A COMPARATIVE STUDY OF COURTSHIP BEHAVIORS ACROSS THE GENUS *APHELINUS* (APHELINIDAE, HYMENOPTERA) AND THEIR ROLE IN SPECIATION

by

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I would like to dedicate my thesis to my parents, thank you for your love and support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Studies of courtship behavior often focus upon one or a few species, with few implication about the effects of phylogenetic similarity or geographical distribution. Understanding isolating mechanisms among closely related species is important to ensure successful biological control introductions and reduce risk to native species. *Aphelinus*, a genus of aphid endoparasitoids, has been used to control invasive species of aphids as well as greenhouse pests. Courtship behavior within the *varipes* and *mali* species complex includes two types of antennae movements displayed by males after mounting females: simultaneous dipping and alternate waving. Courtship behaviors of 9 *Aphelinus* species across multiple species groups revealed strong selection for divergent simultaneous dipping and alternate waving durations in two separate pairs of closely related sympatric species, *A. coreae* and *A. rhamni*, and *A. hordei* and *A. kurdjumovi*. Within these pairs, the species that displayed long durations (*A. hordei* and *A. rhamni*) also displayed polymorphisms for most antennae position characters, while the other member(*A. kurdjumovi* and *A. coreae,* respectively) displayed moderate simultaneous dipping and alternate waving durations and, fixed traits for antennae spread characters. Three closely related allopatric species, *A. certus,*

A. varipes and *A. atriplicis,* displayed moderate simultaneous dipping and alternate waving durations and usually displayed fixed antennal position characters. Females of species whose males display moderate simultaneous dipping and alternate waving durations may rely upon antennal position for mate discrimination to a larger extent than females of species with long durations. Conversely, females of species with long durations may rely more heavily upon them, instead of antennal position characters. Simultaneous dipping and alternate waving durations, combined with antennal position characters were used to create a multinomial logistic regression model that fit the data well, suggesting it is possible to distinguish the species of a male based on his courtship display.

Chapter 1

STATISTICAL TESTING OF MALE COURTSHIP BEHAVIOR COMPONENTS OF 9 SPECIES OF *APHELINUS* (CHALCIDOIDEA, HYMENOPTERA)

1.1 Introduction

Aphelinus Dalman, 1820 (Aphelinidae, Chalcidoidea) is genus of solitary, synovigenic endoparasitoids that show much promise for use in biological control of invasive aphid species. *Aphelinus* species have been used to combat Russian Wheat Aphid(*Diuraphis noxia*) in South Africa and North America, as well as Soybean Aphid (*Aphis glycinis*) in North America. There are currently 93 described species of *Aphelinus* (Noyes, 2014). Understanding of taxonomy and species distributions have been hindered by the insects small size (about 1mm in length) and the high level of morphological similarity within species groups. Host range experiments on species in the *asychis*, *varipes* and *mali* species groups have shown that some species are generalists, parasitizing many aphid species, while others are specialists, parasitizing only a few species of related aphids, usually within a particular tribe or genus (Hopper, unpublished data).

Understanding the mechanisms that prevent gene flow between species is an important component of biological control involving introduction of exotic

agents. Hybridization between species reduce the efficacy of biological control introductions by producing progeny of lower fitness. Host range shifts could also occur, resulting in negative effects on native species and ineffective biological control of the targeted pest species. (Hopper et al. 2006) In the laboratory, temporal, geographical, and ecological pre-zygotic barriers are circumvented; only behavioral and physiological mechanisms of isolation remain. Potential pre-zygotic barriers include mate recognition, differences in mating behavior and physical incompatibility of genitalia (Hopper et al. 2006).

Very little is known about *Aphelinus* mate recognition. Male antennae are believed to be important during the courtship of some chalcidoid wasps because they contain pheromone-releasing secretary organs that are used in female mate acceptance (Gordh & Debach, 1978; Isidoro et al.1996; Romani et al. 2008). The scapes of male antennae in the *varipes* species complex contain glands that may emit pheromones involved in mate recognition. Similar glands are also seen on males from the *mali* species complex but are absent from the *asychis* species complex. *Aphelinus asychis* males lack glands on their antennae and have been observed to wing fan, a courtship component not present in the *varipes* and *mali* groups. Wing fanning may be used to waft male pheromones to the female and provide auditory and visual cues to the female.

1.1.1 Typical courtship sequence observed in the *varipes* and *mali* species complexes

Aphelinus species in the *varipes* and *mali* groups have similar male courtship components and sequences. The male approaches the female and follows her closely. Fauvergue et al. (1995) demonstrated with *Aphelinus asychis* that males find potential mates using trail pheromones produced by females. *Aphelinus* primarily search on foot for mates, therefore utilizing trail pheromones is effective for males to pursue females. Olfactometer testing suggested that volatile pheromones are not used by males to locate females. (Fauvergue et al. 1995) Upon male approach, the female may stop and remain motionless, allowing the male to determine her orientation and climb upon her dorsum. The male's hind legs are placed upon the middle of the female's forewing, the mid-legs are held outward and the forelegs are placed on the female's head near the apex of each eye. Upon mounting, the male immediately begins courtship behaviors.

Aphelinus courtship behavior consists of simultaneous dipping and alternate waving of the male's antennae. Simultaneous dipping often occurs first, the male lowers its head and both antennae towards the female antennae, sometimes past the female antennae along the sides of her head. Once the male antennae have reached the bottom of the movement they are lifted back to the top

of the movement, completing one full “dip”. This single movement, here referred to as a “Simultaneous Dip”, is repeated numerous times, usually at a steady rate. The second movement consists of alternatively lowering and raising the individual male antennae, here referred to as “Alternate Waving”. Alternate waving is usually not as dramatic as simultaneous dipping. These two movements are provide a stark contrast to one another in most species, providing a unique sequence for the female to discriminate whether the male is hetero- or con-specific. The male courts until the female lifts her abdomen, exposing her genital plate and allowing the male to mate with her, or until the female walks away, causing the male to dismount. The male will then move backwards and copulate with the female for about three seconds and then immediately return to courting the female, here referred to as “ Mate Guarding.” During mate guarding, the same sequence of male courtship movements are shown. Mate guarding may prevent other males from mounting and mating with the female during a window of receptivity when females are still producing trail pheromone. Mate guarding may also persuade the female to store the sperm in her spermatheca. The entire series of events from the original approach to copulation or rejection is considered one courtship bout. If the male is rejected before copulation the entire series is repeated.

The purpose of this investigation is to quantify the courtship behavior of 9 species of *Aphelinus* and determine whether significant differences occur and can be used to recognize species. *Aphelinus* courtship movements are very complex and females likely use multiple characters when selecting a mate. Therefore, when an individual's courtship characters are combined into a model, it should be possible to predict which species the individual belongs to.

1.2 Materials And Methods

Nine species of *Aphelinus* were collected in the field (Table 1) by Kim Hoelmer, Keith Hopper, Dominique Coutinot, Natalie Ramualde, Dave Ragsdale, and George Heimpel. Parasitoid cultures were reared in laboratory conditions at 20C and 16 light:8 dark photo-period. Multiple cages of each culture were turned over by Keith Hopper and Kathryn Lanier every 3 weeks, transferring 200 wasps per cage to ensure genetic diversity. Gestation period for *Aphelinus* is typically 21 days, seven days after oviposition, the parasitized aphid turns black and these mummified aphids are easy to spot with the naked eye. In the last week before the anticipated emergence date, mummies were isolated from one another to prevent matings and determine exact age of the wasps. Individual mummies and a small drop of honey water solution were placed individually in the wells of

microtiter plates and the wells were sealed with a rubber stopper. The microtiter plates were placed in plastic bags with a moist paper towel to prevent desiccation. Emergence was scored daily to accurately identify wasp age.

All observations consisted of a recently emerged (<3 days old) unmated conspecific pair in a behavior arena for 5 minutes or until mating had occurred and mate guarding had ended. Behavior arenas consisted of two pieces of weatherstripping foam (approximately two inches in length, half an inch in width) adhered to two glass slides, one with a hole approximately 1 cm in diameter and 0.5 cm in height punched in the weatherstripping foam, which is the area in which the wasps were placed. After each observation where adeagal intromission was observed, the female was dissected to ascertain if sperm transfer had occurred. Courtship bouts that lead to adeagal intromission and sperm transfer were considered successful. Mating frequency, female choice and unsuccessful male courtship behaviors were not the focus of the current research. Therefore, only successful courtship bouts were analyzed. The experimental unit used was the entirety of the successful courtship bout.

Observations were performed with a Nikon Dissecting Scope at 10X power. An Optronics camera system was used to record the observations which were written to DVD using a Panasonic DVD writer. Handbrake version

0.9.9.5530 was used to convert ripped DVD observation files from .VOB to .MP4 format. Kinovea version 0.8.15 was used for playback of the observations and to measure the lengths of courtship components. R version 3.0.1 and SAS version 9.4 were used for statistical analysis.

1.3 Observation Annotation and Analysis

Total courtship duration was measured from the start of male antennation until adeagal intromission. Male antennation was composed of two primary movements: simultaneous dipping and alternative waving. Each movement's duration was measured to the nearest tenth of a second. Movement amplitude relative to the female's antennae was determined by the location of the male's antennae during simultaneous dipping; either below, equal to or above the female's antennae. The antennal spread was determined at the top and bottom of male antennal movements, relative to the width of the head. These ordinal states are (from narrow to wide): intraocular, mideye, head and greater than head (Figure 1). Antennae movement during simultaneous dipping resembles an inverted V shape. Typically the antennae spread at the top of the movement is narrow and widens as the antennae are dipped towards the female. Inverted V shape was measured as the change in width between the top and bottom of the

movement; straight(0 change in state), slight(1 change in state) or strong(>1 change in state) For example, if top and bottom antennae spread were the same width, the inverse V shape would be straight, due to a difference of 0 character states. If the top were mideye width and the bottom head width, then inverse V shape would be slight due to a 1 character change in state. If the top were mideye width and the bottom greater than head width, then inverse V shape would be strong due to a 2 character state change.

Values from 63 individuals across 9 species were used to perform multiple comparison testing of simultaneous dipping and alternate waving durations using Tukey HSD. Seven behavioral variables were analyzed (average simultaneous dipping duration, average alternate waving duration, antennae spread at top of simultaneous dipping, antennae spread at bottom of dipping, antennae spread during alternate waving, inverted V shape of antennae during simultaneous dipping, and the position of male antennae at the bottom of simultaneous dipping relative to female antennae). The average number of simultaneous dips was excluded for two reasons. First, there were missing data for *A. glycinis.* In addition rates of all other species except *A.* near *daucicola*, were very similar, averaging about 7 dips per second. The model was chosen based on optimal AIC scores, R^2 values and percentage of concordant pairs(percent of concordant pairs

is a measure of the ability of the model to correctly distinguish between a pair of different observations). Other models with fewer number of species and variables were tested but always resulted in worse models.

1.4 Results

Significant differences were seen for most variables; mating frequency (χ^2 =42.9, df=8, p=0.00) (Table 2), average simultaneous dipping (df=8, F=73.5, p=0.00)(Figure 4) and alternate waving (df=8, F=35.4, p=0.00) (Figure 5) durations, male antennae spread at top of simultaneous dipping movement (χ^2 = 78.6 df=16 p=0.00) (Table 3), male antennae spread at bottom of simultaneous dipping movement (χ^2 = 83.3 df=24 p=0.00) (Table 4), male antennae spread during alternate waving movement (χ^2= 39, df=8, p=0.00) (Table 5), inverted "V-shape" of antennae during simultaneous dipping, (χ^2= 60.1 df=16 p=0.00) (Table 6), male antennae position at bottom of simultaneous dipping, relative to female antennae (χ^2=90.3, df=16, p=0.00) (Table 7) and comparison of male antennae spread at top of simultaneous dipping to alternate waving spread (χ^2=75.3, df=16, p=0.00) (Table 8). Number of simultaneous dips per second was similar for all species except *A.* near *daucicola*, (Table 9). Tukey HSD multiple comparison revealed three groups of species for both average simultaneous dipping and

alternate waving durations (Table 10, Figures 2 and 3). For all species, if a male was to be successful, it was usually during the first courtship bout, but occurred as late as the 4th bout (mode 1, median 1, range 4). The logistic regression model had a strong fit to the data(R^2=0.83). The model successfully predicted the species to which a male belongs based on male courtship components (93.1 concordant, 6.8 discordant).

1.5 Discussion

The purpose of this investigation was to quantify the courtship behavior of 9 species of *Aphelinus,* determine whether significant differences occur and if they can be used to recognize species. Almost all variables were significantly different, suggesting that courtship behaviors vary widely across these species. The multinomial logistic regression demonstrated that it is possible to determine species membership using only courtship behavior.

The two major components of male courtship behavior differed greatly among the 9 species. Multiple comparison testing of both revealed three groupings: short, intermediate and long. For both movements, the members of these three groups were not specific to species group; both species groups displayed multiple durations. Interestingly, simultaneous dipping and alternate

waving showed identical patterns with the exception of *A.glycinis*. The behavior of *A.glycinis* is unique, simultaneous dipping is a very brief period during which the male shakes violently to such an extent that accurate counting of simultaneous dips was not possible. Both *A. glycinis* and *A.* near *daucicola* have unique courtship behaviors, high mating frequency and short courtship bouts. This suggests that these specialized courtship behaviors may prevent hybridization and allow for females to efficiently determine the species and fitness of a courting male.

The current study used many of the same cultures as Heraty et al. (2007). In this study, mating frequency varied greatly between species. When compared to Heraty et al. (2007), mating frequencies for the other varipes complex species observed in this experiment were varied and overall quite low. This may be due to different exposure durations. Heraty et al. (2007) used an exposure period of 24 hours before dissecting the female to observe sperm presence in the spermatheca. All 62 dissections had sperm present in the spermathca, consistent with previous studies, suggesting that sexual isolation hinges on pre-mating mechanisms (Heraty et al., 2007; Wu et al., 2004).

The multinomial logistic regression model predicts species based on male courtship behavior, suggesting females may be able to identify a male as con- or

heterospecific based on courtship display. This investigation has demonstrated that statistical differences among 9 species, which vary in phylogenetic and geographic distance, does occur. Investigating the differences of courtship behavior in both a phylogenetic and geographical context may demonstrate the effects of these variables upon courtship behavior and the isolation mechanisms that prevent hybridization between *Aphelinus* species.

Chapter 2

THE EFFECTS OF GEOGRAPHIC AND PHYLOGENETIC DISTANCE ON *APHELINUS* COURTSHIP BEHAVIOR

2.1 Introduction

Many courtship behavior studies have focused upon one or a few species. (Ardehet al. 2004; Barstock & Manning, 1955; Mackauer, 1969; Niehuis et al., 2013; Steiner & Ruther, 2009; Tallamy et al. 2003; Tallamy 1996). Studying many species that vary in geographical distribution and derive from different phylogenetic lineages allows investigation of current mechanisms that prevent hybridization and may elucidate the past evolutionary processes responsible for those differences. Several systems have been investigated in a similar manner: the Hawaiian *Drosophila* (Drosophilidae, Diptera) (Kaneshiro, 2000, 2006), the scale parasitoid *Aphytis* (Aphelinidae, Hymenoptera) (Rao et al. 1969, Khasimuddin & DeBach, 1975; Gordh & Debach, 1978), the lepodopteran egg parasitoid *Trichogramma* (Trichogrammatidae, Hymenoptera) (Pinto, 1992, Pinto et al. 2003) and European *Pardosa* (Lycosidae, Araneae) spiders (Chiarle et al. 2013).

Aphelinus is a genus of parasitic wasps that have been used in biological control of invasive aphid species in North America as well as in South Africa. *Aphelinus* species are also commercially available for biological control of aphid

pests in greenhouses. Understanding the mechanisms that prevent gene flow between closely related parasitoid species is an important component of biological control efforts. Hybridization between species can reduce the efficacy of biological control introductions. Host range shifts could also occur, resulting in negative effects on native species and ineffective biological control of the intended pest species. (Hopper et al. 2006)

Although it is difficult to determine whether speciation happened in sympatry versus allopatry, we can use current geographical distributions to test current effects of geography upon courtship behavior and reproductive isolation. Kazmer et al. (1996) found that populations in the *asychis* complex from China, Kazakhstan and the Mediterranean basin were usually reproductively isolated by pre-zygotic barriers. Males would court heterospecific females, but females would not copulate with them. However, 17% of the crosses between Chinese males and Kazakhstani females showed sperm transfer, although this cross did not produce female progeny, indicating a post-mating barrier. Heraty et al.(2007) found that species in the *varipes* species complex were almost always reproductively isolated. However, reciprocal crosses of *A. certus* and *A. atriplicis* produced viable female progeny, although backcrosses had lower fecundity (Wu et al., 2004). Further experiments have shown that isolation is pre-zygotic in

almost all cases; males court heterospecific females, but sperm is not transferred. However, *A. atriplicis* and *A. varipes* do occasionally mate, although crosses with female progeny are quite rare, indicating post-mating barriers. Closely related species were usually reproductively isolated, but heterospecific matings of allopatric species with post-mating problems did occur in both Kazmer et al. (1996) and Heraty et al. (2007).

Hopper et al. (2012) developed a key for the *mali* species complex and described three new species, *A. glycinis*, *A. rhamni* and *A. coreae*. *A. rhamni* and *A. coreae* are morphologically similar, differing in male antennal club length and the coloration of scape and mesotibiae (Hopper et al. 2012). These two species are reproductively isolated from each other in laboratory crossing experiments (Hopper 2012). Molecular methods have placed them sister to one another, with very little divergence. Host range testing has shown that all three species are host specific to *Aphis glycines* and *Aphis gossypii*(soybean and cotton aphid, respectively). *A. rhamni* and *A. coreae* provide an opportunity to determine if selection for divergent courtship behaviors in the presence of similar life traits and geographical distribution occurs.

The purpose of this investigation is to investigate the effects of differing phylogenetic and geographical distances upon courtship behavior. If courtship

behavior is an important component of reproductive isolation between *Aphelinus* species, then closely related species that exist in sympatry should differ more than closely related allopatric species. If courtship behavior is genetically constrained, then closely related species should have more similar courtship behaviors than more distantly related species.

2.2 Methods

Nine species of *Aphelinus* were collected in the field (Table 1) by Kim Hoelmer, Keith Hopper, Dominique Coutinot, Natalie Ramualde, Dave Ragsdale, and George Heimpel. Parasitoid cultures were reared in laboratory conditions at 20C and 16 light:8 dark photo-period. Multiple cages of each culture were turned over by Keith Hopper and Kathryn Lanier every 3 weeks, transferring 200 wasps per cage to ensure genetic diversity. The culture of *Aphelinus* near *daucicola* has not been identified to species yet, so distribution information is unknown, although it should be noted that the species it most closely resembles, *Aphelinus .daucicola,* is distributed across Europe (Noyes, 2014).

A molecular phlyogeny was constructed using branch and bound methods based on single nucleotide polymorphisms(SNPs) of putative genes (Figure 6) (Hopper unpublished data). The molecular phylogeny reveals three closely

related species pairs: (1)*A. certus* and *A. varipes*,(2) *A. hordei* and *A. kurdjumovi* , and, (3)*A. coreae* and *A. rhamni.* *Aphelinus atriplicis* is basal to both *A. certus* and *A. varipes* but not distant from them and, when used as an out group, allows another level of comparisons. *Aphelinus hordei* and *A. kurdjumovi* form the species pair that displays the most phylogenetic distance between each other, but they are still relatively closely related. Lastly, *A. coreae* and *A. rhamni* are the species pair that displays the smallest distance between one another and is relatively distant to the other species in the *mali* complex, *A. glycinis*.

Maximum parsimony was used to map traits to the molecular phylogeny (Mesquite version 2.75). Seven antennation characters (Tables 3-8, Figures 4 and 5) were mapped (Figures 7-14). Continuous trait values were calculated for average simultaneous dipping duration and average alternate waving duration by finding each individual's average value and then finding the species average among individuals. Unfortunately, Mesquite cannot take into account frequencies of polymorphic traits. Therefore, multiple character states for a species were all handled as occurring at equal frequency, regardless of observed frequency.

2.3 Results

2.3.1 Geographic Distribution

Culture collection and known distribution information were used to determine which species exist in sympatry or allopatry (Table 1). Exact knowledge of geographical distribution is difficult due to misidentifications and low sampling. *A. certus*, and *A. varipes* are probably allopatric, being separated by great distance and several mountain ranges. The level of contact between *A. varipes* and *A. atriplicis* is difficult to categorize; the reported distribution of *A. varipes* is very wide, across all of Europe (Japoshvili & Abrantes, 2006; Japoshvili, 2009), while *A. atriplicis* has only been reported in Georgia, Hungary, Russia and Ukraine. The Carpathian mountain range may prevent some contact, but it is possible that some geographic overlap does occur. For the purposes of this study, *A. varipes* and *A. atriplicis* will be considered to exist in allopatry. *Aphelinus hordei* and *A. kurdjumovi* are sympatric in Georgia and Ukraine. The close proximity and lack of geographical barriers between the collection sites for *A. coreae*, *A. rhamni* and *A. glycinis* suggest that these species are sympatric. Furthermore, it is very likely that these three species all come into contact with *A. certus*, which is widely distributed across much of far eastern Asia.

2.3.2 Ancestral State Reconstruction

Closely related species that are sympatric (*A. coreae* and *A.rhamni*, and *A. kurdjumovi* and *A. hordei*) differed greatly in courtship behaviors. Closely related species that are allopatric (*A. certus*, *A. varipes*, and *A. atriplicis*) displayed similar courtship behaviors. Phylogenetic distance also seemed to affect courtship behavior, e.g. *A. glycinis* differed greatly from *A. coreae* and *A. rhamni*, although this may also be compounded by all three species being allopatric. Courtship behaviors of the distantly related *A.* near *daucicola* are different from other species. It is difficult to attribute this solely to the large phylogenetic distance separating *A.* near *daucicola* from both the *varipes* and *mali* complexes because of its unknown geographical distribution. If this species is *A. daucicola,* which has been reported across Europe, it is sympatric with *A. varipes, A. hordei*, and perhaps *A. kurdjumovi* and *A. atriplicis*.

2.4 Discussion

Closely related species that exist in sympatry differed more than closely related allopatric species. Courtship behavior is likely genetically constrained, closely related species displayed more similar courtship behaviors compared to distantly related species.

A. varipes, *A. certus* and *A. atriplicis,* the three closely related allopartic species, displayed similar durations for simultaneous dipping and alternate waving. Categorical variables were fixed within species but some times differed among species. The same antennae position trait was displayed for alternate wave spread and bottom of simultaneous dipping relative to female antennae in all three species. Similar antennae position traits between these three closely related species suggests that parts of courtship behavior may be phylogenetically constrained. However, several traits related to antennae spread during simultaneous dipping differed among these species.

A. certus and A. atriplicis differed in *s*imultaneous dipping top spread, inverted "V" shape, and antennal spread at the top of simultaneous dipping and alternate waving. Most of these characters are rooted in simultaneous dipping top spread behavior. *A. certus* and *A. varipes* differ in two related variables: bottom spread of simultaneous dipping and inverted "V" shape of simultaneous dipping. *.A. certus* and *A. atriplicis* further in both phylogenetic distance and in number of courtship variables. However, these species hybridize at a higher rate than *A. certus* and *A. varipes* in laboratory crossing experiments (Heraty et al., 2007), the spread between male antennae at the bottom of simultaneous dips may

be an important component for female selection. *A. varipes* and *A. atriplicis* differed antennnal spread at the top and bottom of simultaneous dipping and during alternate waving. These species do not hybridize in laboratory crossing experiments (Heraty et al., 2007), possibly due to the differences in antennae spread at the bottom of simultaneous dipping.

A. kurdjumovi almost always displayed fixed traits for antennae position, while *A. hordei* frequently displayed polymorphisms. *A. hordei*'s long durations of simultaneous dipping and alternate waving may be important for mate discrimination, leading to little selection on antennal position traits. In contrast, *A. kurdjumovi* has simultaneous dipping and alternate waving durations similar to other species in the *varipes* complex. Thus its fixed traits for antennae position may be a response to geographical overlap with *A. varipes* and lack of difference in movement durations. A similar pattern is also seen with *A. rhamni* and *A. coreae*. *Aphelinus coreae* typically displayed fixed traits for antennae position and has simultaneous dipping and alternate waving durations similar to other species. *A. rhamni* was polymorphic for most antennae position traits and longdurations of simultaneous dipping and alternate waving.

Long durations of simultaneous dipping and alternate waving in *A. hordei*

and *A.rhamni* may have been selected for, allowing for effective conspecific partner identification. These characters may be the primary means of mate selection used by *A. hordei* and *A.rhamni*, which would explain why other traits related to antennae spread are polymorphic. Differences between *A. varipes*, *A. certus* and *A. atriplicis*, closely related species allopatric species, are smaller than those associated with closely related sympatric species. Due to the lack of extreme durations of simultaneous dipping and alternate waving, antennae position traits are likely under strong selection, explaining the fixed traits associated with all species that have moderate simultaneous dipping and alternate waving.

Comparison of species in the *varipes* and *mali* complex with *A.* near *daucicola* is difficult due to large phylogenetic distance and unknown geographocal distribution of A. near *daucicola*. It is interesting that the short durations of movements associated with high mating frequency, a pattern also seen with *A.glycinis*.

Aphelinus hordei and *A. glycinis* are being used against *Diuraphis noxia* and *Aphis glycines*, respectively. Their courtship behaviors of these parasitoid species are very different from closely related species. *A. glycinis* has a unique courtship display, involving short movement durations with violent body shaking

and extremely fast antennation during simultaneous dipping. *A. hordei* courtship behavior has long durations of simultaneous dipping and alternate waving which are much different from other species in the *varipes* complex. These novel courtship behaviors reduce the chance of hybridization.

TABLES

Table 1. Culture origin, collection date and reported distribution of species used. Material collected by Kim Hoelmer, Keith Hopper, Dominique Coutinot, Natalie Ramualde, Dave Ragsdale, and George Heimpel.

Species	Culture origin	Culture collection date	Reported distribution
A. atriplicis	Tbilisi, Republic of Georgia	2000	Georgia, Hungary, Russia and Ukraine
A. certus	South Korea	2009	China , Japan, Western Russia
A. varipes	Montpellier, France	2006	Eastern and Western Europe
A. hordei	Montpellier, France	2000	Austria, Georgia, Russia, and Ukraine
A. kurdjumovi	Tbilisi, Republic of Georgia	2000	Republic of Georgia and Ukraine
A. rhamni	Beijing, China	2005	Beijing, China
A. coreae	South Korea	2009	South Korea
A. glycinis	Liaoning province, China	2005	Liaoning province, China
A.* near *daucicola	Delaware, USA	2013	Delaware, USA

Table 2. Results of 233 observations among 9 species of *Aphelinus,* including number and frequency of matings, presence of sperm in successful matings and total number of observations.

Species	**Number of matings**	**Total number of observations**	**Sperm present in spermatheca**
A. atriplicis	9	16	8 of 8
A. certus	3	57	1 of 1
A. varipes	8	24	4 of 4
A. hordei	6	16	6 of 6
A. kurdjumovi	8	25	6 of 6
A. rhamni	10	24	6 of 6
A. coreae	6	39	5 of 5
A. glycinis	10	13	10 of 10
A.* near *daucicola	7	19	6 of 6

Table 3. Male antennae spread at top of simultaneous dipping movement. Width measured relative to eye and head width. $\chi^2 = 78.6$ df=16 p=0.00

Species	**Intraocular**	**Mideye**	**Head**	**Wider than head**
A. atriplicis	9	0	0	0
A. certus	0	0	3	0
A. varipes	0	0	6	0
A. hordei	2	3	0	0
A. kurdjumovi	4	0	0	0
A. rhamni	1	8	0	0
A. coreae	0	2	0	0
A. glycinis	0	4	4	0
A.* near *daucicola	6	0	0	0

Table 4. Male antennae spread at bottom of simultaneous dipping movement. Width measured relative to eye and head width. (χ^2 = 83.3 df=24 p=0.00

Species	Intraocular	Mideye	Head	Wider than head
A. atriplicis	0	0	9	0
A. certus	0	0	3	0
A. varipes	0	0	0	6
A. hordei	1	2	0	2
A. kurdjumovi	4	4	0	0
A. rhamni	1	1	7	0
A. coreae	0	0	1	0
A. glycinis	0	1	6	0
A.* near *daucicola	0	0	0	7

Table 5 Male antennae spread during alternate waving movement. Width measured relative to eye and head width. $\chi^2 = 39$, df=8, p=0.00

Species	Intraocular	Mideye
A. atriplicis	0	9
A. certus	0	3
A. varipes	0	6
A. hordei	5	0
A. kurdjumovi	4	0
A. rhamni	1	8
A. coreae	0	2
A. glycinis	0	6
A.* near *daucicola	4	2

Table 6. Inverted "V-shape" of antennae during simultaneous dipping. Units reflect the change of width between top and bottom of simultaneous dip. Straight denotes no change, slight denotes a change of one unit and strong denotes a change of two units. $\chi^2 = 60.1$ df=16 p=0.00

Species	Straight	Slight	Strong
A. atriplicis	0	9	0
A. certus	1	0	0
A. varipes	0	6	0
A. hordei	1	3	2
A. kurdjumovi	0	4	0
A. rhamni	2	5	0
A. coreae	0	1	0
A. glycinis	5	2	0
A.* near *daucicola	0	0	6

Table 7. Male antennae position at bottom of simultaneous dipping, relative to female antennae.
χ^2=90.3, df=16, p=0.00

Species	Below	Equal	Above
A. atriplicis	0	9	0
A. certus	0	3	0
A. varipes	0	6	0
A. hordei	0	2	3
A. kurdjumovi	0	0	7
A. rhamni	2	5	1
A. coreae	1	3	0
A. glycinis	10	0	0
A. near daucicola	6	0	0

Table 8. Comparison of simultaneous dipping and alternate waving spread. χ^2=75.3, df=16, p=0.00

Species	Simultaneous Wider	Equal	Alternate Wider
A. atriplicis	0	0	9
A. certus	3	0	0
A. varipes	6	0	0
A. hordei	0	2	3
A. kurdjumovi	0	4	0
A. rhamni	0	8	0
A. coreae	0	2	0
A. glycinis	2	4	0
A.* near *daucicola	0	4	2

Table 9. Average and standard deviation of male courtship behavior components. Simultaneous dips per second is the quotient of the average number of simultaneous dips and the average length of simultaneous dipping(dividend and divisor, respectively).

Species	**Average number of simultaneous dips**	**Simultaneous dips per second**
A. atriplicis	25.34±4.21	6.76
A. certus	17.63±3.08	6.56
A. varipes	20.12±4.66	5.65
A. hordei	42.60±4.56	7.04
A. kurdjumovi	23.5±3.26	7.1
A. rhamni	36.28±2.6	5.9
A. coreae	23.47±1.49	6.48
A. glycinis	-	-
A.* near *daucicola	2±0.0	2.28

Table 10. Tukey HSD multiple comparison p-values of average simultaneous dipping (shaded cells) and alternate waving (unshaded cells) durations.

	A. certus	***A. varipes***	***A. atriplicis***	***A. hordei***	***A. kurdjumovi***	***A. rhamni***	***A. coreae***	***A.glycinis***	***A.* near *daucicola***
A. certus		0.97	0.2	0	0.86	0	0.41	0	0
A. varipes	0.99		0.47	0	0.99	0	0.82	0	0
A. atriplicis	0.21	0.27		0	0.86	0	0.99	0	0
A. hordei	0	0	0		0	0.99	0	0	0
A. kurdjumovi	0.1	0.12	0.99	0.01		0	0.98	0	0
A. rhamni	0	0	0	0.03	0		0	0	0
A. coreae	0.6	0.83	0.99	0	0.95	0		0	0
A. glycinis	0.03	0.2	0.99	0.01	0.99	0	0.78		0.99
A.* near *daucicola	0.43	0	0	0	0	0	0	0	

FIGURES

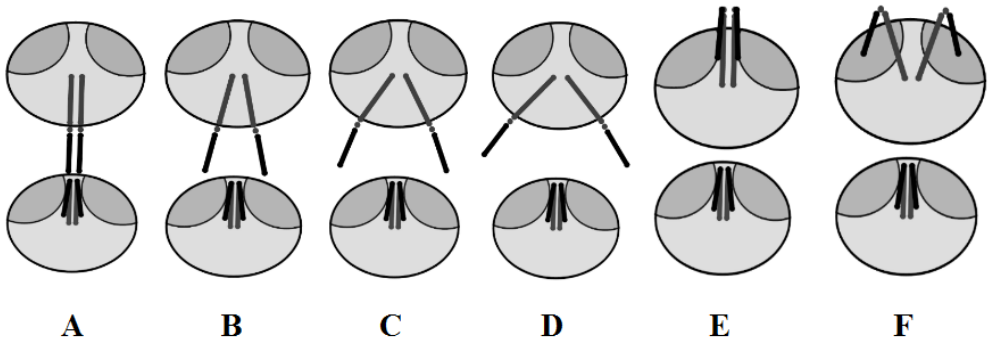

Figure 1. Diagram of antennae spread during courtship behavior while male is mounted on top of female: (A) bottom of simultaneous dipping interocular, (B) bottom of simultaneous dipping mideye, (C) bottom of simultaneous dipping head width, (D) bottom of simultaneous dipping wider than head, (E) top of simultaneous dipping intraocular, and (F) top of simultaneous dipping mid eye.

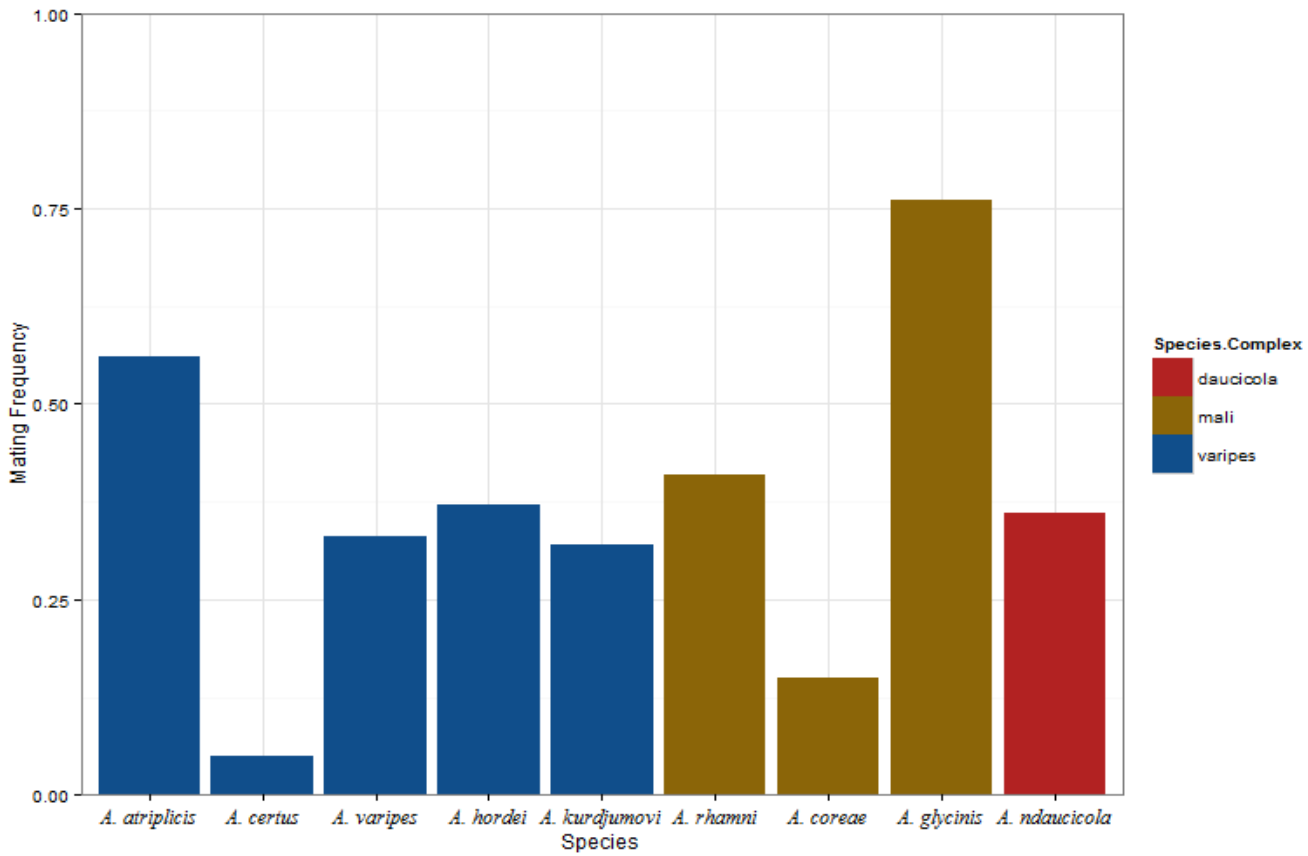

Figure 2. Mating frequency of 9 *Aphelinus* species across three species complexes.

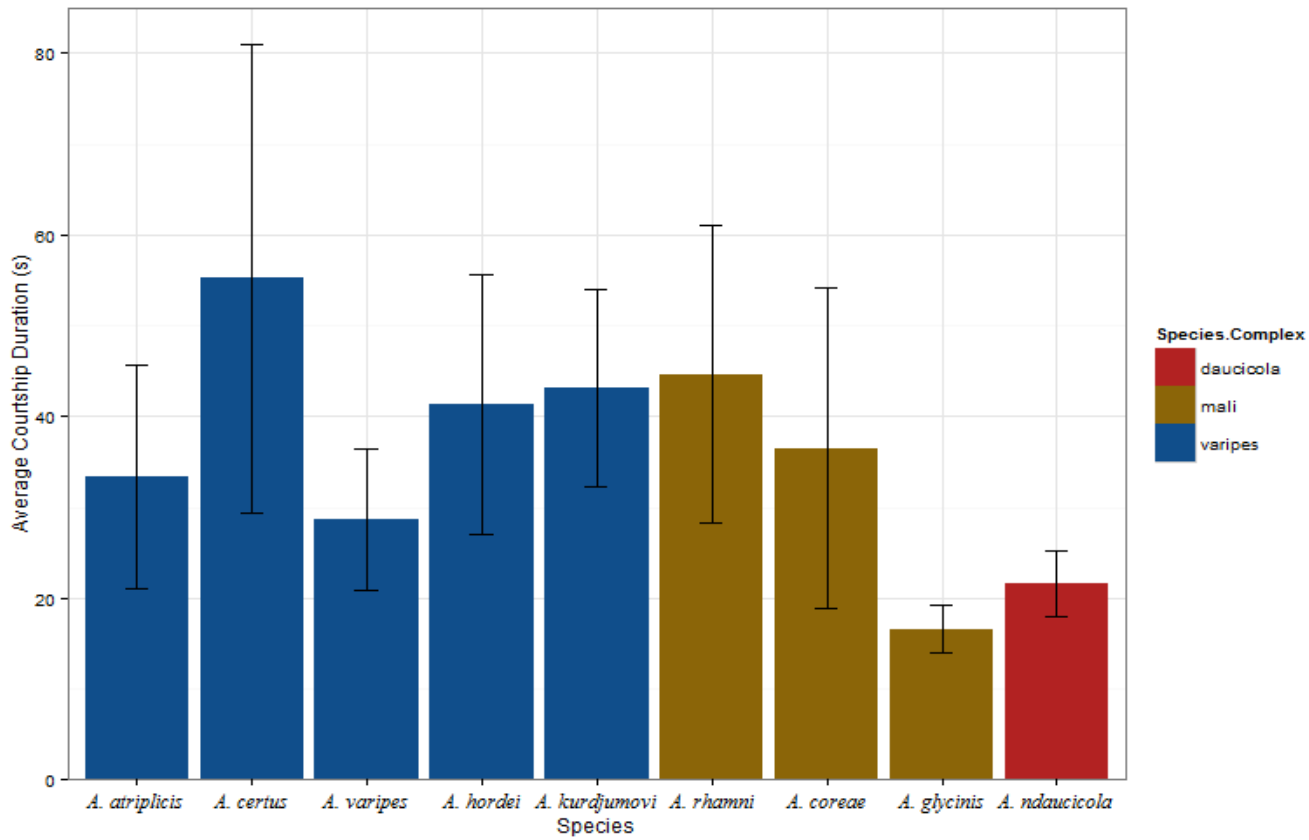

Figure 3. Average courtship duration for 9 *Aphelinus* species across three species complexes.

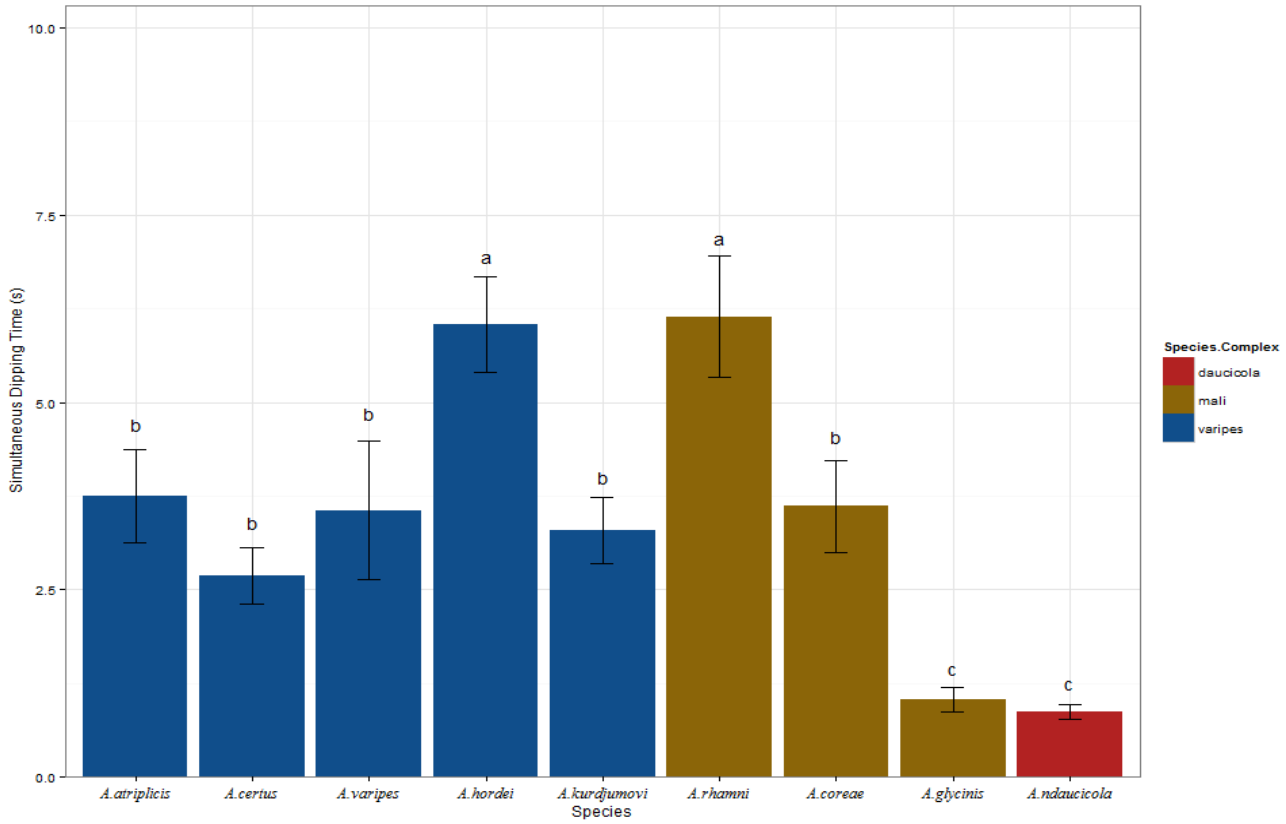

Figure 4. Simultaneous dipping duration, standard deviation and significance based on multiple comparisons of 9 species of *Aphelinus*, across three species complexes.

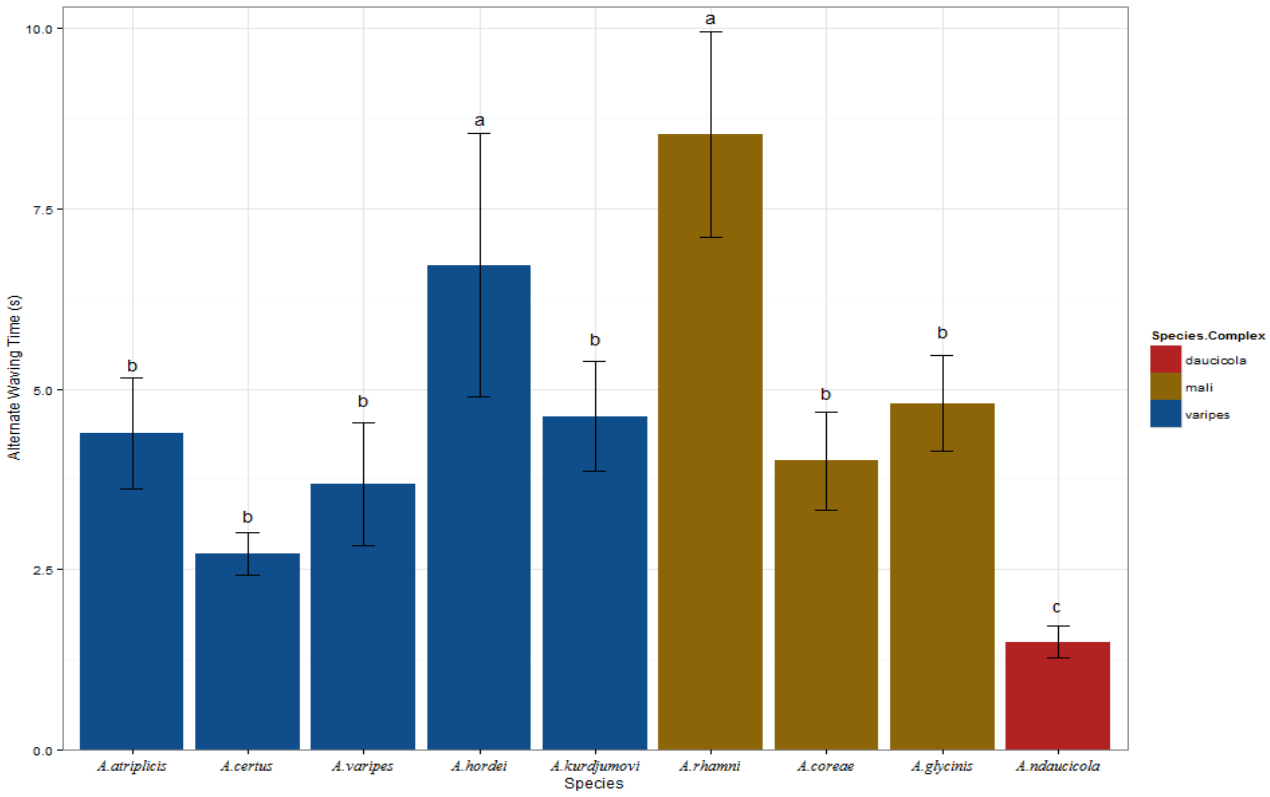

Figure 5. Alternate waving duration, standard deviation and significance based on multiple comparisons of 9 species of *Aphelinus*, across three species complexes.

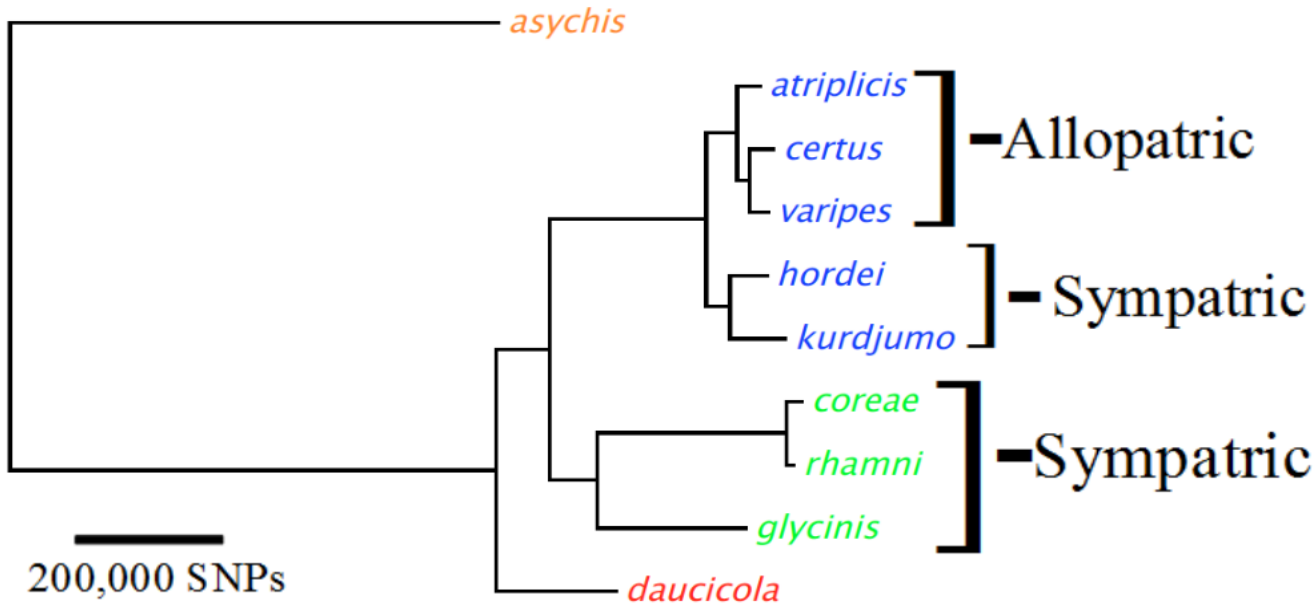

Figure 6. Molecular phylogeny of *Aphelinus* species, across species groups: blue- *varipes* complex and green- *mali* complex, red- *daucicola complex*, orange – *asychis* complex Molecular phylogeny was constructed using branch and bound technique from putative gene SNP data.

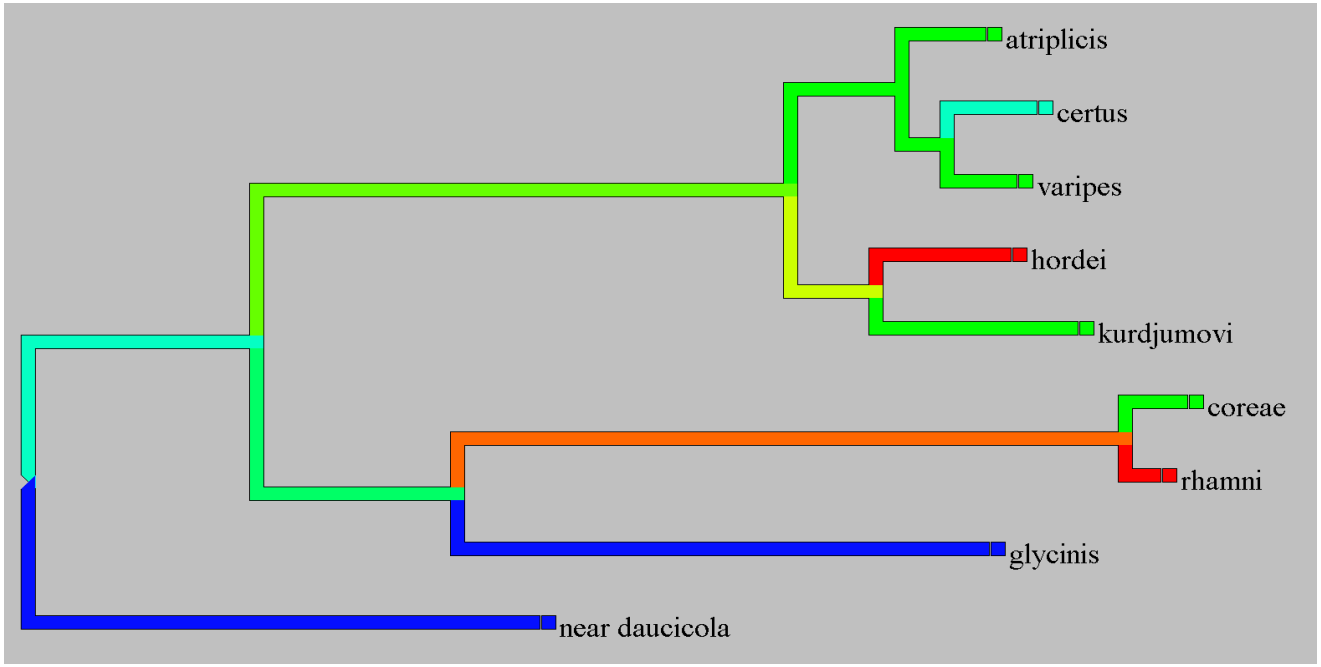

Figure 7. Mapping average simultaneous dipping duration to the phylogeny using maximum parsimony.

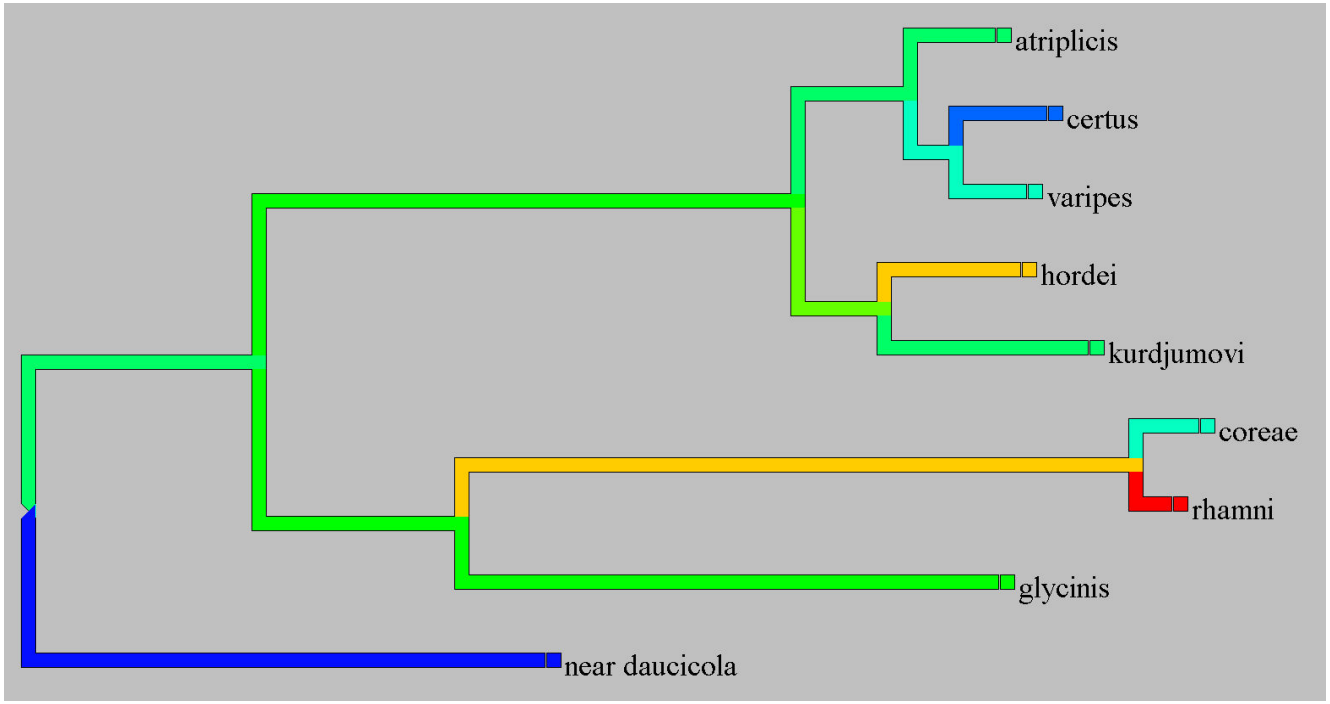

Figure 8. Mapping average alternate waving duration to the phylogeny using maximum parsimony.

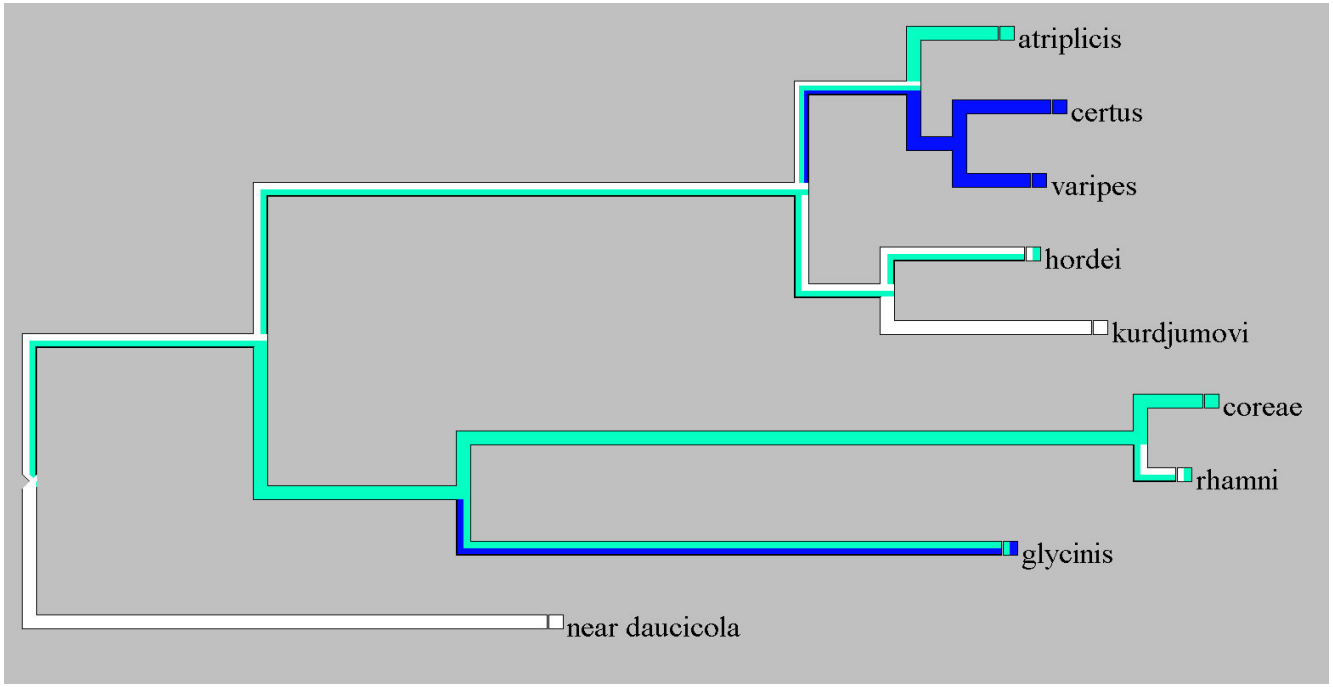

Figure 9. Mapping Simultaneous Dipping Top Spread to the phylogeny using maximum parsimony. White – intraocular, Light Blue – mideye, Dark Blue – head.

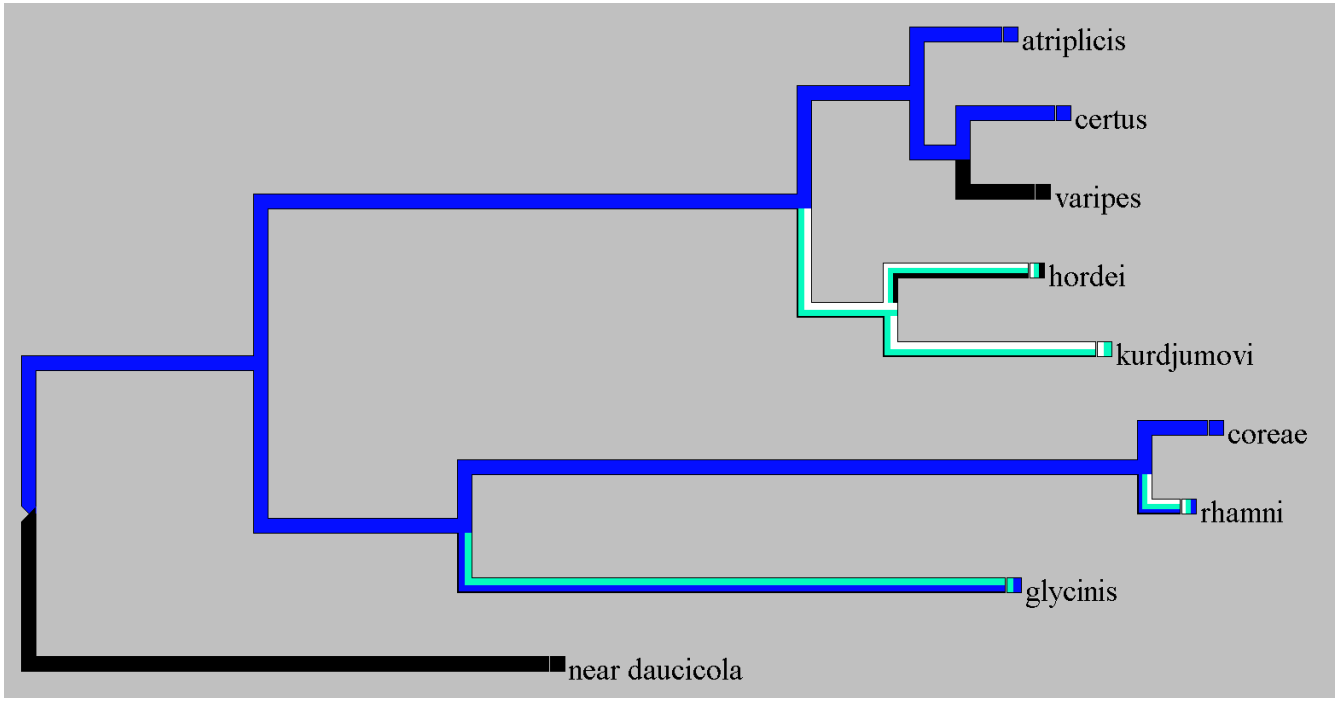

Figure 10. Mapping Simultaneous Dipping Bottom Spread to the phylogeny using maximum parsimony. White – intraocular, Light Blue – mideye, Dark Blue – head, Black – wider than head.

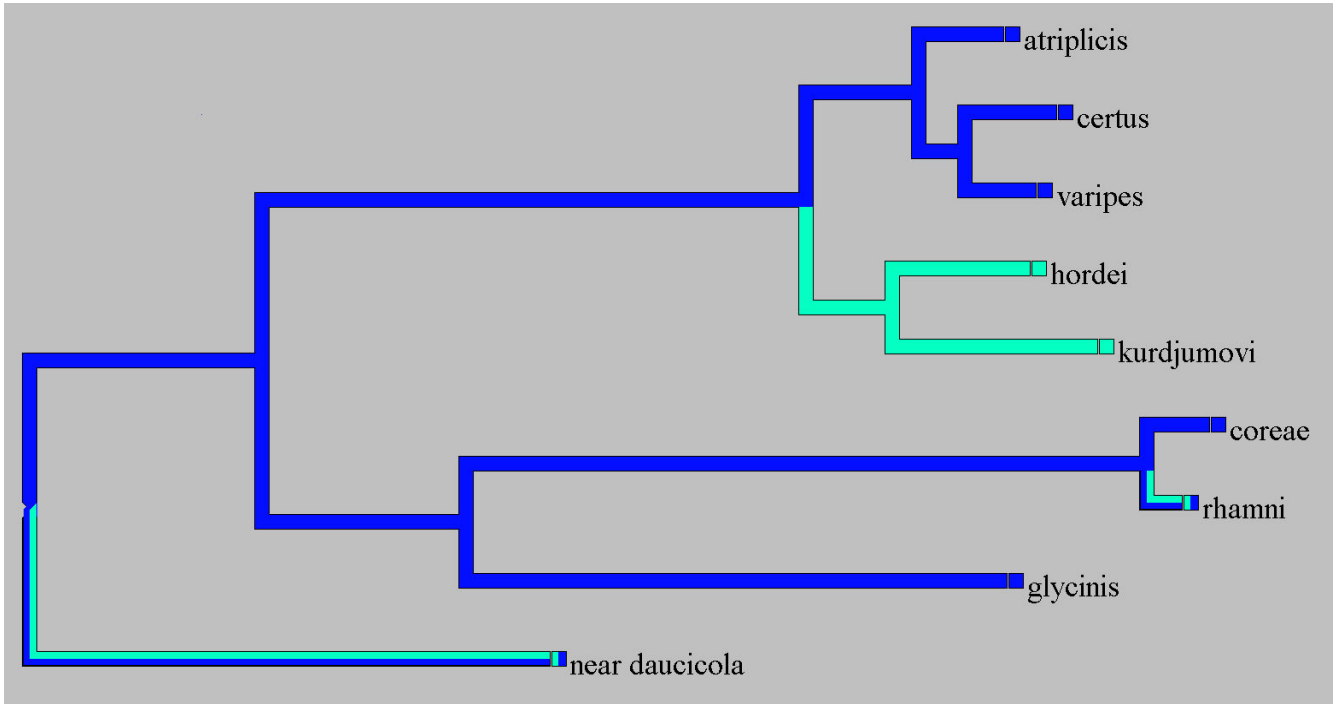

Figure 11. Mapping alternate waving spread to the phylogeny using maximum parsimony. Light Blue – intraocular, Dark Blue — mideye.

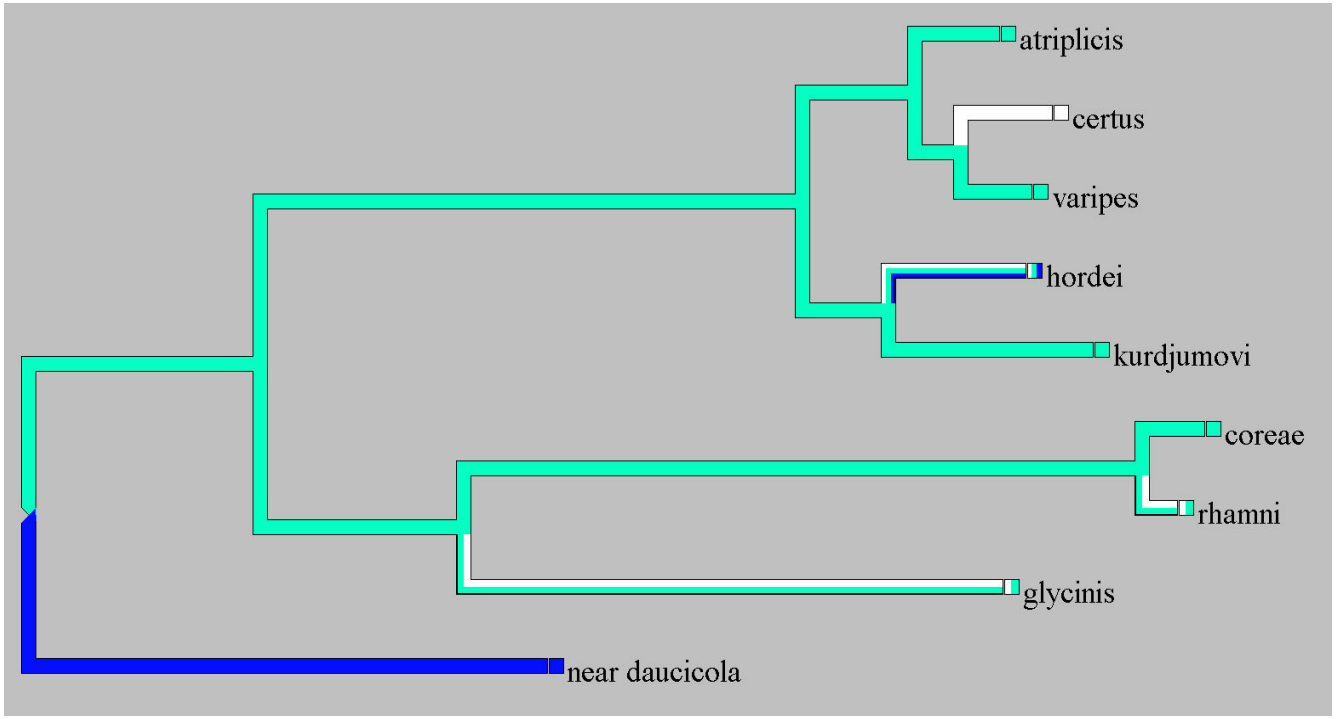

Figure 12. Mapping simultaneous dipping inverted "V" shape to the phylogeny using maximum parsimony. White – straight, Light Blue – slight, Dark Blue – strong.

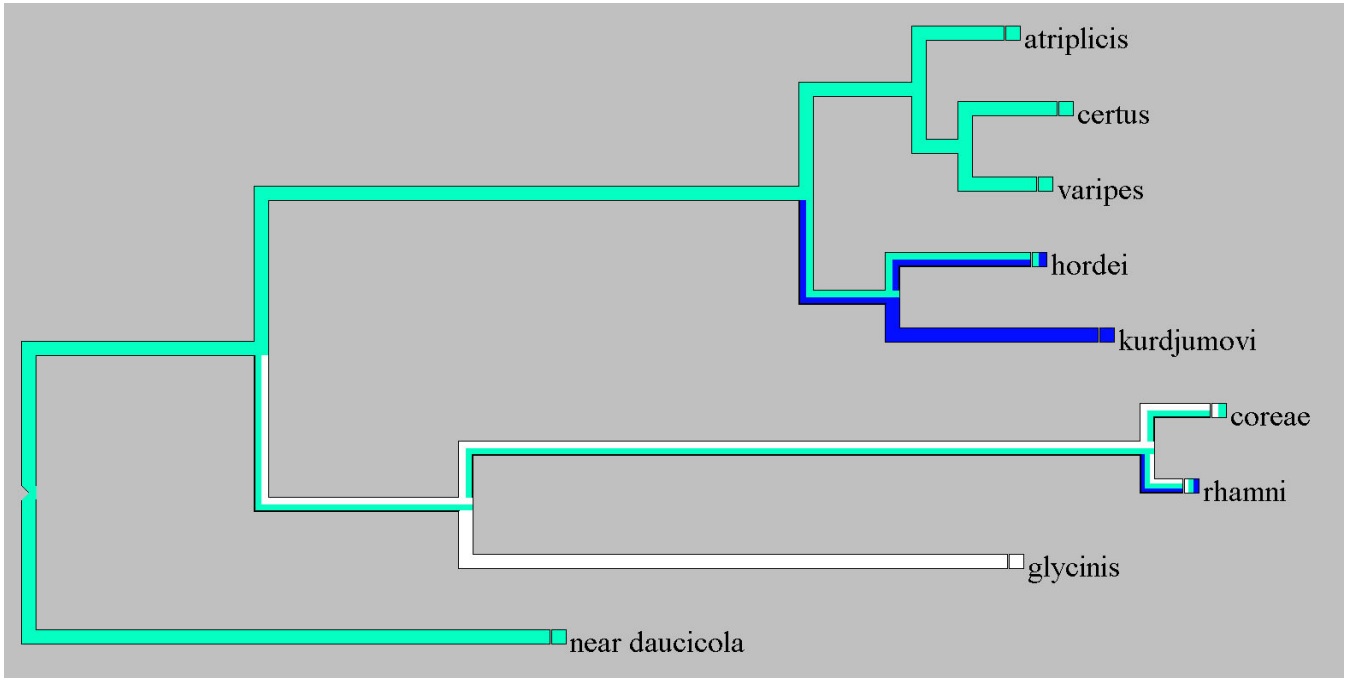

Figure 13. Mapping Bottom of Simultaneous Dipping Relative to Female Antennae. White –below, Light Blue – equal, Dark Blue – above.

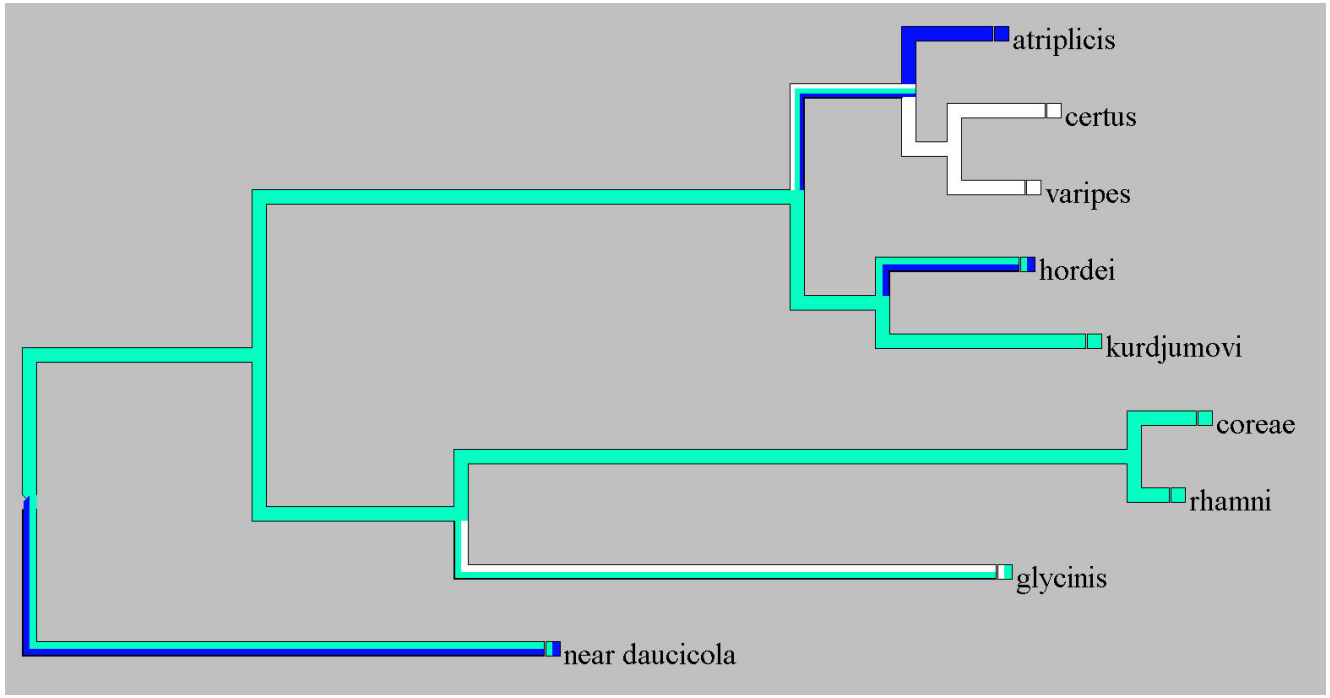

Figure 14. Comparison of antennae spread at the top of simultaneous dipping and during alternate waving spread, mapped to the phylogeny using maximum parsimony. White – simultaneous dipping wider, Light Blue – equal, Dark Blue – alternate waving wider.

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