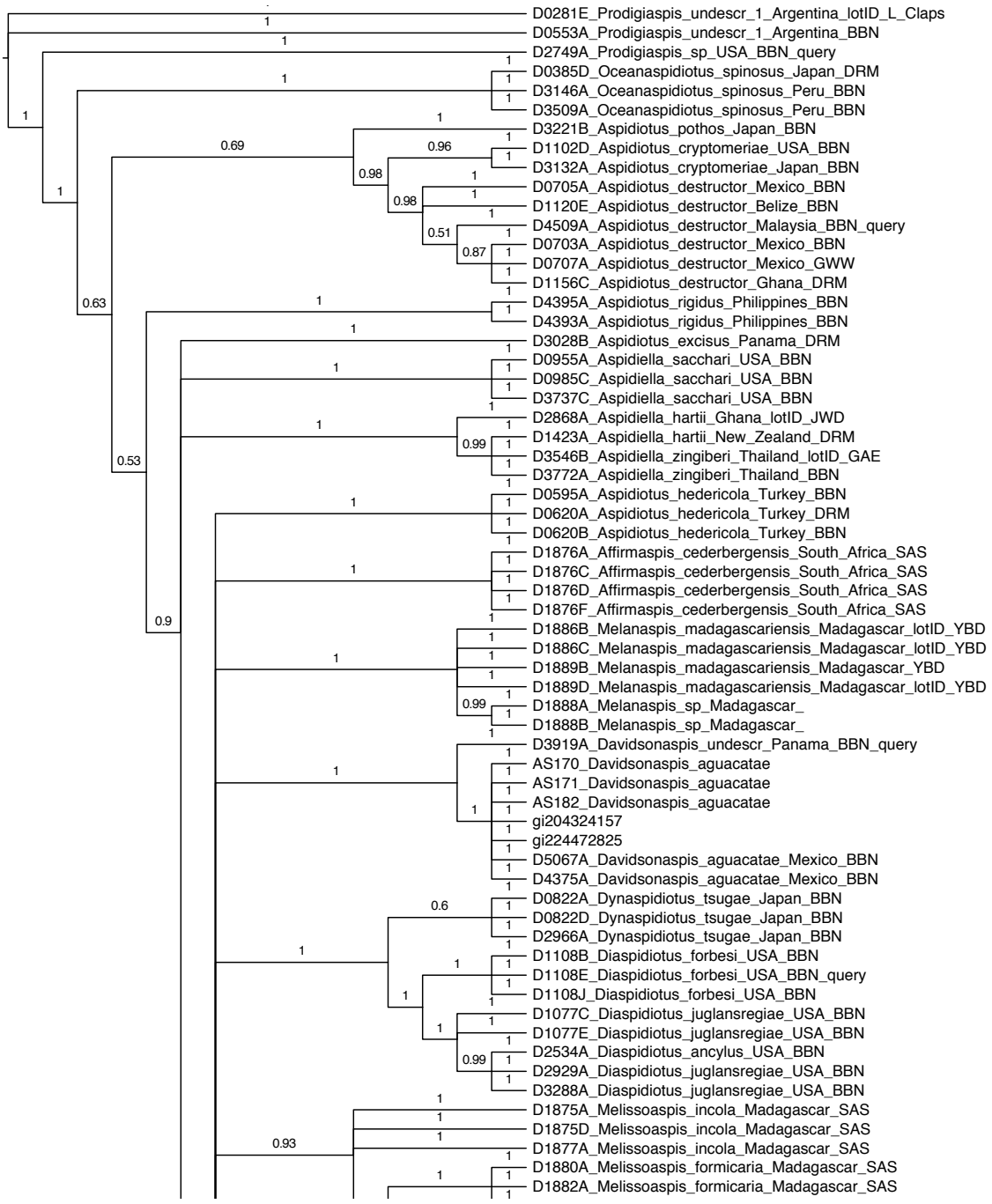
The prevalence of predatory mutualisms is a subject for future study. Yucca plants associate with pollinators that also act as seed predators (Pellmyr and Huth 1994), *Dictyostelium* slime molds practice bacterial husbandry (Boomsma 2011), and other ants besides *Melissotarsus* consume mutualist partners (Cushman and Addicott 1991, Sakata 1995, Ivens et al. 2012). We run the risk of underappreciating potential contributions of predation to mutualisms if we assume that predation only serves to "cheat" the system. My work on associations between *Melissotarsus* ants and armored scale insects suggests that predation can play a more fundamental role in the establishment and maintenance of mutualisms.

3.6 Summary

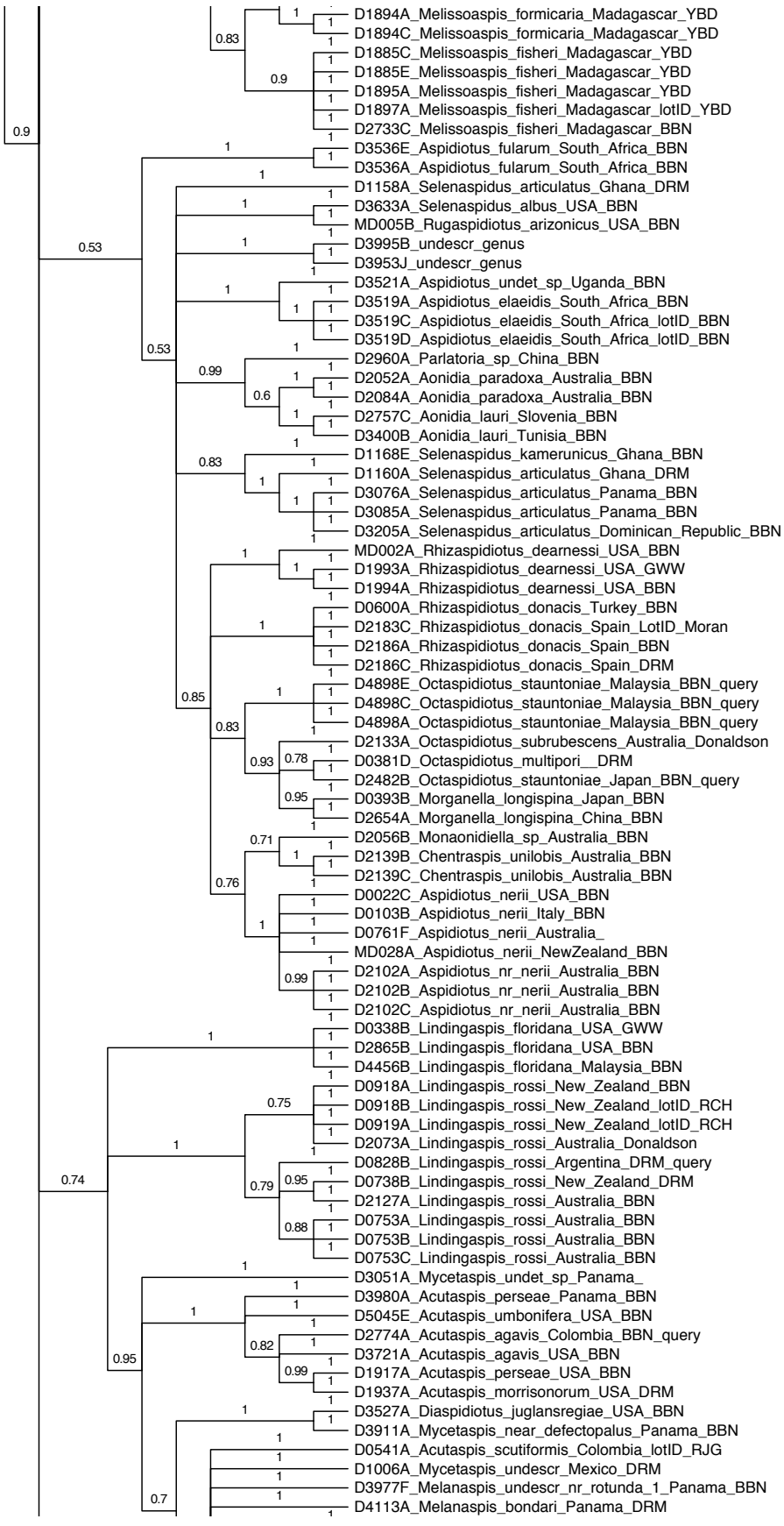
The objective of this study was to investigate the potential occurrence of mutualistic interactions between predator and prey populations, which I refer to herein as predatory mutualisms. Diaspidids are clearly an important food source for associated *Melissotarsus* ants. Diaspidids also appear to benefit greatly from associating with their primary predators. More in-depth comparative studies of trophobiotic ant diets are needed to fully understand how predation can influence ant/insect relationships.

3.7 Acknowledgements

I wish to thank the Zoology Department at Stellenbosch University for providing access to laboratory space during my visit to South Africa. Special thanks go to Bennie and Carina Bezuidenhout for their gracious hospitality on Nardouw Farm and for their enthusiasm regarding this project. Thank you to members of the UMass Stable Isotope Laboratory group and Guang Xu at the UMass Genomics Research Laboratory for technical assistance. Funding was provided by the National Institute of Food and Agriculture (2009-02310), NIFA Hatch Fund (2013-1000785), National Science Foundation (DEB-1258001), Department of Biology - UMass, and by The Natural History Collections, University of Massachusetts Amherst: David J. Klingener and Jane H. Bemis Endowments.



S1: 28S genealogy – The majority-rule consensus tree resulting from Bayesian analysis of the D2 expansion segment of the large subunit ribosomal RNA gene (28S). Branch support is indicated as posterior probabilities.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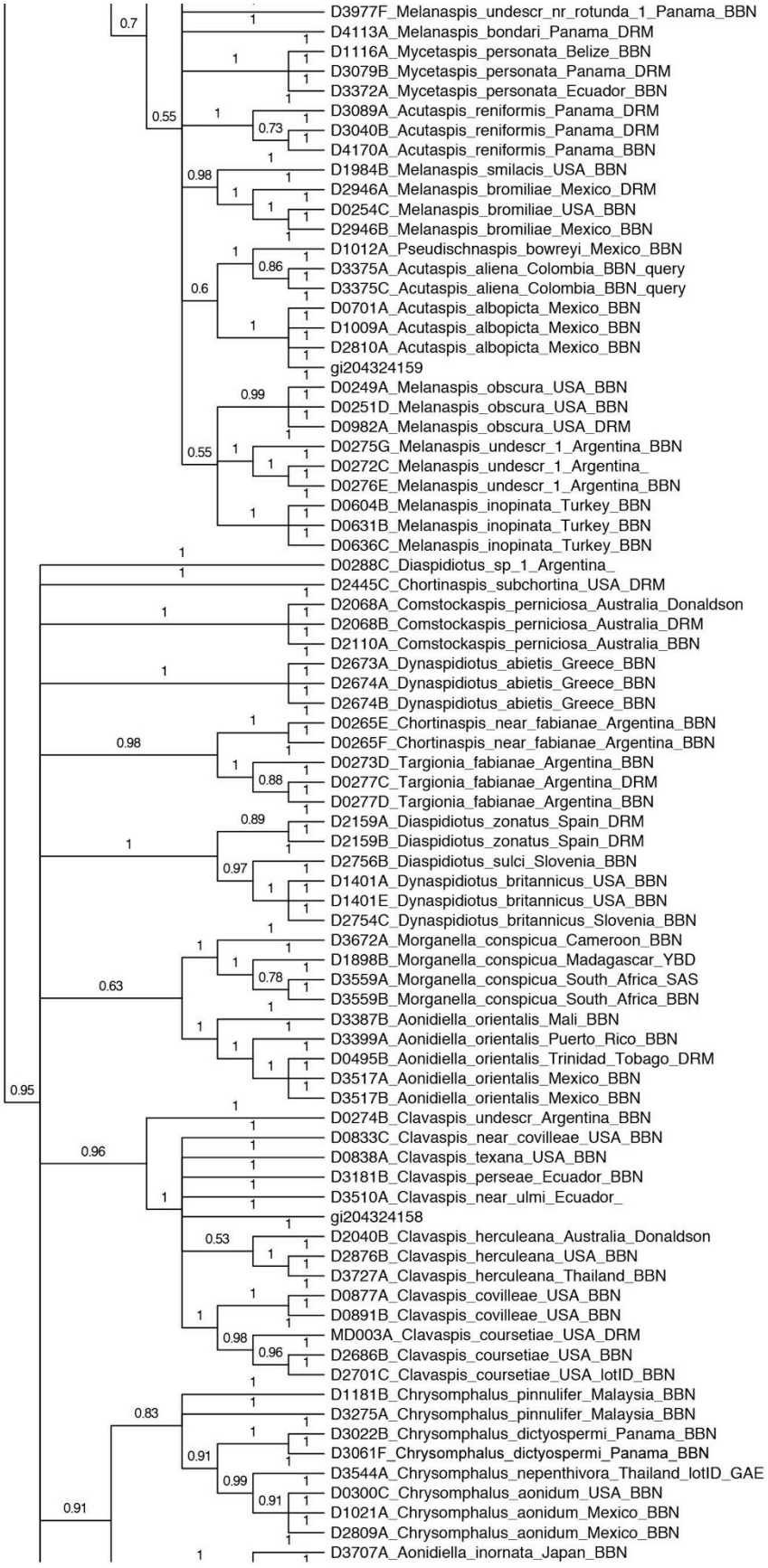
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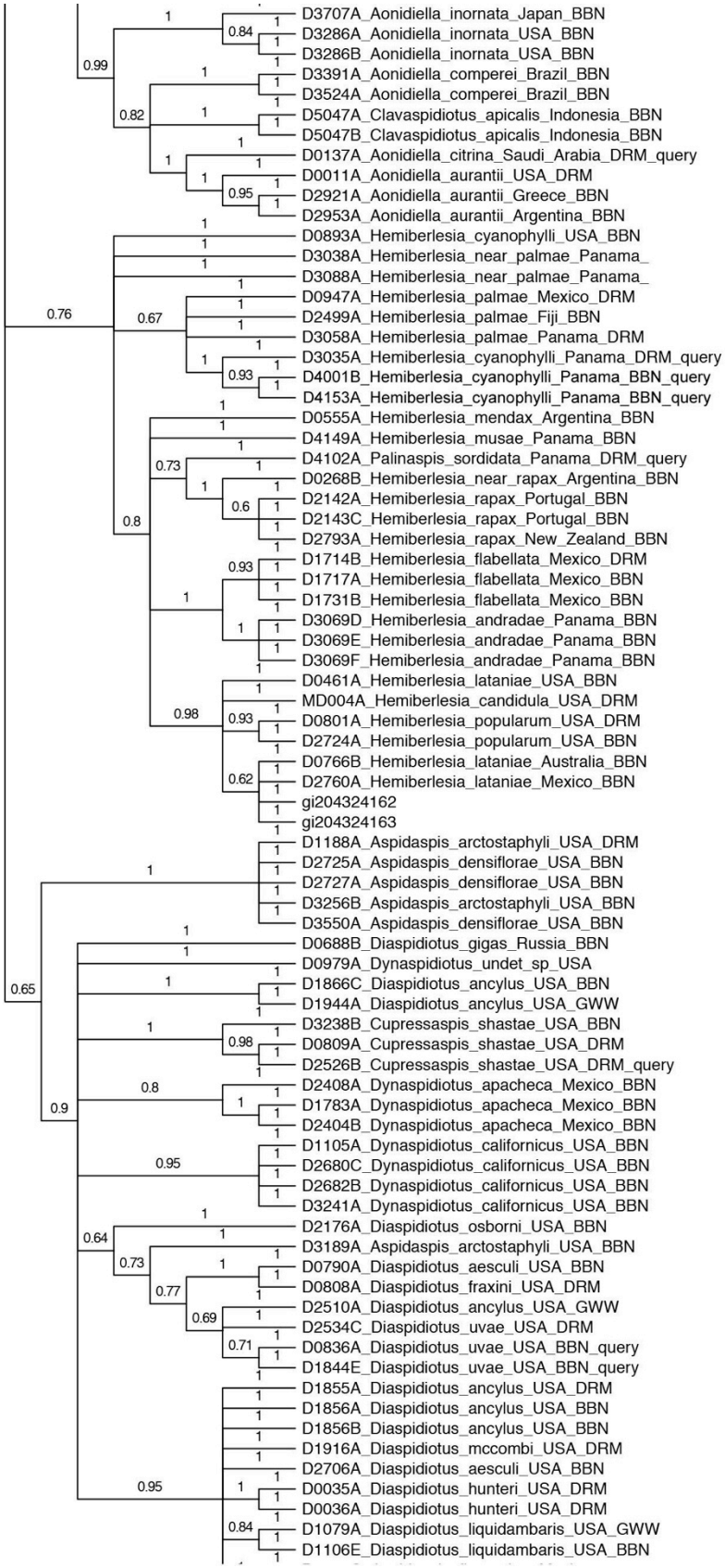
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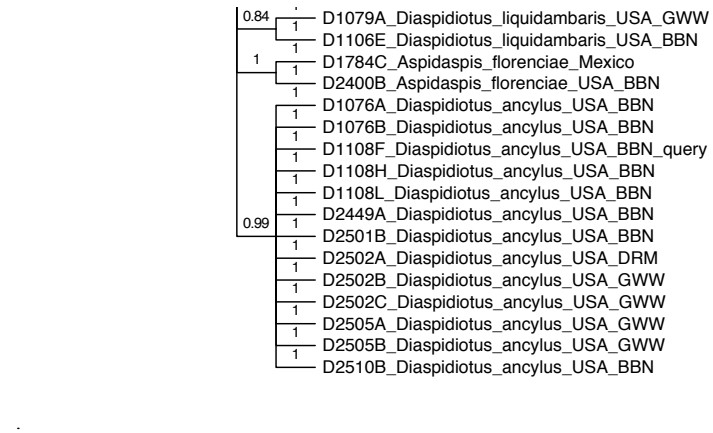
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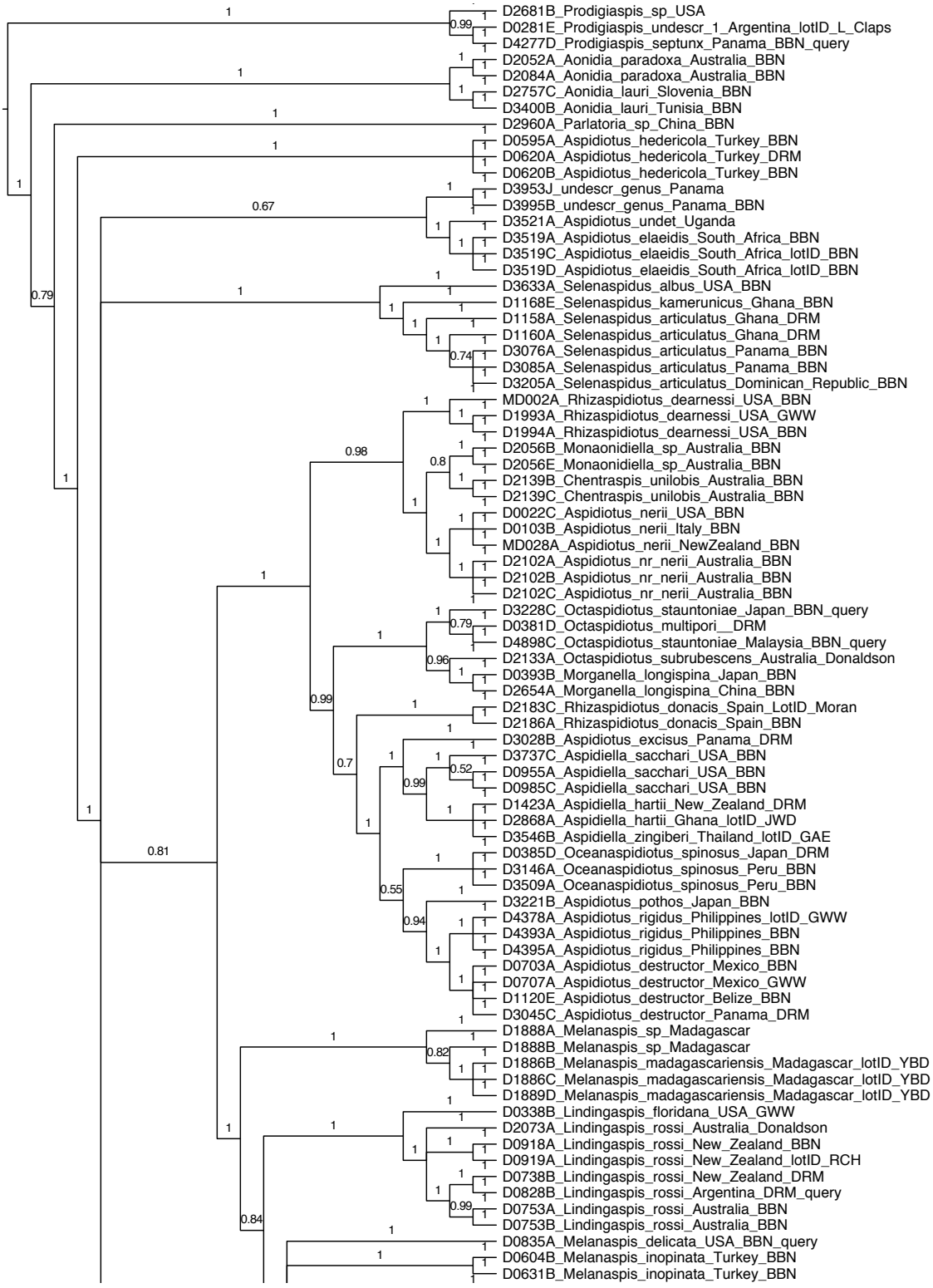
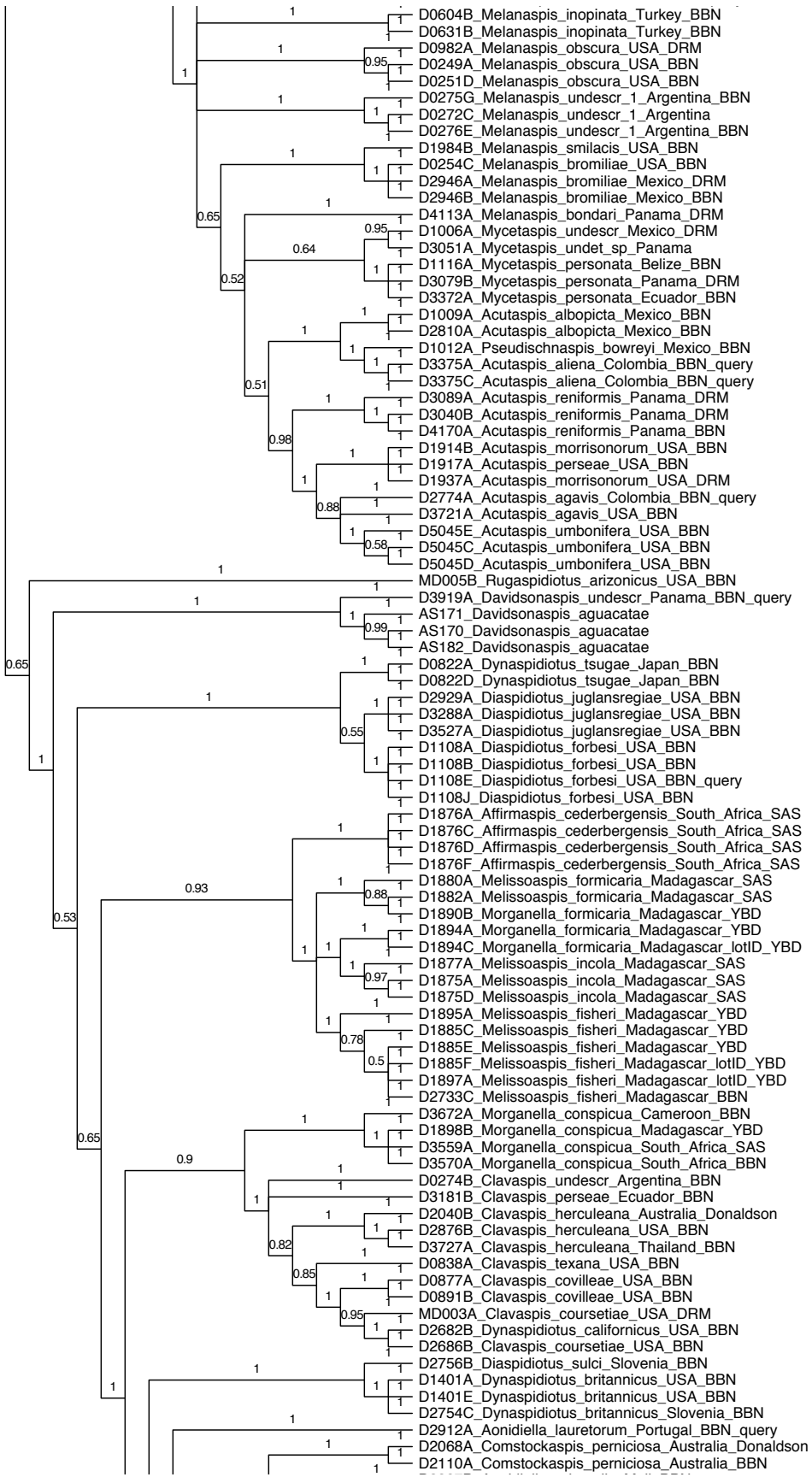


S2: EF-1α genealogy – The majority-rule consensus tree resulting from Bayesian analysis of the nuclear protein-coding gene Elongation Factor-1α (EF-1α). Branch support is indicated as posterior probabilities.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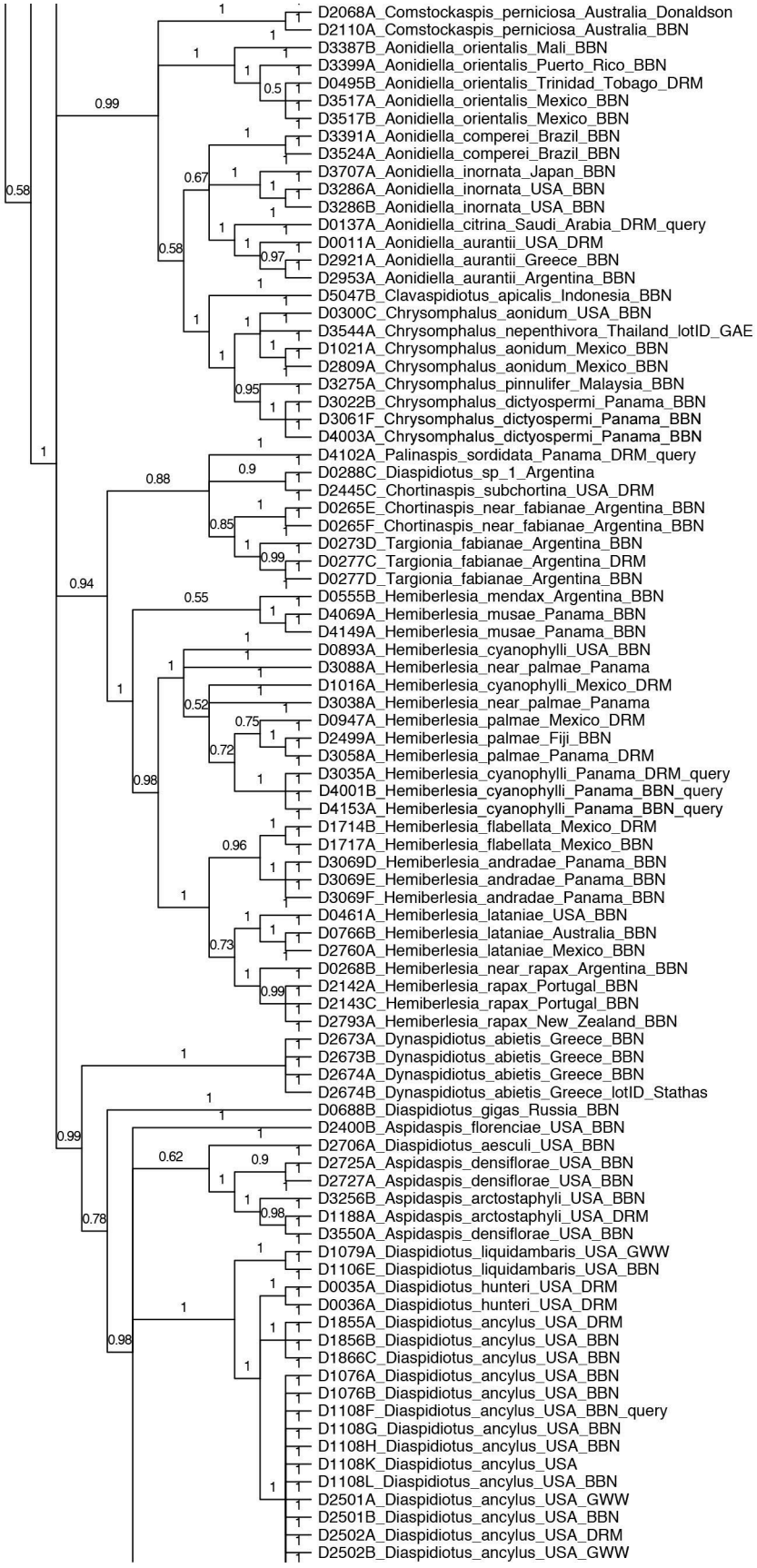
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D1866C_Diaspidiotus_ancylus_USA_BBN
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D1076B_Diaspidiotus_ancylus_USA_BBN
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D1108H_Diaspidiotus_ancylus_USA_BBN
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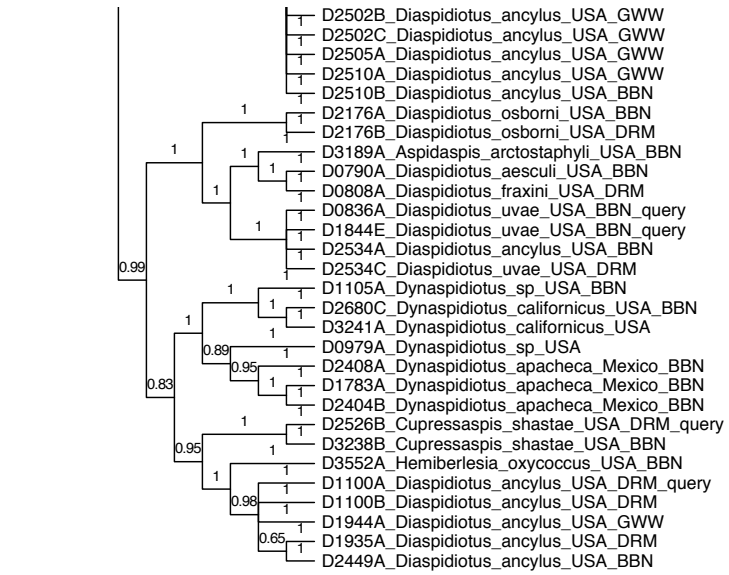
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D2510B_Diaspidiotus_ancylus_USA_BBN
D2176A_Diaspidiotus_osborni_USA_BBN
D2176B_Diaspidiotus_osborni_USA_DRM
D3189A_Aspidaspis_arctostaphyli_USA_BBN
D0790A_Diaspidiotus_aesculi_USA_BBN
D0808A_Diaspidiotus_fraxini_USA_DRM
D0836A_Diaspidiotus_uvae_USA_BBN_query
D1844E_Diaspidiotus_uvae_USA_BBN_query
D2534A_Diaspidiotus_ancylus_USA_BBN
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D1105A_Dynaspidiotus_sp_USA_BBN
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D0979A_Dynaspidiotus_sp_USA
D2408A_Dynaspidiotus_apacheca_Mexico_BBN
D1783A_Dynaspidiotus_apacheca_Mexico_BBN
D2404B_Dynaspidiotus_apacheca_Mexico_BBN
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D3552A_Hemiberlesia_oxycoccus_USA_BBN
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D1944A_Diaspidiotus_ancylus_USA_GWW
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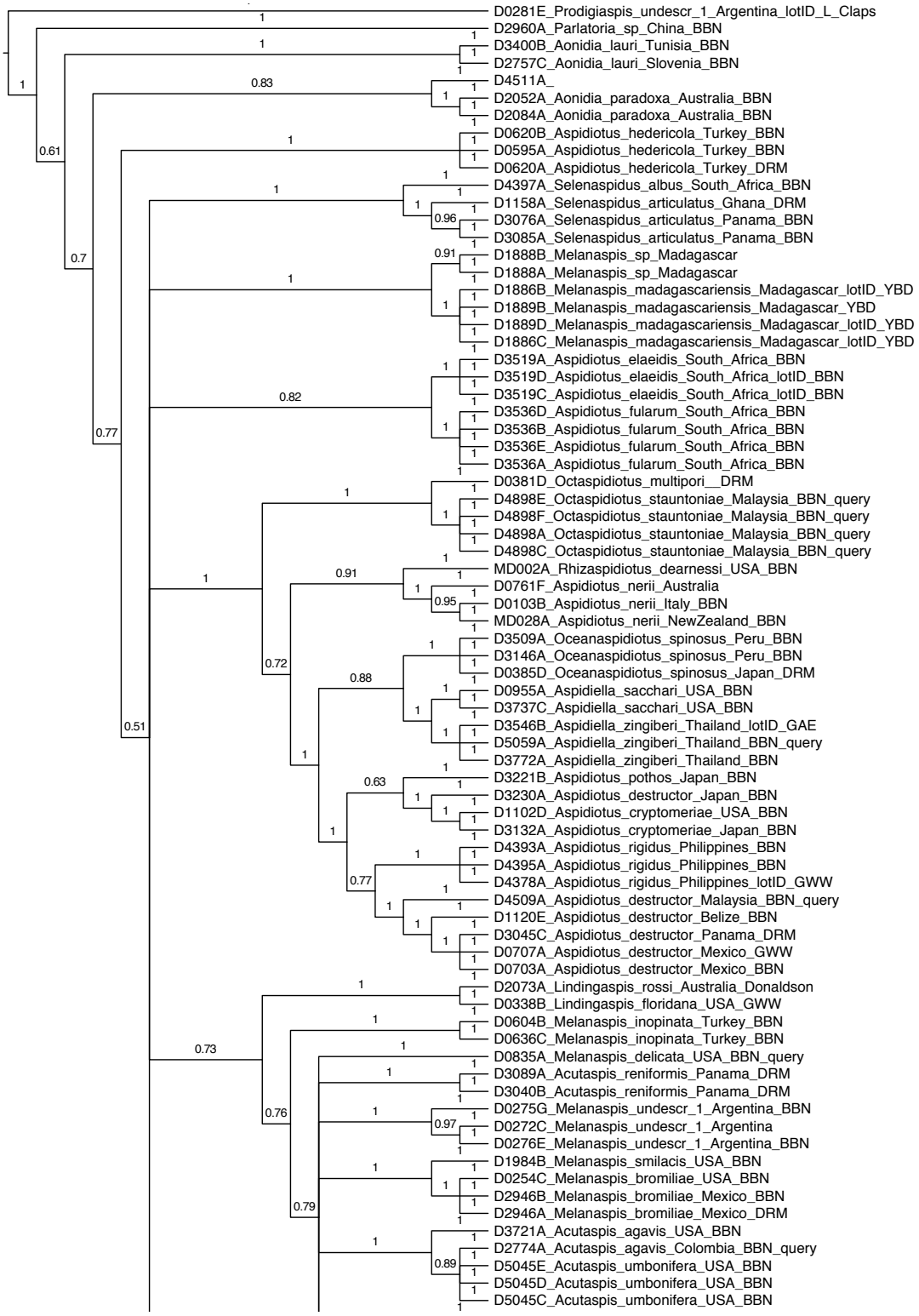
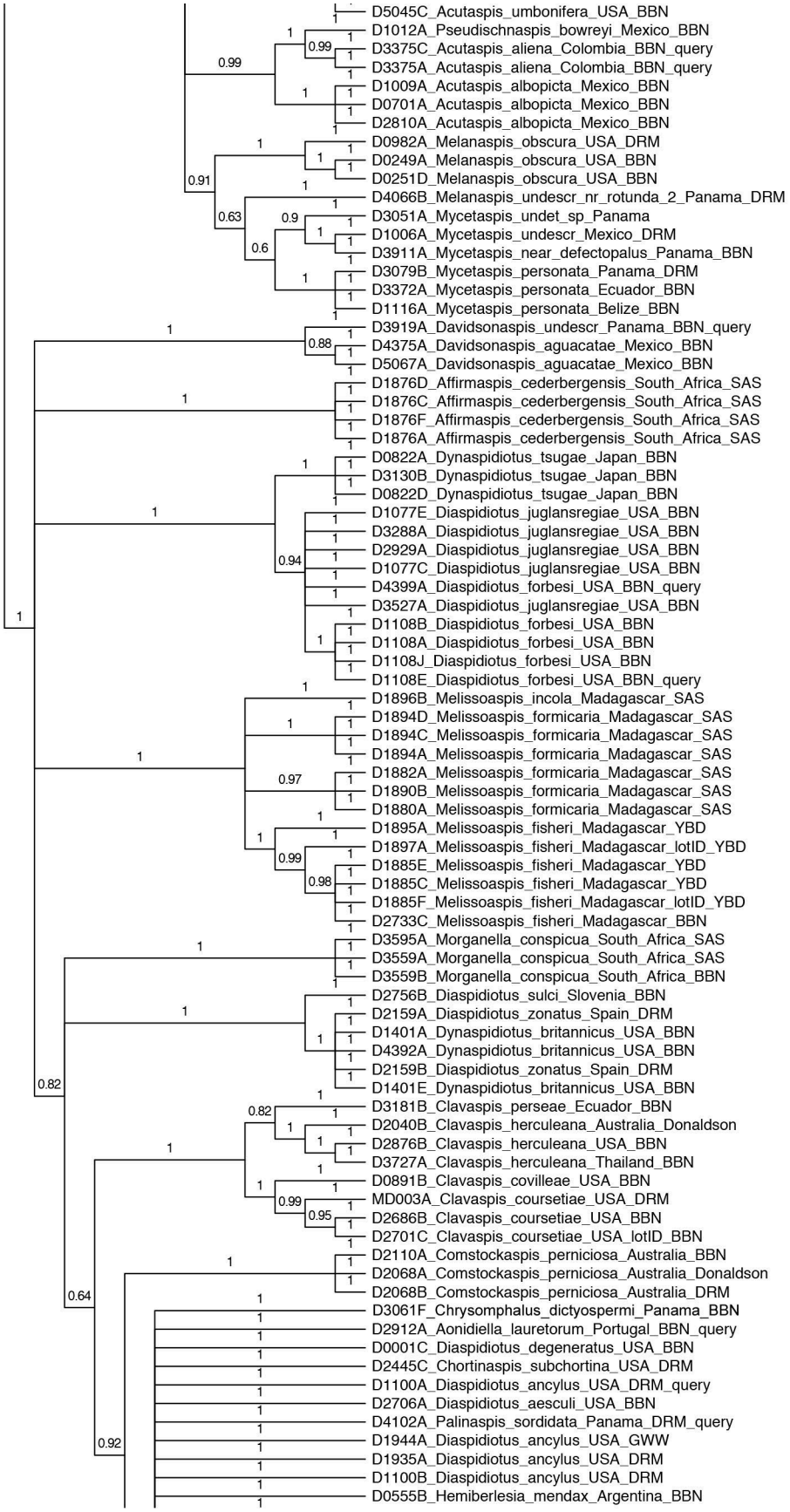
0.65
0.04



S3: CAD genealogy – The majority-rule consensus tree resulting from Bayesian analysis of a segment of the nuclear protein-coding gene Carbamoyl-phosphate synthetase (CAD). Branch support is indicated as posterior probabilities.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D0701A_Acutaspis_albopicta_Mexico_BBN
D2810A_Acutaspis_albopicta_Mexico_BBN
D0982A_Melanaspis_obscura_USA_DRM
D0249A_Melanaspis_obscura_USA_BBN
D0251D_Melanaspis_obscura_USA_BBN
D4066B_Melanaspis_undescr_nr_rotunda_2_Panama_DRM
D3051A_Mycetaspis_undet_sp_Panama
D1006A_Mycetaspis_undescr_Mexico_DRM
D3911A_Mycetaspis_near_defectopalus_Panama_BBN
D3079B_Mycetaspis_personata_Panama_DRM
D3372A_Mycetaspis_personata_Ecuador_BBN
D1116A_Mycetaspis_personata_Belize_BBN
D3919A_Davidsonaspis_undescr_Panama_BBN_query
D4375A_Davidsonaspis_aguacatae_Mexico_BBN
D5067A_Davidsonaspis_aguacatae_Mexico_BBN
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D1876C_Affirmaspis_cederbergensis_South_Africa_SAS
D1876F_Affirmaspis_cederbergensis_South_Africa_SAS
D1876A_Affirmaspis_cederbergensis_South_Africa_SAS
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D0822D_Dynaspidiotus_tsugae_Japan_BBN
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D2929A_Diaspidiotus_juglansregiae_USA_BBN
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D1894D_Melissoaspis_formicaria_Madagascar_SAS
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D1894A_Melissoaspis_formicaria_Madagascar_SAS
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D0891B_Clavaspis_covilleae_USA_BBN
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D4102A_Palinaspis_sordidata_Panama_DRM_query
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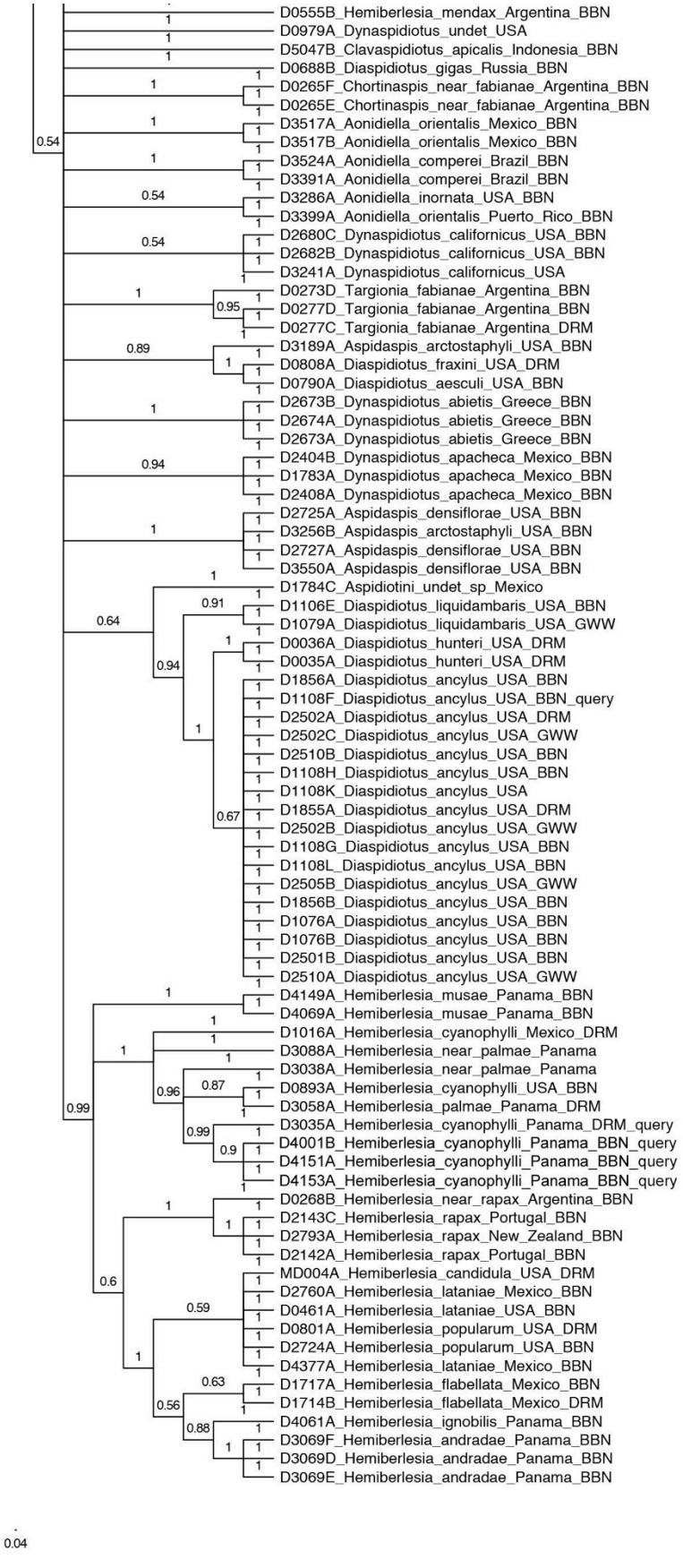
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D3399A_Aonidiella_orientalis_Puerto_Rico_BBN
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D0808A_Diaspidiotus_fraxini_USA_DRM
D0790A_Diaspidiotus_aesculi_USA_BBN
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D1783A_Dynaspidiotus_apacheca_Mexico_BBN
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D2725A_Aspidaspis_densiflorae_USA_BBN
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D1079A_Diaspidiotus_liquidambaris_USA_GWW
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D2724A_Hemiberlesia_popularum_USA_BBN
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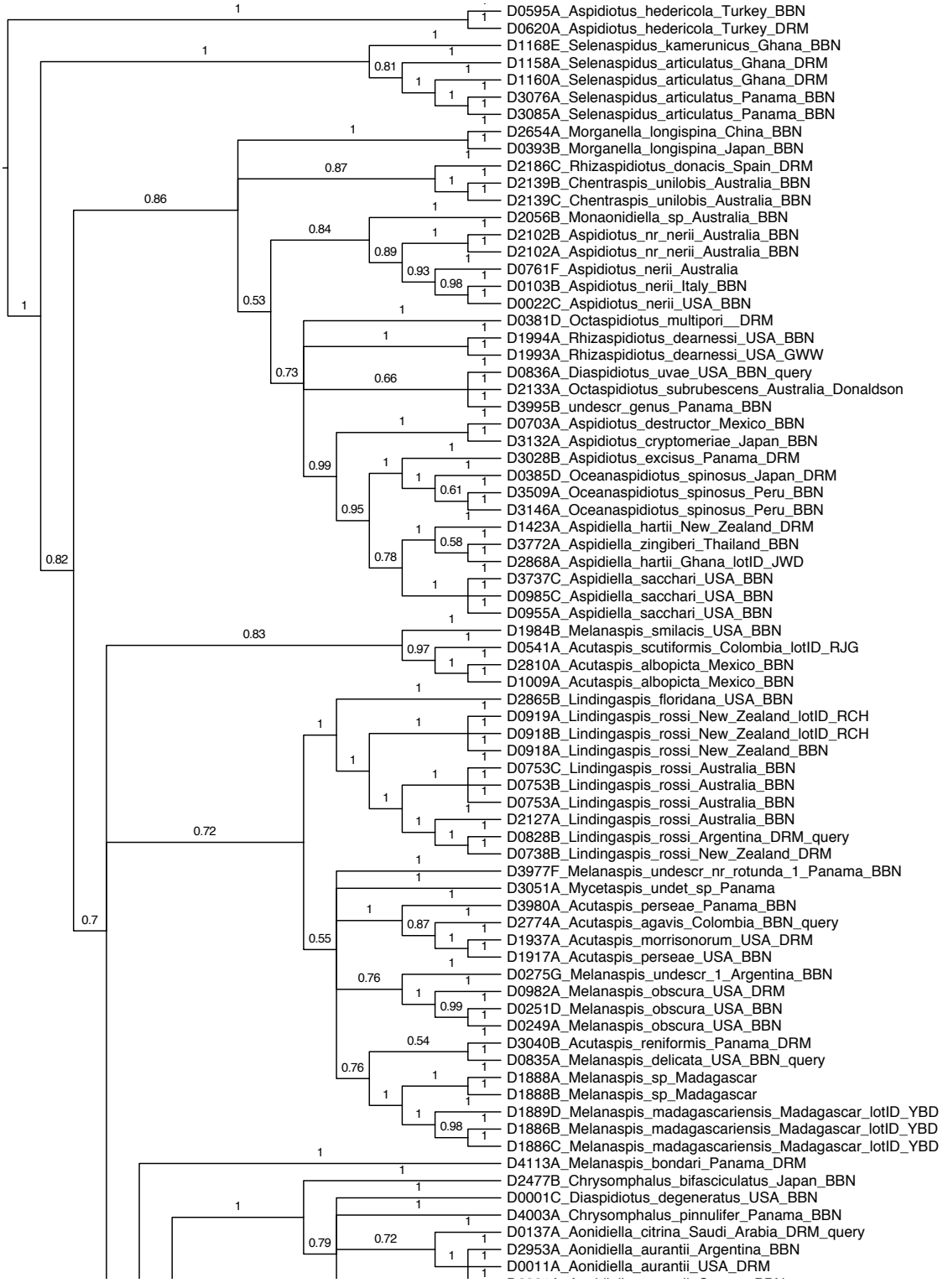
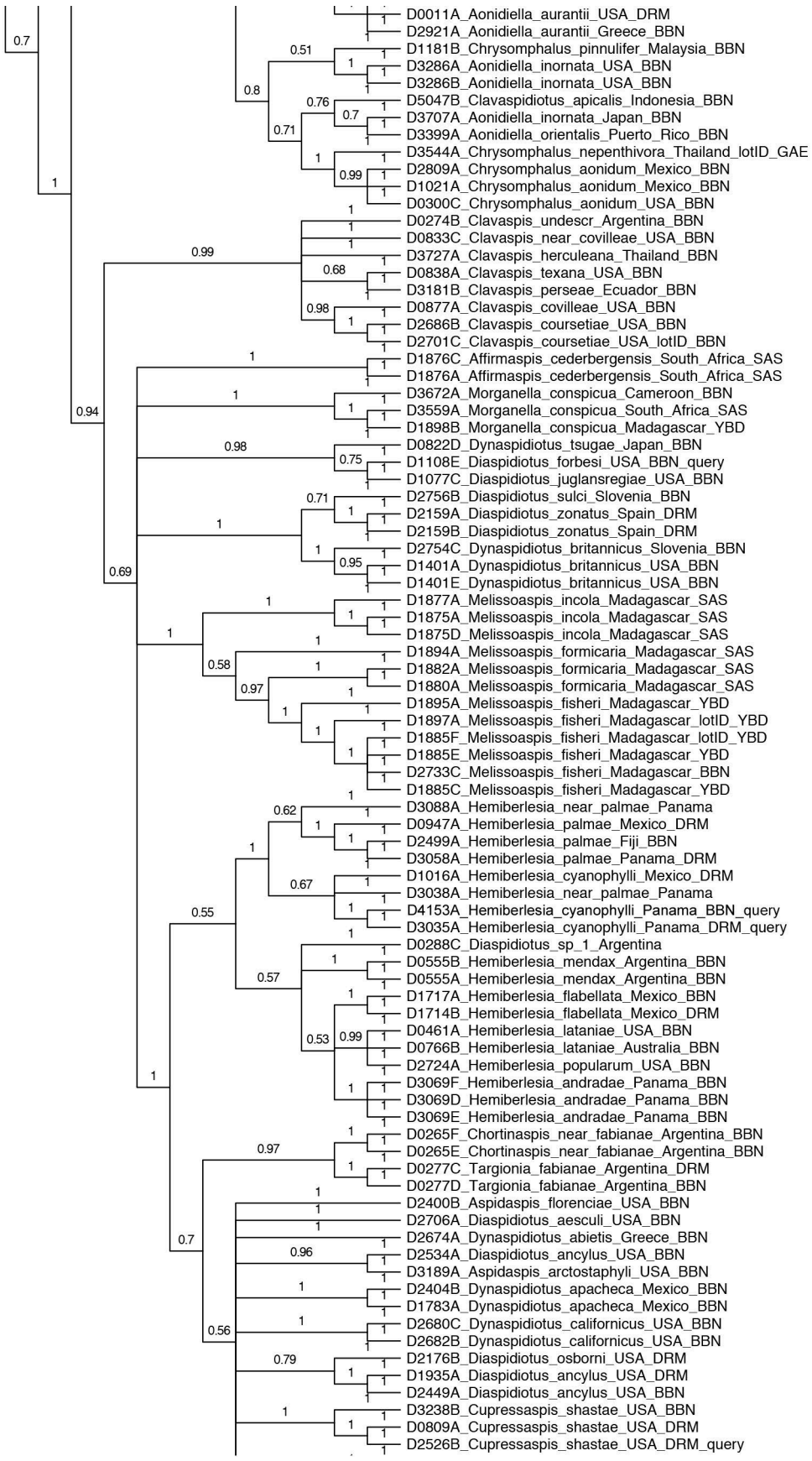


S4: COI-COII genealogy – The majority-rule consensus tree resulting from Bayesian analysis of a region of mitochondrial DNA encompassing the 3′ portion of cytochrome oxidase I (COI) and the 5′ portion of cytochrome oxidase II (COII). Branch support is indicated as posterior probabilities.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D3286B_Aonidiella_inornata_USA_BBN
D5047B_Clavaspidiotus_apicalis_Indonesia_BBN
D3707A_Aonidiella_inornata_Japan_BBN
D3399A_Aonidiella_orientalis_Puerto_Rico_BBN
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D2809A_Chrysomphalus_aonidum_Mexico_BBN
D1021A_Chrysomphalus_aonidum_Mexico_BBN
D0300C_Chrysomphalus_aonidum_USA_BBN
D0274B_Clavaspis_undescr_Argentina_BBN
D0833C_Clavaspis_near_covilleae_USA_BBN
D3727A_Clavaspis_herculeana_Thailand_BBN
D0838A_Clavaspis_texana_USA_BBN
D3181B_Clavaspis_perseae_Ecuador_BBN
D0877A_Clavaspis_covilleae_USA_BBN
D2686B_Clavaspis_coursetiae_USA_BBN
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D3559A_Morganella_conspicua_South_Africa_SAS
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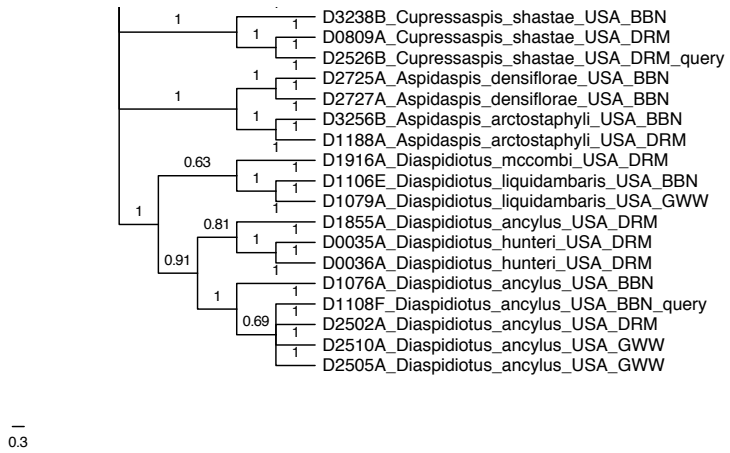
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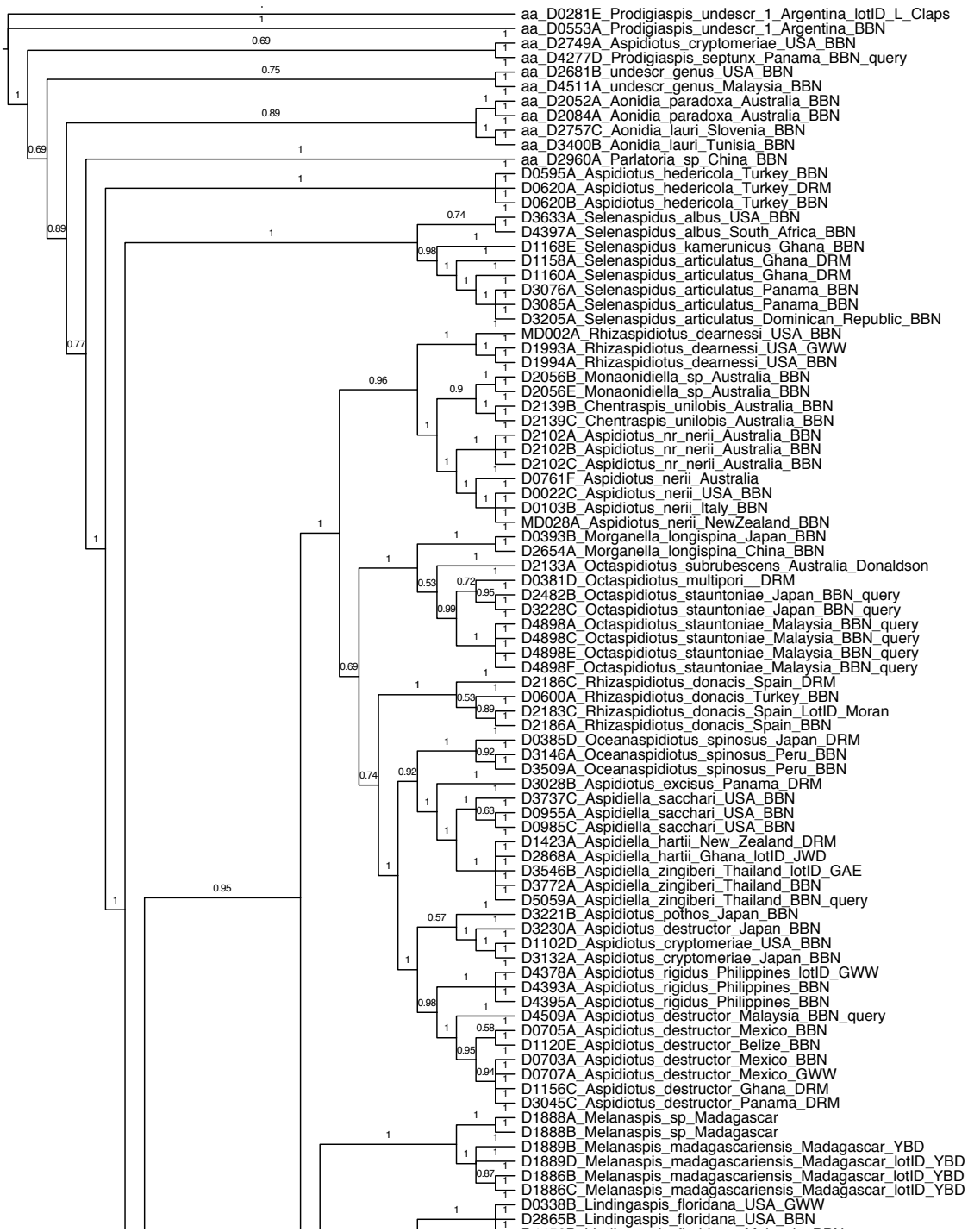
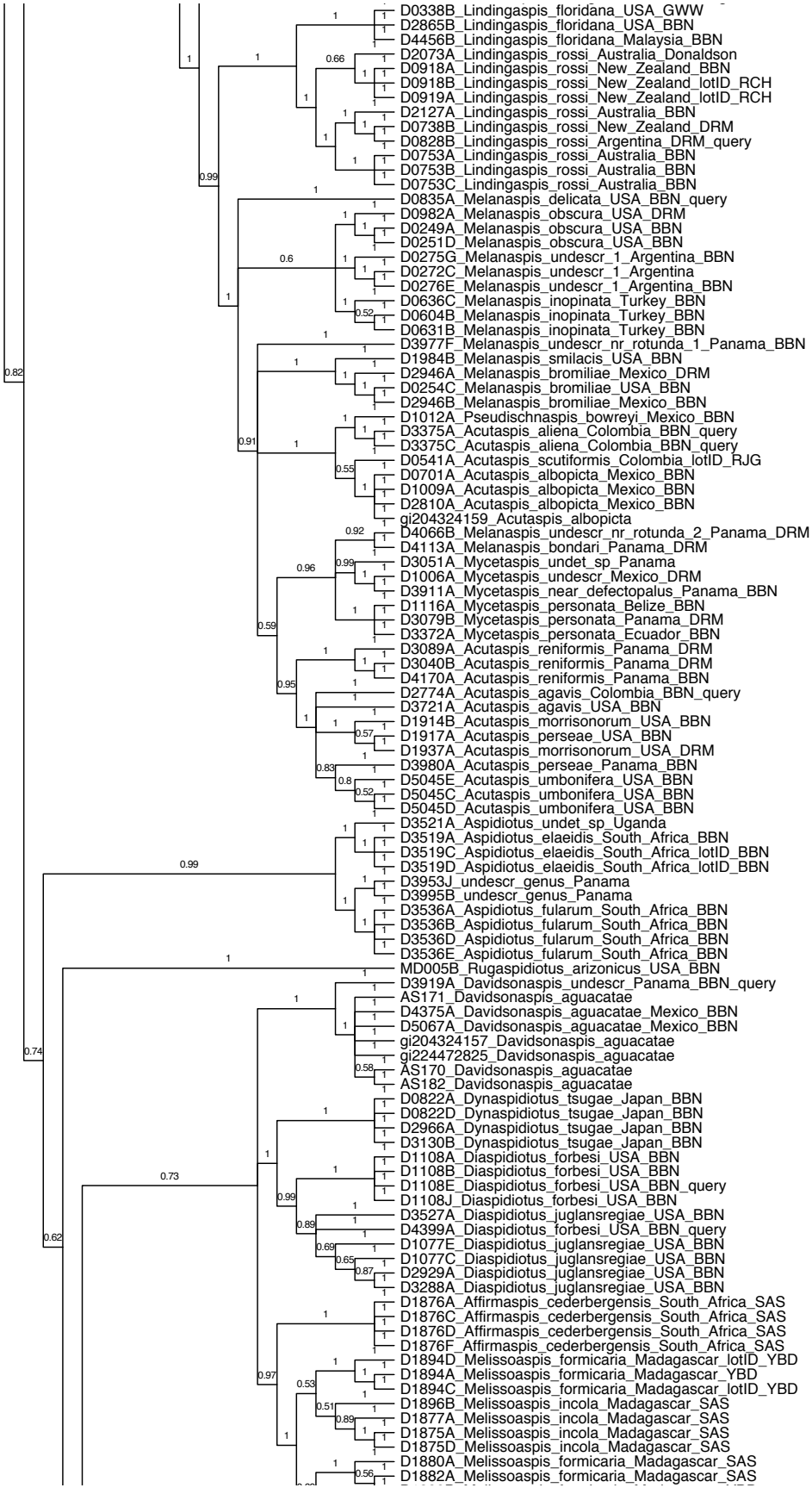


S5: Phylogeny of the tribe Aspidiotini − full taxon set – The majority-rule consensus tree resulting from Bayesian analysis of the concatenated dataset for four gene regions (28S, EF-1α, CAD, COI-COII) and the full taxon set of 127 species, 356 total taxa. Branch support is indicated as posterior probabilities.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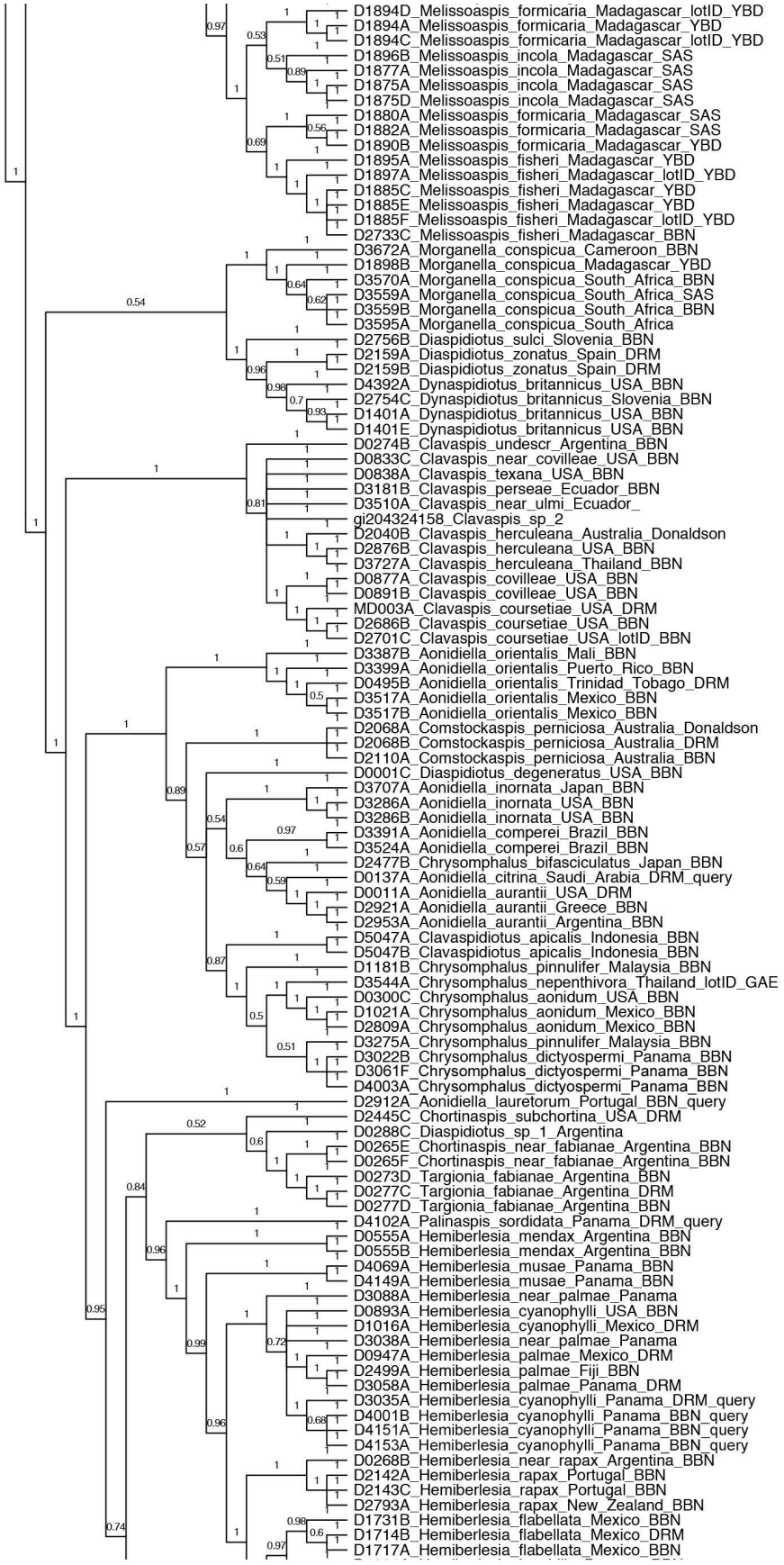
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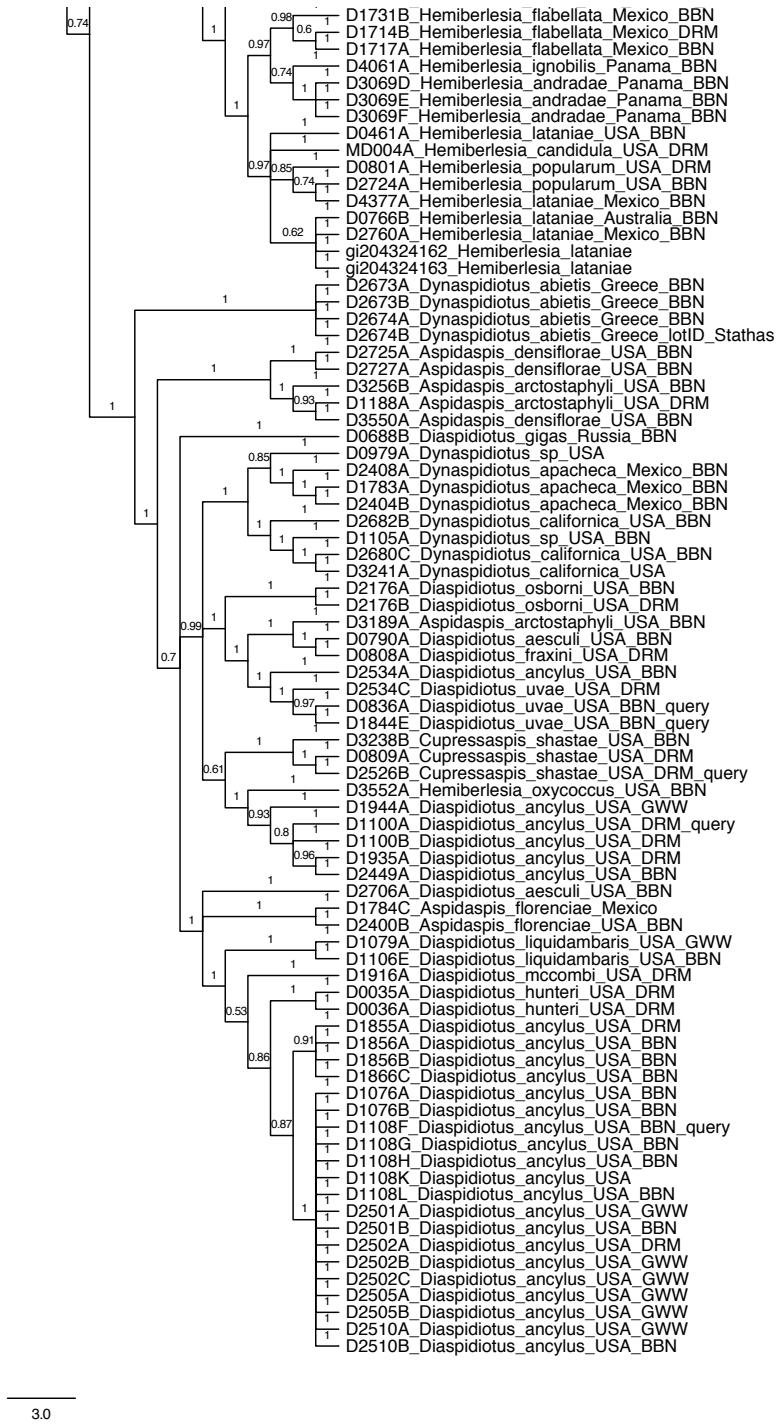
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D1784C_Aspidaspis_florenciae_Mexico
D2400B_Aspidaspis_florenciae_USA_BBN
D1079A_Diaspidiotus_liquidambaris_USA_GWW
D1106E_Diaspidiotus_liquidambaris_USA_BBN
D1916A_Diaspidiotus_mccombi_USA_DRM
D0035A_Diaspidiotus_hunteri_USA_DRM
D0036A_Diaspidiotus_hunteri_USA_DRM
D1855A_Diaspidiotus_ancylus_USA_DRM
D1856A_Diaspidiotus_ancylus_USA_BBN
D1856B_Diaspidiotus_ancylus_USA_BBN
D1866C_Diaspidiotus_ancylus_USA_BBN
D1076A_Diaspidiotus_ancylus_USA_BBN
D1076B_Diaspidiotus_ancylus_USA_BBN
D1108F_Diaspidiotus_ancylus_USA_BBN_query
D1108G_Diaspidiotus_ancylus_USA_BBN
D1108H_Diaspidiotus_ancylus_USA_BBN
D1108K_Diaspidiotus_ancylus_USA
D1108L_Diaspidiotus_ancylus_USA_BBN
D2501A_Diaspidiotus_ancylus_USA_GWW
D2501B_Diaspidiotus_ancylus_USA_BBN
D2502A_Diaspidiotus_ancylus_USA_DRM
D2502B_Diaspidiotus_ancylus_USA_GWW
D2502C_Diaspidiotus_ancylus_USA_GWW
D2505A_Diaspidiotus_ancylus_USA_GWW
D2505B_Diaspidiotus_ancylus_USA_GWW
D2510A_Diaspidiotus_ancylus_USA_GWW
D2510B_Diaspidiotus_ancylus_USA_BBN
3.0

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