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Weak Olfactory Preferences of the Gall Midge Asphondylia borrichiae, Associated Fungal Endophytes and Implications on Gene Flow and Host Range Expansion

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Abstract

Asphondylia borrichaie is a small fly (Diptera: Cecidomyiidae) that is currently undergoing host-associated sympatric divergence. *Asphondylia borrichaie* is an ambrosia galler, these insects utilize a host plant for oviposition, but its offspring also rely on a symbiotic fungus (or fungal community) to promote the formation of the gall as well as serve as a food source for the developing larvae. Previous studies indicate that *A. borrichaie* consists of two host- associated populations based on its original host plant *Borrichia frutescens* (Asterales: Asteraceae), and another one from the two *Iva* species (*I. frutescens* (Asterales: Asteraceae) and *I. imbricata* (Asterales: Asteraceae)). Differences in development time suggest allochronic isolation as the primary mechanism promoting host-associated sympatric divergence of midge populations and *A. borrichiae* has been shown to display fidelity to its natal host plant genus (e.g. *Borrichia* vs. *Iva*). Effects of host range expansion on the endophytic fungal community of the galls the midge's two primary host plants (*B. frutescens* and *I. frutescens*) have never been compared. In the current study, olfactometery trials utilizing field-collected galls/midges demonstrated a weak association for a midge's natal host; overall midges chose their natal host plant 56.1% percent of trials compared to 43.9% for the alternative (novel) plant. However, no significant differences were found in the time taken for a midge to choose natal vs novel host. Moreover, male midges showed a stronger preference for their natal host genus than females suggesting that gene flow between host-associated populations is more likely to be limited through males than females. Although clones of *B. frutescens* (collected from different localities) exhibited

significant differences in leaf size (but not number of serrations after more 18 months of being cultured in a common greenhouse environment, suggesting at least a partial genetic control for leaf morphology, no association was detected for midges to their specific clone of *B. frutescens* from the midge's natal locality. Lastly, this study successfully cultured and sequenced the ITS region of fungi extracted from gall chambers which could then be identified it down to fungal genera using BLAST searches. These data suggest a possible association of fungal genera with each host plant: specifically, with *Cladosporium* spp. which was more commonly found from galls collected from *B. frutescens,* while *Fusarium* spp. was more likely to be found in galls collected from *I. frutescens*.

INTRODUCTION

Fungi and Relationships to Plants

Fungi are numerous and diverse eukaryotes that include molds, rusts, yeasts, mildews, and mushrooms. Fungi as a group encapsulate a vast array of different physiologies and life histories, but overall are known mostly for being decomposers in ecosystems; that is, most are considered saprotrophs similar to many soil bacteria such as *Streptomyces*. Prior to the development of microscopy, fungi were thought to be plants due to their sessile nature and the similarity of fungal hyphae to plant roots. However, unlike plants, fungi are heterotrophs that lack chlorophyll in their cells, while containing chitin within their cell walls. Genetic analysis has additionally uncovered that fungi share closer similarities with animals than they do with plants (Wainright et al., 1993).

Fungi are involved in numerous symbiotic interactions with other organisms that range from mutualistic to neutral (communalistic) to pathogenic or parasitic. Pathogenic fungi are known to infect humans as well as many agriculturally important plants. For instance, one of the most destructive global rice pathogens is the cryptic species complex of *Magnaporthe grisea* (Magnaporthales: Magnaporthaceae)(M.E. Barr, 1977) and *M. oryzae* (Magnaporthales: Magnaporthaceae) (Couch and Kohn, 2002) (Ou, 1980). Conversely, many fungi exhibit mutualistic interactions with plants, such as mycorrhizal associations that are established when fungi colonize plant roots. Mycorrhizal fungi may be extracellular or intracellular (also known as arbuscular), but key to all mycorrhizal fungi is that they provide their host plant with increased access to water and minerals by increasing surface area of the roots in exchange for carbohydrates. Mycorrhizal fungi not only assist in water and nutrient uptake but they provide

defenses against pathogens within the rhizosphere. Arbuscular fungi in particular have demonstrated prophylactic properties against pathogenic fungi and nematodes (Azcón-Aguilar and Barea, 1997). These mycorrhizal associations can be so vital that some plant families, such as the Orchidaceae (Alexander and Hadley, 1987) have seeds that are unable to germinate without the necessary associated fungi. While mycorrhizal fungi are found within the rhizosphere of plant roots, fungi may also colonize or be associated with other plant structures and tissues, although in many cases these endophytic fungal associations are not so overtly mutualistic.

Endophytic Fungus

Like mycorrhizal fungi, endophytic fungi live within plant tissues, but unlike mycorrhizal fungi endophytes are not restricted to the roots and rhizosphere of the plant and are limited to microscopic fungi. Although the majority of endophytic fungi are known to be either basidiomycetes or ascomycetes their ecology is poorly understood. Originally believed to be completely parasitic (Hardoim et al., 2015), endophytic fungi are now believed to have little effect on plant tissues and they do not cause any visible disease for at least part of the life cycle of the fungi. The term “endophyte” may also include bacteria and viruses, but when it comes to fungi despite over 100 years of research the role fungal endophytes play in their ecosystems still remains unclear despite the diversity of species involved (Rodriguez et al., 2009).

One of the difficulties in understanding the role of endophytes is identifying the constituent species in the environment. These fungi can take very different appearances dependent on their what nutrients they are provided during cultivation (Griffith et al., 2007). While standards for

cultivating and sampling the mold-like endophytic fungi for microscopic visual identification exist and can assist in making these visual identifications (Rajendran and Sandle, 2011), the nature of these fungi can still complicate this method. Basidiomycetes and ascomycetes have several different reproductive stages, the sexually reproductive teleomorph, the asexual anamorph, and some fungi are holomorphs that have both these stages while others may only have an anamorph. Complicating this matter further, prior to 2011 anamorphs could be given different taxonomic names from their teleomorphs (Hawksworth, 2011). Because of this, molecular methods may be a preferable method of identification.

Sequencing of DNA for taxonomic identification requires a careful selection of a region to amplify for PCR. Regions must be relatively easy to isolate and amplify with available primers, and be useful for making accurate and precise identifications within the target taxa. Due to millions of years of mutation and divergence, one region of DNA useful in one kingdom or phyla may be extremely difficult to duplicate or use as an identifying region. For example, the mitochondrial the cytochrome c oxidase subunit 1 is frequently used as a barcode in animals, but is not only difficult to amplify in fungi, it also lacks sufficient variation and contains a large number of introns, rendering it phylogenetically uninformative (Schoch et al., 2012). Thus, the region with the most variation for the broadest range of fungus has been determined as the internal transcribed spacer (ITS) within the ribosomal cistron (Schoch et al., 2012).

The internal transcribed spacer is located between the large-subunit at the 3’ end and small-subunit at the 5’ end within the ribosomal tandem repeat gene cluster of the nuclear genome. In fungi it is approximately 600 base pairs and subdivided into ITS1 and ITS2 regions by the 5.8S subunit. As a non-coding region it has not been under as strong selection as coding regions

which provides usable interspecies variation for DNA barcoding. Additionally, as the ribosomal gene cluster that contains the ITS region is arranged in tandem repeats, PCR amplification can be performed from relatively small samples of the organism (Wurzbacher et al., 2019). However there do remain some shortcoming to using the ITS region for fungal DNA barcoding, which has led to considerations of other regions of the fungal genome to work as a secondary diagnostic markers (Stielow et al., 2015). Still, the ITS region remains a useful diagnostic region that can assist in understanding the ecological functions of fungal endophytes.

Ecology of Endophytic Fungus

Endophytes enter plant tissues through a variety of mechanisms either using hydrolytic enzymes to actively enter plant tissues or entering them through wounds or natural opening in the plant (Suman et al., 2016). Such wounds can occur incidentally as the plant grows or may be caused by herbivores. Once the host's tissue or the plant itself begins to senesce, eruption of endophytes from the host's tissues typically occurs (Stone et al., 2004) which often leads to sporulation. Most fungal endophytes then live within the tissues of their host plants without causing disease, despite many known fungal endophytes being closely related to known plant pathogens (Carroll, 1988). While many endophytes have a mutualistic relationship with their host, whether they become beneficial to the plant or a harmful pathogen can be dependent on the environmental conditions of the host and/or levels and types of damage caused by herbivores (Richardson, 2000). Thus, an endophyte useful in deterring grazing can become nothing more than a parasitic carbohydrate sink to the plant if herbivory is low or absent.

While fungal endophytes gain benefits from their plant hosts through access to carbohydrates, plants can still indirectly gain defensive benefits from their fungal infestations. These acquired defenses can be strong enough to be significantly detrimental to insect parasites. For instance fungal infections of California live oaks (*Quercus agrifolia* Née 1801) lowered the success and increased morbidity in the cynipid wasp *Dryocosmus dubiosus* (Hymenoptera: Cynipidae) (Fullaway, 1911) (Taper et al., 1986).

There are two primary mechanisms of defense plants can gain from the presence of fungal endophytes: alteration of nutrient content and production of secondary metabolites by the fungus. In the former, fungal endophytes, which consume carbohydrates and sometime nitrogen from their plant hosts, produces a nutrient sink which reduces the availability of nutrients to potential herbivores thereby limiting their growth and development, potentially reducing feeding damage. For example, Gange and Nice (1997), reported that for the aster *Cirsium arvense* (Asterales: Asteraceae), the presence of mycorrhizal fungi lowered the concentration of nitrogen in the plant stem which subsequently reduced performance of the fly *Urophora cardui* L (Diptera: Tephritidae). The other potential defense mechanism involves the production of secondary metabolites contributed by fungi, including alkaloids, which are often produced and used in plants as defenses against herbivores. In some fungi, alkaloids increase in concentration after plant wounding including attacks by grazing animals or after oviposition by insects (Zhang et al. 2009). The impact of these secondary metabolites and their detrimental effects against herbivorous insects, nematodes, and plant pathogens can be significant enough that endophytic fungi have been considered biocontrol agents in agriculture (Vega et al. 2008). While plants can incur some benefits from hosting fungi within their tissues to the point of

gaining defenses against herbivores, not all interactions between fungi and insects are detrimental to the insect. Phytophagous insects can also have symbiotic relationships with fungi that assist them in their utilization of the plants; for instance, especially the relationship between gall-making insects and their host plants.

Galls, Induction, and Effects on Host Physiology

Galls are abnormal swellings or outgrowths of plant tissue that are comparable in some ways to warts or tumors in animals, although they differ in their creation and physiology. Gall induction can be stimulated by several biologically disparate groups including viruses, bacteria, fungi, nematodes, and insects (Mani, 2013); however, even some parasitic plants such as the hemiparasitic plants of order Santalales (commonly known as mistletoe) can induce galls on plants (Anselmo-Moreira et al., 2019). Galls are most frequently induced by attacking the meristematic tissue of the plant. The galling organism takes advantage of tissue already holding the potential for cell division and alters nutrient requisition and gene expression to manipulate the plant into producing a tumor-like growth. This may be a means of encapsulating the galler or simply as a plant reaction to infection. Induction of galls is likely optimal during times of growth and cellular division and galling insects usually attack one specific region where cell division still occurs such as the stem apical meristems, growing roots, or developing fruit and they often have very limited host range (i.e. diet breadth).

For insects, gall making behaviors are an example of parasite and host co-evolution as most galling insects are monophagous to one or a few closely related host plants. Galling in arthropods is an ancient lifestyle, the earliest record of an arthropod-induced gall being found

on the cone stalk of *Aethophyllum stipulare* (Brongniart, 1828) from the Triassic (Grauvogel-Stamm, 1978). To date it is estimated that about 15,000 vascular plants are host to galling arthropods (Meyer, 1987) and several hypotheses have been proposed to explain the wide range of arthropods that independently evolved a galling lifestyle. Reflective of its independent evolution in several taxa, stimuli for gall induction can vary extensively; for instance, some insect orders directly inject phytohormones from their salivary glands into the plant during feeding (Raman, 2012), while others secret phytohormones within their ovipositional fluid (Barnewall and Rosemarie, 2012), or through symbiotic relationships with gall-inducing fungi (Stiling et al., 1992). However, in many systems gall induction remains poorly understood (Shorthouse and Rohfritsch, 1992). Similarly, the appearance and internal structures of galls can vary by taxa, but most commonly they reflect a usage of both habitat and food source for the insect, as well as what plant structure the insect exploits for gall formation.

Six major hypotheses on the origins of the evolution of insect galls have been proposed including: nonadaptive, plant protection, mutual benefit, nutrition, microenvironment, and the enemy hypotheses. (Price et al., 1987) A long-lasting prevalence of gall-making behaviors that have evolved several times independently in multiple taxa, rather than once or in a limited or narrow range of species, suggests that the nonadaptive hypothesis may be discounted. Moreover, the plant protection and mutual benefit hypotheses face difficulty because of the parasitic relationship galling insects have with the plant; galls act as physiological sinks for the plant (Larson and Whitham, 1991) likely weakening it and not providing the plant with any reproductive or survival advantages. For example, a detailed field study by Spirko and Rossi (2011) using the system of the flowering aster *Borrichia frutescens* L (de Candolle) and the gall

midge, *Asphondylia borrichiae* (Rossi and Strong, 1990), found that galling may increase stem production on *B. frutescens* potentially resulting in more flowers, but this effect (release of apical dominance) becomes weaker over the growing season; ultimately providing no fitness benefit to the plant. That galls provide a source of nutrition for the insect as well as a microenvironment for developing larvae has been well supported; for example, galls on goldenrod (*Solidago* spp.) have been found to provide insulation for the galling fly, *Eurosta solidaginis* (Diptera: Tephritidae) (Fitch, 1855), larvae during winter (Irwin and Lee, 2003). Moreover, cynipid gall-formers have demonstrated the ability to manipulate host nitrogen levels for their own nutritional benefit (Hartley and Lawton, 1992). The enemy hypothesis (i.e. galls provide shelter from predators) may be more tenuous. Although galls provide a layer of plant tissue for protection from natural enemies, it comes with a cost: larvae and pupae become sessile within the gall, which is often a large conspicuous overgrowth, that may visually alert potential predators and parasitoids. Natural enemies, especially parasitoids, can provide a strong selective pressure; for instance, in *A. borrichiae* parasitism has even been found reach 100% causing local extinctions of the midge (Stiling et al., 1992). Indeed, selective pressures imposed by natural enemies may have strong influences on gall size. For instance, gall size of the goldenrod gall fly, *Eurosta solidaginis,* shows upwards size selection against the wasp, *Eurytoma gigantea* (Hymenoptera: Eurytomidae)(Walsh, 1870), that attack small larvae and downward size selection against bird predators that are more attracted to larger galls (Weis and Kapelinski, 1994.) Additionally, parasitism rates and parasitoid guild composition of *A. borrichiae* galls are largely determined by gall diameter, which is primarily a function of host plant species and quality as well as a non-random distribution of the two major parasitoids of the midge

within patches of sea oxeye daisy (Stiling and Rossi, 1996; Rossi et al., 2006; Orta and Rossi, in review).

Concealed feeders and endophagous insects such as gall-makers are of particular interest to humans because many are pests of agriculturally important plants, such as the wheat pest *Mayetiola destructor (*Diptera: Cecidomyiidae) (Say, 1817) or the rice pest *Orseolia oryzae* (Diptera: Cecidomyiidae)(Wood-Mason, 1889). Galls siphon and redirect nutrients from other tissues often to the detriment of the plant which can affect crop yield. For the insect, this is to its benefit because the gall provides a protective microhabitat and it acts as a resource sink, potentially providing the insect (or fungus) access to starches and other nutrients from the plant. But this redirection of nutrients to the gall can lead to decreases in the health and reproductive fitness of the plant. For example, the chestnut gall wasp, *Dryocosmus kuriphilus* (Hymenoptera: Cynipidae)(Yasumatsu, 1951), which is native to China and now an introduced pest in much of the Northern hemisphere, attacks several species of *Castanea* spp. and is considered its most devastating pest species. Females oviposit within developing buds of the tree and attacks by this wasp have resulted in reduced fruit yields by 50 to 70% (Payne et al., 1983.) Heavy infestations can cause even more drastic health effects resulting in reduced tree vigor and wood production and even death (Kato and Hijii, 1997; Moriya et al., 2003).

Most galling insects are host and site-specific, with each species of insect often preferring and thriving on a single host plant and structure. However, introductions of new plant species that are closely related to the original host can lead to a transference of the insect to the introduced plant and potentially lead to sympatric speciation. For phytophagous insects, host transference events start as ovipositional mistakes and divergence is further exacerbated

through host-fidelity (Dethier, 1959). Some of these host range expansions, if establishment is successful, may result in novel pest species to humans. One of the most well-studied of these systems is that of the apple maggot fly, *Rhagoletis pomonella* (Diptera: Tephritidae)(Walsh, 1867); this insect successfully expanded its host range from its native hawthorn host (*Crataegus spp.*) to introduced domesticated apples (*Malus domestica* Borkh) (Bush, 1996). Due to differences in development time within the two plant species, fly populations have become allochronically isolated, which has reduced gene flow between the two host-associated populations, promoting divergence based on host plant species within only a few hundred years (i.e. since the recent introduction of domesticated apples to North America) (Feder and Filchak, 1999)(Hood et al., 2019). Poorer survivorship in apples was compensated by lower competition (Feder et al., 1995) and reduced parasitoid attacks on the derived host (Feder, 1995). Such examples of host range expansion provide useful systems for investigating sympatric speciation, especially at the genomic level (Egan et al., 2015), but they also pose potential challenges to human agriculture. The apple maggot fly has since diversified and expanded its range to include numerous introduced plants such as cherries, rose hips, apricots, and pears (Niazee and Penrose, 1981)(Yee and Goughnour, 2008) and fruit loss in apples alone can reach 100% in untreated plants (Glass and Lienk, 1971). Given the risk to human interests, endophagous insects, especially gallers, it is essential to understand the mechanisms of host acquisition and fidelity.

As detailed previously, endophages, especially galling insects, provide some of the best systems for studying the modes and tempo of speciation. The concept of host range expansion was suggested by Walsh (1894), who first proposed that sympatric speciation may occur when

host-specific phytophagous insects shift or adapt to a new host plant (Walsh, 1864). In such situations, speciation occurs and gene flow is restricted not by a geographic or other physical barrier but by combining factors of independent regulation of populations into specific host-plant niches, developmental differences leading to allochronic isolation and/or sensory bias in mate choice, host-assortative mating, and disruptive selection (Berlocher and Feder, 2002).

Fungal Associations with Galls

While many insects manipulate the phenotypic expression of plants by using phytohormones such as auxins and cytokinins (Mapes and Davies, 2001), numerous galling insects instead rely on a mutualistic relationship with fungus, where the fungus both induces the gall and often serves as a food source for the midge. Such a close association and reliance on the fungus for food, shelter, and propagation, and on the midge for dispersion of the fungus suggests numerous opportunities for co-evolution. Even in non-gallers, mutualistic relationships between insects and fungi can be observed. The ambrosia beetles of the subfamilies Scolytinae and Platypodinae (Coleoptera: Curculionidae) create and tend to fungal gardens in their homes of wood, rotting or live. While the wood itself lacks any nutritional availability due to the beetle's inability to digest it, the fungus breaks down and concentrates nutrition in a form available to the beetle (Kasson et al., 2016). In return, the beetles spread the fungal spores as they excavate galleries in trees. Another example involves leafcutter ants of the genera *Atta* and *Acromyrmex* (Hymenoptera: Formicidae) that collect leaves to feed and cultivate various fungi of family Lepiotaceae (Pinto-Tomas et al., 2009) which concentrate nutrients for consumption by the ants.

Ambrosia gallers draw their name from their similarities to the habits of ambrosia beetles. These galls are created by an insect with the assistance of a fungal symbiote. These fungal associations induce or assist in the formation of the gall and the mycelia can provide nutrition for the insect, while the insect propagates the fungi through the environment and transports it between host plants on their mycangia. A great number of ambrosia gallers are midges (Diptera) from the family Cecidomyiidae. These flies infest and attack a number of plant genera from multiple families including *Asteromyia carbonifera (*Diptera: Cecidomyiidae)(Osten Sacken, 1862) which attack goldenrod, *Solidago altissima* L (Asterales: Asteraceae) (Heath and Stireman 2010), *Daphnephila* midges which attack the Magnoliid *Machilus zuihoensis* (Laurales: Lauraceae) (Hayata, 1911)(Chao and Liao, 2013), and *Asphondylia borrichiae* which primarily attacks the salt marsh aster *Borrichia frutescens* (Rossi and Strong, 1990). Associations between *A. carbonifera* and the fungus, *Botryosphaeria dothidea* (Botryosphaeriales: Botryosphaeriaceae) (Ces. & De Not., 1863), have additionally been found as the female midge carries the fungal conidia on the mycangia (Heath and Stireman, 2010), although detailed life histories of these associations remain unknown or incomplete.

Olfaction in Gall Makers

Galling insects are typically considered to be phytophagous, although they may feed only on the fungal symbiont and/or consume plant tissue because development occurs within the plant's tissue; however, the resulting gall draws nutrients and may damage the host plant (e.g. senescence of affected meristems). Herbivory is a common specialization among animals and it has been estimated that between 24-40 percent of all animal species are phytophagous

insect specialists that are host-specific in nature (Bush and Butlin, 2004). In many systems herbivorous insects use plant volatiles to locate their host plants (Bernays and Chapman, 1994) and gall making insects are no exception.

Primary olfactory organs in insects are the antennae, although some olfactory capabilities are possible with the maxillary palps or through receptors in the tarsi of some insects (e.g. flies and lepidopterans). Olfactory reception in the antenna is conducted via numerous sensory hairs known as the sensilla which are enervated with olfactory receptor neurons (Keil, 1999). These receptors can be adapted to respond to a single odorant or respond to a more general array of odorants. Chemical sensory cues can range from pheromone signals to locate mates or plant volatiles used to locate food and hosts. Olfactory detection can even extend to detecting volatiles associated with fungi, be it attraction to certain yeasts (Davis and Landholt, 2013) or avoidance of fungal-infected host plants (Tasin et al., 2011). Limited neural capacity in detecting and responding to odorants can explain the evolutionarily development of phytophagous specialists (Bernays, 2001). Bernays and Funk (1999) note specialist populations of the aphid *Uroleucon ambrosiae* (Thomas, 1878) were found to have faster decision time when it came to host finding, selection, and acceptance compared to generalist populations. Specialization enables insects to adapt to host defenses more effectively and may facilitate host-plant selection and oviposition because specialists should evolve a greater ability to discriminate between plants and increase their ability to locate suitable hosts; imperative for an insect with a short lifespan. However, olfactory coding and preference is not constant throughout the lifespan of all insects. Mating in cotton leafworm (*Spodoptera littoralis* (Boisduval, 1833)) females change olfactory preference from the food plant *Syringa vulgaris* L.

to the larval host plant *Gossypium hirsutum* L. (Saveer et al., 2012). While gall midges, which often have short adult lifespans, rarely have a food plant to locate which would take them away from host plants, plasticity of olfactory responses still exist and can explain speciation within the clade.

Boddum (1999) found that females of the crucifer specialist *Contarinia nasturtii* (Diptera: Cecidomyiidae)(Kieffer, 1888) not only exhibited the ability to use olfactory cues to differentiate between host plant species, but also host plants at different times of development. Moreover, attraction to these plants remained even when they lacked the green leaf volatiles or the crucifer-specific glucosionlates indicating that host location is more complex than specific combinations of the right volatiles. Additionally, female *C. nasturtii* exhibited positive olfactory responses to a greater range of plants than would be suitable as hosts; specifically, while there was a clear olfactory preference for *Arabidopsis*, *C. nasturtii* did not oviposit on this crucifer, probably due to plant defenses to which *C. nasturtii* is not adapted. However, the presence of these olfactory responses demonstrates a level of plasticity that could influence how the midge locates and chooses host plants, which may reduce gene flow across host plants and facilitate divergence and, ultimately, speciation. This contention is supported by Boddum (1999), who investigated the antennal response study on 12 species of gall midges to a blend of 45 plant compounds, which found some interspecies variation in responses to certain compounds. These individual differences could explain how speciation events are possible in phytophagous specialists and how they can be reinforced. One such species of interest undergoing host-associated sympatric speciation is the gall midge *A. borrichiae*.

Life History of Asphondylia Borrichiae

Asphondylia borrichiae is a slender mosquito-sized fly native to the coastal regions of Florida (Rossi and Strong, 1990). Adult *A. borrichiae* lack functional mouthparts, which is likely an adaptation to maximize fitness given their short adult stage lasting only a few days; lack of functional mouthparts likely helps ensure that they spend their energy finding a mate and suitable host plant for oviposition rather than expending time and energy foraging for food. Most of *A. borrichiae*'s life cycle is spent in the juvenile stages (egg, larva and pupa); each midge develops individually within its own chamber and the largely spherical galls average 4-5 chambers per gall (Rossi and Stiling, 1995; Rossi et al., 1998). Depending upon the time of year, development from egg to pupa accounts for approximately 95-98% of the midge's life cycle and takes approximately six weeks during which time the immature stages remain sessile within their individual chambers (Rossi and Stiling, 1998). *Asphondylia borrichiae* attacks the apical meristems of three closely related salt marsh plants: sea oxeye daisy (*B. frutescens*) and two species of elder, *Iva frutescens* L (Asterales: Asteraceae) (marsh elder) and *I. imbricata* (Asterales: Asteraceae)(Walter 1788) (dune elder). All three species are closely related halophytic asters and *B. frutescens* and *I. frutescens* have similar distributions and are common members of marsh communities. However, *I. imbricata* is a beach dune species and it is less likely to occur with the other two plants in all locations.

After emergence from the gall, adults live for about 48 hours during which time they mate, and females find and oviposit within suitable hosts (Stiling et al., 1992). Although galls are typically present year-round, especially on *B. frutescens*, population densities can fluctuate more than 100-fold. In Florida, large population pulses typically occur in spring, midsummer

and early fall although smaller pulses may be present (Rossi and Stiling, 1995). Although juvenile *A. borrichiae* are endophagous and completely enclosed within the gall, four species of specialized parasitic wasps (parasitoids) attack the larvae and pupae of the midge. On *B. frutescens* (the midge's primary host), gall populations decline during the winter due to slower development rates, coupled with high parasitism rates, but gall midge populations rebound during the subsequent spring as the parasitoid population crashes (Stiling et al., 1992).

Although concealed, immature stages (i.e. larvae and pupae) of *A. borrichiae* are attacked by four species of hymenopteran parasitoids: *Galeopsomyia haemon* (Eulophidae) (Walker, 1847), *Rileya cecidmyiae* (Eurytomidae) (Ashmead, 1888), *Tenuipetiolus teredon* (Eurytomidae) (Walker, 1843), and *Torymus umbilicatus* (Torymidae) (Gahan, 1919) (Stiling et al. 1992). Of these four, *T. umbilicatus* and *G. haemon* are typically much more common because they are hyperparasites – that is, they also parasitize other members of the parasitoid guild in addition to *A. borrichaie* (Hawkins and Goeden, 1984; Stiling et al. 1992; Stiling and Rossi 1994). *Galeopsomyia haemon*, which is gregarious and the smallest member of the guild, has an advantage in small galls, while *T. umbilicatus*, owing to its much larger size and longer ovipositor, is more likely to dominate large galls because it can oviposit later in the gall's development when gall diameter is too thick for the other species effectively penetrate (Stiling et al., 1992; Stiling and Rossi, 1994; Rossi et al., 2006).

Size and/or location of the galls within a patch may influence parasitoid search image or preferences. For instance, differential gall size and parasitoid community composition from *Borrichia* and *Iva* galls may provide insight as to the persistence of *Asphondylia borrichiae* on the less suitable host plants (*Iva* spp). Moreover, Rossi et al. (2006) found a non-random

distribution of *G. haemon* and *T. umbilicatus* using artificial galls. Specifically, *G. haemon* was found on galls near the top of the canopy patch, while *T. umbilicatus* was significantly more abundant on large galls compared to small- or medium-sized galls. Parasitism rates on *B. frutescens* galls are lowest in the spring and rise to nearly 100% in some areas around the early summer and during the fall (Stiling et al. 1992). Parasitism rates on *I. frutescens* and *I. imbricata,* however, are relatively consistent throughout the year (Rossi and Stiling, 1995). The pattern of parasitism throughout the year where local extinctions may occur in *Borrichia* flies provides a selective advantage for flies that oviposit on *Iva* despite the disadvantages of a longer generational time and smaller body size. This "acquisition of enemy-free space" is believed to contribute to host range expansion of *A. borrichiae* to *Iva*.

Sympatric speciation does not require a physical barrier to prevent gene flow between sub-populations. As such, it has been typically considered less likely than divergence in allopatry where geographic barriers do exist. However, unique circumstances such as internal development on its host plant and a short-lived adult stage that restricts gene flow between host-associated populations of *A. borrichiae*, that may facilitate speciation even in sympatry. Although the midge initially evolved on *B. frutescens*, it appears to have expanded its host plant range via ovipositional mistakes on two closely related species of aster (*I. frutescens and I. imbricata*) (Rossi et al. 1999; Stokes et al. 2012). Previous research has indicated that the host-associated midge populations (at the level of plant genus) exhibit genetic divergence (Stokes et al., 2012). Field studies utilizing reciprocal transplants of *B. frutescens* clones and common garden experiments found significant effects of both plant genotype and local environment on midge populations (Stiling and Rossi, 1996; Rossi and Stiling, 1998) and midges showed high

levels of host fidelity (when presented with all three hosts), for both *B. frutescens* and *I. frutescens* (Rossi et al., 1999). Additionally, it has been observed that divergence is in part driven by both the differential development times (phenology) of the midges on the various host plants and the activity of gall-midge parasitoids (Rossi et al., 1999). While much of the divergence between these two populations can be explained by differences in development times with *Iva* midges taking longer to develop than those from *Borrichia* (Rossi et al., 1999), it is unclear if other factors such as host plant fidelity contribute to this divergence by further reducing gene flow between host-associated populations of the midge.

While many well-known (including some agriculturally devastating) instances of host range expansion and divergence typically involve an introduced plant, *Asphondylia borrichiae* and its associated host plants provide a look into a completely native system which in turn could provide a useful model in understanding the mechanisms of sympatric speciation in phytophagous insects. This system has several characteristics that make it a good candidate for investigating speciation such as long-lived host plants, prolonged endophagy, and association with its host plants (see Rossi 2004 for a complete list).

Evidence for host-range expansion facilitated by acquisition of enemy-free space has been found in several systems where an herbivorous insect escapes the search image of predators and parasites by becoming established on another host plant. Experiments involving the transfer of larvae of the leaf-mining midge *Liriomyza helianthi* (Diptera: Agromyzidae) (Spencer, 1981) from its native host plant *Helianthus annuus* L. (Asteraceae) to several novel host plants found significant effects of host plant identity on the midge's ability to avoid predation which varied based on the concentration of predators in the environment (Gratton

and Welter, 1999). In this same study, there was significant evidence that changing host plants to avoid parasites and predators did provide a selective advantage that outweighed any selective disadvantage of switching to a novel host. However, in other systems fluctuations in parasitism rates both temporally and spatially suggests that acquisition of enemy-free space may be less important (Heard et al. 2006). While divergence between host plants may not always be driven by natural enemy avoidance, it may play a contributing factor of phytophagous insect diversification that combines with several other environmental and biotic factors. diversification that combines with several other environmental and biotic factors.

Asphondylia borrichiae gall chambers become lined with endophytic fungus which likely provides nutrition for the developing larva (Gagne, 1989). It is likely that midge-fungal associations are co-evolved to overcome plant defenses, as gall-induction by *A. borrichiae* requires fungal conidia. Moreover, the full identity and biology of the of the fungal community of galls induced by *A. borrichiae* has not been established, although an initial survey was conducted by Te Strake et al (2006). As *A. borrichae* has been suggested to be diverging in sympatry into host-associated populations (Rossi et al., 1998; Stokes et al., 2011), a logical extension of gall midge isolation through the development of phenological differences may restrict both gene flow and exchange and survival of the associated fungi as well. However, like many plants, *Borrichia frutescens* and *Iva frutescens* are naturally hosts to many endophytic fungi that are present regardless of the presence of the gall or midge (Te Strake et al., 2006). Fungal spores have been observed on the mycangia of wild midges, and those spores are thought to be inserted along with the eggs during oviposition. However, in newly emergent midges no spores have been observed in the mycangia (Te Strake et al., 2006) which calls into

question how and where *A. borrichiae* females acquire the fungus necessary for gall-induction between emergence and oviposition. Suspected methods of fungal acquisition by galling insects include accidental collection from leaves and leaf litter (Borkent & Bissett 1965) or fecal contamination (Haridass 1987). While the acquisition of the fungus by the fly has not been ascertained, the method of fungal introduced into the gall also remains unknown but likely occurs when fungal conidia are deposited into the host plant along with eggs during oviposition.

Due to the natural pre-existence of fungal endophytes in and on plant tissue it may be difficult to ascertain the origins of all members of the gall's fungal microenvironment. Complicating matters further, there exists the possibility of fungus not only being introduced by the midge, but via contamination by *A. borrichiae*'s parasitoids. The fungus that induces the galling response by the plant may not even be of a single species. In addition, nutrition concentration due the gall acting as a sink as well as the wound resulting from oviposition and the gall's microhabitat may promote establishment of plant pathogens, further expanding the range of potential fungal biodiversity possible within the gall.

A previous fungal survey conducted by Te Strake et al. (2006) of *A. borrichiae* galls on *B. frutescens* including fungus found on stems, leaves, and the apex of galled and non-gall *Borrichia*, identified at least eight fungi down to genera using standard light microscopy. Studies on food preferences of fungivores indicated a preference for darkly pigmented fungi (Shaw, 1992); as a result, *Alternaria* sp. and *Bipolaris* sp. are theorized by Te Strake et al. (2006) to play a part in the nutritional system of *A. borrichiae*. Other identified species associated with galls include *Verticillium lecanii* (Viegas)(Now *Lecanicillium lecanii*, Zare and Gams, 2001), *Acremonium strictum* (W. Gams, 1971) (Now *Sarocladium strictum* (W. Gams) Summerbell

2011), *Humicola grisea* (Traaen, 1914) (now Trichocladium griseum, X. Wei Wang and Houbraken, 2018) *Monocillium indicum* (S.B. Saksena, 1955), and *Fusarium sp.* However, a genetic analysis on the fungal communities within *A. borrichiae* galls has not been conducted; identifying and understanding interactions between fungal symbionts may help determine if there is a difference between the endophyte communities in *A. borrichiae* galls on *Borrichia* vs. *Iva*. In turn, adaptation to different fungal communities may lead to a greater understanding in the differences in fitness and persistence of host-associated populations of *A. borrichiae* on its host plants. Additionally, ascertaining how *A. borrichiae* responds in olfactometry trials involving leaves of potential host plants will better assist in understanding how host fidelity is maintained and reinforced.

MATERIALS AND METHODS

Collection of galls

Galls induced by *Asphondylia borrichiae* were collected from the midge's two primary host plants, *Borrichia frutescens* and *Iva frutescens* from four previously used study sites (e.g. Stokes et al., 2011); two in north Florida and two in central Florida. The two northern sites are in Duval County, Florida; the first is located near the Sawpit Creek boat ramp at Big island Talbot State Park (30°30.6' N, 81°27.6' W) the second Round Marsh at Timucuan Ecological & Historical Preserve for Jacksonville (30°22.8' N, 81°30' W), these two sites are approximately 48 km apart. The two central Florida sites are near opposite ends of the Courtney Campbell Causeway in Tampa, Florida (27°57.6' N, 82°42' W); these two site are approximately 7 km apart. Galls collected from the northern sites near University of North Florida (UNF) were taken back to the lab and immediately placed in labeled individual plastic dram vials ranging 1.88oz to 3.13oz. Because Tampa is approximately 320 km from Jacksonville (and UNF), galls collected from the two central Florida sites were placed on ice in a cooler and taken to the lab where they were also placed in labeled vials. Galls were monitored daily and as adult gall midges emerged, they were collected and used for olfactometry trials (see below).

Collection of Host Plants

During spring 2017, several hundred individual *B. frutescens*, (which are clonal (see Stiling and Rossi, 1996; 1998)) stems and a few dozen *I. frutescens* (which are shrub-like) were collected from each northern site and a single southern site. At the time of collection, all leaves were trimmed from the plants and native soils were gently washed from the roots. These

trimmed plants were then cultured in the university's greenhouse. To minimize differences in plant quality caused by variation in local site conditions (i.e. maternal effects), cuttings of both *B. frutescens* and *I. frutescens* were grown in a common potting soil and under identical (ambient greenhouse) conditions for one-and-a-half years prior to their use in olfactory trials and measurements of leaf characteristics (see below). Plants were watered twice daily.

Testing Leaves for Phenological Differences Between Locality

To determine if plants from each population exhibit genetically-based phenological differences, ten fully-expanded leaves were haphazardly sampled from each plant and the following characteristics were recorded: length (mm), width (mm), and number of serrations on the left vs. right side of the midvein. Leaf length and width were measured using a pair of digital dial calipers (to the nearest 0.1 mm). Leaf length was measured along the midvein from the base of the petiole to the apex of the leaf, while width was taken recorded across the middle (widest point) of the leaf. Leaf serrations were counted from both the right and left side of the leaf. Right and left was oriented by taking an overhead view of the leaf with the tip distal and the petiole proximal to the researcher.

Data were analyzed by separate one-way ANOVAs (followed by a Tukey's HSD to compare group means after a significant main effect) for each plant species using SPSS (IBM Corp., Armonk, NY, USA) and which was used to perform ANOVAs on all three locations simultaneously for each data category. Number of serrations were compared using chi-square tests.

Olfactometry

Olfactometry experiments were performed using a glass Y-tube olfactometer with a 2-channel ADS air delivery system (Figure 1) (Analytical Research Systems, Inc., Gainesville, FL, USA). Air flow was 1.0 L/min and both channels were double-filtered and used both charcoal and distilled water to remove outside volatiles from the environment. The central tube (B) including the starting chamber (A) was 22cm, and without the starting chamber was 16.5 cm. The directional tubes were 17cm from the bend of the "Y" to the wire mesh separating subjects from olfactory samples, and a "finish line" denoted by the meeting of two separate glass pieces at 12cm (Figure 1.) During trials, leaf samples were held in separate olfactory chambers (D) at the end of the directional tubes. Wire mesh was used to separate the leaf samples from the insect and prevent blocking air flow. Leaf samples were removed from plants cultured in the greenhouse, or frozen leaf samples were used when fresh leaves were limited. Mass of leaf samples used for olfactometry trials ranged between 0.25 - 0.4 g and both samples were kept at equivalent masses during each trial. If a leaf sample had to be trimmed to kept below 0.4g, the other leaf sample was also slightly trimmed with a razor to control for wounding responses.

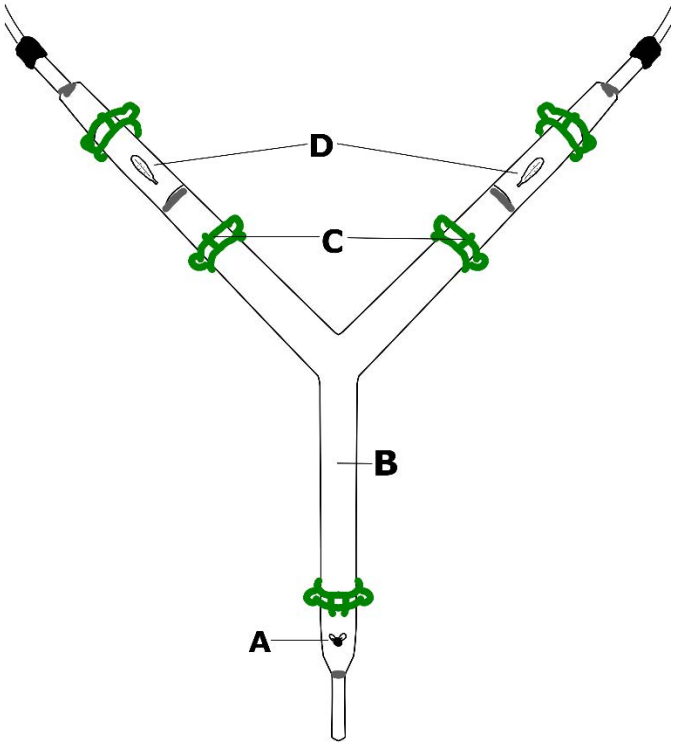


Figure 1: Diagram of the olfactometer used in host fidelity and timing trials.

Three different olfactory tests were presented to newly emerged *A. borrchiae* adults. The first test was performed using the natal and novel host plants with *B. frutescens* and *I. frutescens* samples. The next two comparisons tested preference for natal host plants at natal location vs natal host plant at a novel location, order of the test determined by the midge's natal location (Table 1).

Midge Natal Location	**First Test**	**Second Test**	**Third Test**
Timucuan	*Borrichia* vs *Iva*	Timucuan vs Talbot	Timucuan vs Courtney Campbell
Talbot Island	*Borrichia* vs *Iva*	Talbot vs Timucuan	Talbot vs Courtney Campbell
Courtney Campbell	*Borrichia* vs *Iva*	Courtney Campbell vs Timucuan	Courtney Campbell vs Talbot

Table 1. Olfactometry challenges (by site and host plant species) for newly emerged *A. borrichiae* adults.

The side (left vs right) each leaf sample was placed for each test was randomized via coin flip. The central and side tubes of the olfactometer were cleaned with water and ethanol between each testing session and the central piece of the olfactometer was unclipped and flipped to its other side between individual tests. Midges were removed from their vial and transferred to the olfactometer using an aspirator. The entrance chamber (A) was unattached from the rest of the olfactometer and the vial of the aspirator was attached to the central tube. Timing began as soon as the midge was introduced into the olfactometer and ended as soon as the midge crossed the "finish line" (C) on one side and the choice of the midge was recorded to the nearest second. Lighting was equalized and balanced within the laboratory, with both ambient and artificial light. To encourage traveling from the central tube to the directional tube, an opaque sheet of black paper covered the central tube soon after the midge was introduced into the olfactometer.

Statistical Tests on Olfactometry

Data from the olfactometer was compared using chi-squared tests of independence and ANOVAs were used to compare time trials. Additionally, statistical tests of one proportion were used to check for bias in the directionality of the olfactometer (left/right). The chi-square tests and one proportion tests were conducted using Microsoft Excel (Microsoft Corporation, Albuquerque, NM, USA). ANOVAs were conducted using IBM's SPSS statistics software package. All chi-square tests also included Yate's correction as the degrees of freedom for all tests were only one.

Culturing of fungi

After one week, a minimum of five galls from each location were randomly selected for dissection and fungus extraction. Prior to fungal extraction, galls were externally wiped in 70% ethanol to prevent contamination from fungus on the surface of the gall. Each gall was bisected using a scalpel sterilized by flame and ethanol (provide &) that was re-sterilized between each bisection. Sterile swabs were then inserted into the gall chambers to collect fungus lining the chambers, and then the swab was spread on a petri-dish of Sabouraud Dextrose Agar (SDA), which was selected due to its frequent use in culturing and diagnosing of fungi in clinical laboratories. Additionally, its acidic pH of 5.0 inhibits the growth of several different types of bacteria that could contaminate and complicate our samples. Samples from both sides of the galls were obtained using individual sterile swabs and then plated on their own petri dish. Petri dishes were then individually labeled and then sealed around the rim with Parafilm (Bemis Company Inc, Neenah, WI).

Samples were cultured at room temperature in an area of the lab protected from direct light. After one week, plates were examined to see if they produced suitable (i.e. relatively isolated) fungal colonies for creating isolates. From these colonies, isolates were created using a flame-sterilized inoculation loop and plated again on SDA. These isolates were then placed in the same area and cultured for another week.

PCR and Choosing Primers

Several different primers have been developed to target the ITS region of fungal rDNA that have been sufficiently discriminatory between plant and fungal DNA (Martin and Rygiewicz 2005). However, *in silico* analysis of ITS primers has revealed biases towards basidiomycetes or ascomycetes depending on the specific primer (Bellemain et al., 2010). For this reason, we have selected ITS1 (biased towards basidiomycetes) as our forward primer and ITS4 (biased towards ascomycetes) as our reverse primer to correct for their respective biases.

Cloning

After one week, colonies from the isolates were sampled and DNA was extracted using a DNeasy PowerSoil Kit (Qiagen, Hilden, Germany). Successful DNA extraction was confirmed using gel electrophoresis using 1% agarose/ethidium bromide gels and a UV lamp. Extracted DNA samples were stored at -20°C. After successful extraction, PCR was performed with an annealing temperature of 55°C. PCR product was a total volume of 50μl and prepared with a ratio of 25μl DreamTaq Green PCR Mater Mix, 0.25ul of the forward primer (ITS1), 0.25ul of the reverse primer (ITS4), 14.5ul of nuclease-free water, and 10ul of DNA extract. Thermal cycling was performed using the following parameters: 95 °C for 3 minutes, followed by 34 cycles of 95°C for 30 seconds, 55 °C for 30 seconds, 72 °C for one minute, and a final extension at 72 °C for 5 minutes and held at a temperature of 4 °C until removed from the machine. After amplification 1.5% agarose gels stained with ethidium bromide were used to determine PCR products of varying sizes.

After verification that amplification was successful, the PCR product was cleaned up Qiagen PCR Purification Kit (Qiagen, Hilden, Germany). Concentration of quality DNA was verified using a Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) with a concentration of 10μl of PCR material per 180μl of Qubit working solution. Samples were loaded on to a 96 well-plate with a ratio of 5μl DNA, 2.5μl ITS1, and 2.5μl ITS4 and sequenced (Eurofin Genomics, Kentucky, USA).

Sequencing and Identification

Once sequences were obtained, a BLAST search using the NCBI database was run to compare the obtained internal transcribed spacer (ITS) sequences with identified ITS sequences of related fungal species. The largest Total BLAST score was used to determine the species within the BLAST search. Comparative sequences were found using the Mycobank Database for biogeographical dendrogram construction. ITS1 and ITS4 segments were combined into a single contig per sample using ChromasPro (Technelysium). Sequences were aligned and a biogeographical dendrogram constructed using MEGA 7.0 software (Kumar et al. 2015).

RESULTS

Leaf Measurements

Even after being cultured under identical conditions in the UNF greenhouse for o18 months, there were significant differences in leaf length and width of our *B. frutescens* sample populations by location (Figure 2). However, the two northern populations (Talbot Island and Timucuan) did not differ, but they both produced shorter leaves than the southern population

(Courtney Campbell). Difference in average length was especially significant with Courtney Campell *B. frutescens* leaves being on average almost 15mm longer than those originating at the Jacksonville sites.

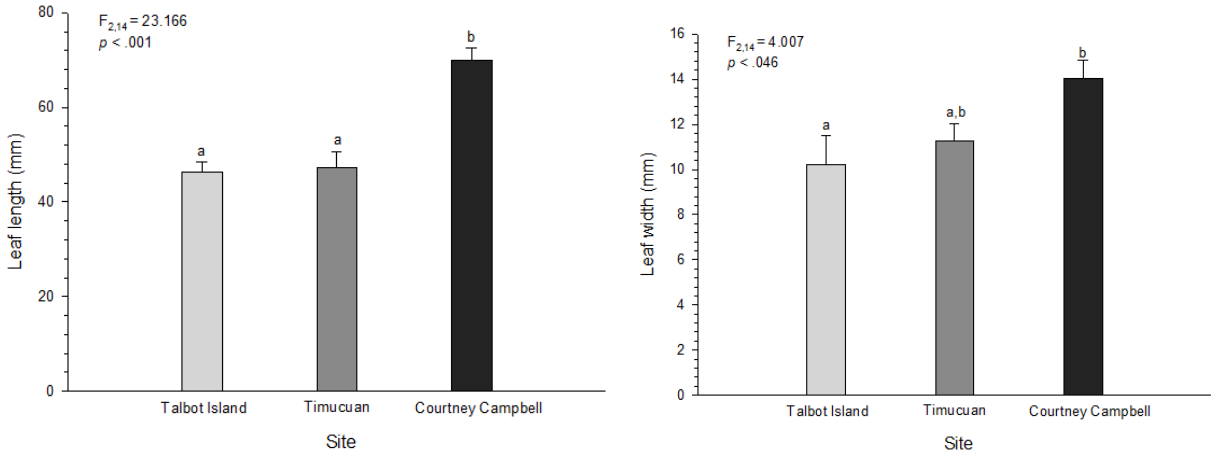


Figure 2(a) and (b): Leaf length (mm) and leaf width (mm) for *Borrichia frutescens* populations from the three study sites (values are mean ± sem; means with different letters are significantly different at $\alpha = .05$)

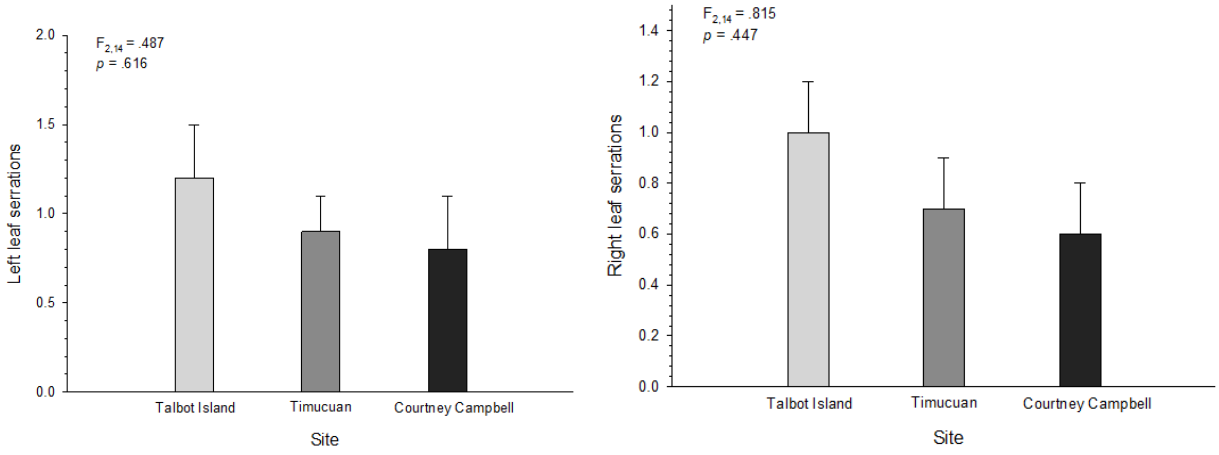


Figure 3(a) and (b): Number of serrations on leaves of *Borrichia frutescens* populations from the three study sites (values are mean ± sem; means with different letters are significantly different at $\alpha = .05$).

Conversely, no significant differences in the number of leaf serrations were found between the Jacksonville sites (Talbot Island and Timucuan) and the Tampa site (Courtney

Campbell) (Figure 3). These results suggest that leaf characteristics of *B. frutescens* are regulated by both environmental and genetic factors which is not surprising given the clonal nature of this coastal halophyte (Antlfinger, 1982; Stiling and Rossi, 1998).

Olfactometry Analysis

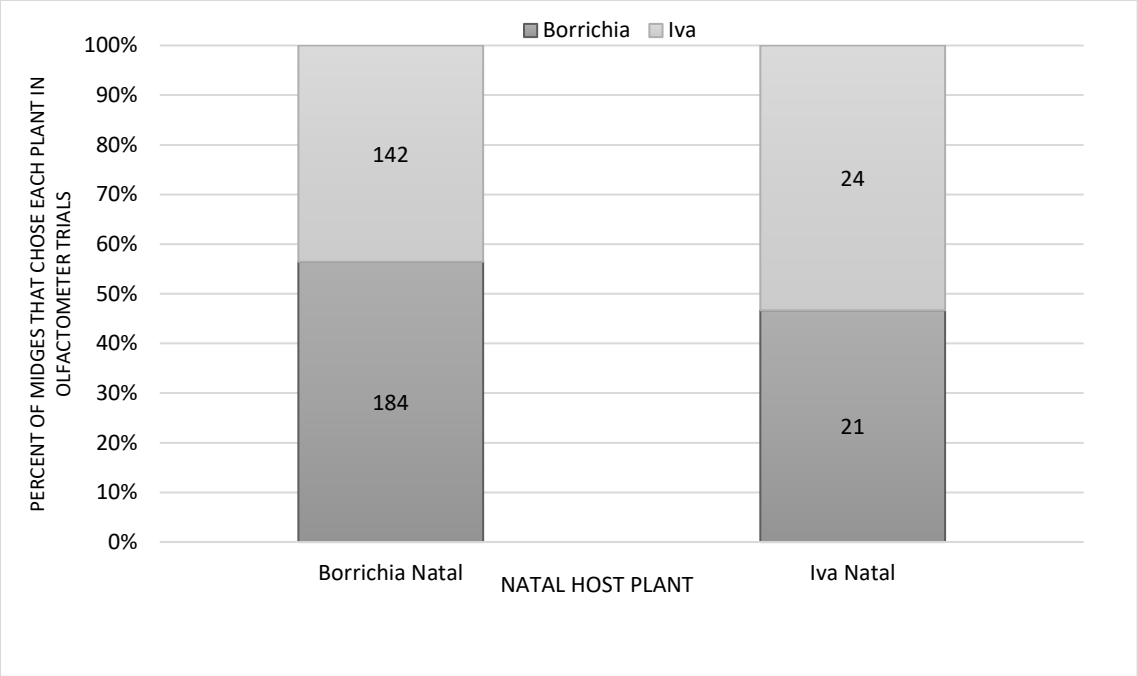
Midges reared from field-collected galls for use in olfactometry trials reflected the ecology of *Asphondylia borrichiae*. Female *A. borrichiae* were far more plentiful; 302 of 371 flies (81.4%) that emerged from galls were female. Additionally, owing to differences in gall abundance, a far greater proportion of midges used in the olfactometry trials came from *B. frutescens* compared to *I. frutescens* (88 vs 12% respectively); mean gall densities are often one-two orders of magnitude greater on *B. frutescens* compared to *Iva* (Rossi and Stiling, 1995; Rossi et al. 1999). Additionally, the two Tampa sites were most productive sites for gall collection and midge emergence compared to the Jacksonville sites. (Tables 2 and 3).

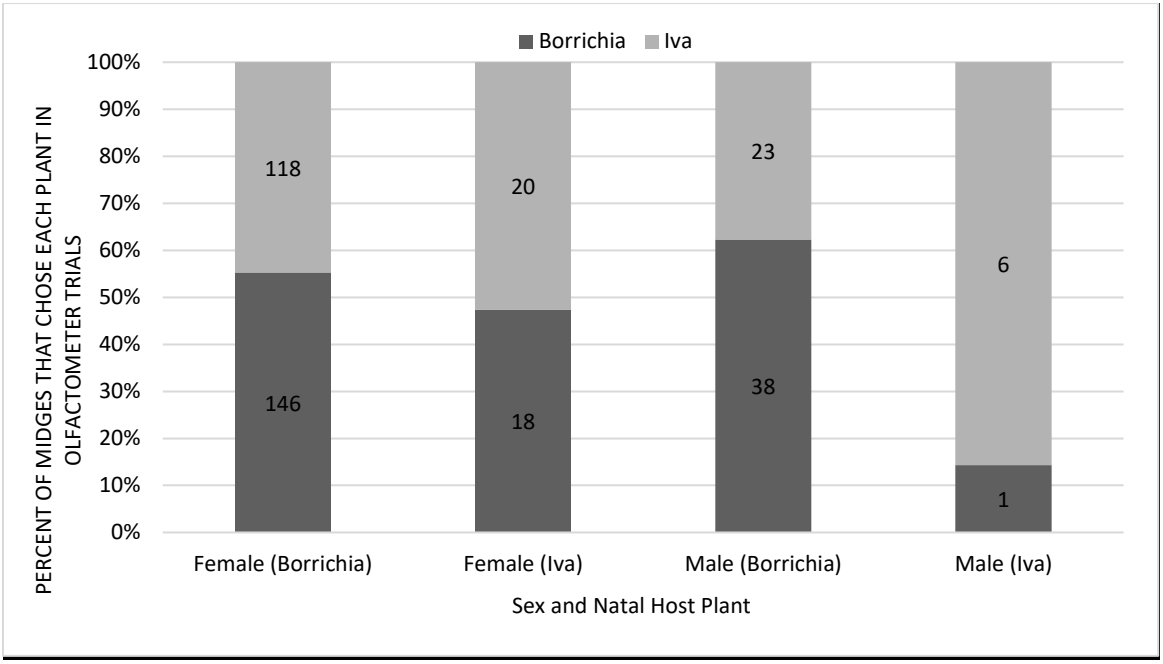
Population Tested	Sample Size
By Natal Host Plant (Species)	**371**
Borrichia frutescens	326
Iva frutescens	45
By Natal Host Plant and Locality (Species and Location collected)	**371**
Timucuan	52
Borrichia frutescens	52
Iva frutescens	0
Talbot	8
Borrichia frutescens	8
Iva frutescens	0
Courtney Campbell (South)	200
Borrichia frutescens	181
Iva frutescens	19
Courtney Campbell (North)	111
Borrichia frutescens	85
Iva frutescens	26
By Sex	**371**
Female	302
Male	68
Indeterminate	1

Table 2: Population Totals of Emerged Midges. Note that while locality is listed, not all midges that emerged from these locations were available to be used in the locality tests due to availability of plants during certain points in the experiments.

Test Analysis	Overall Total	Proportion to Natal	Proportion to Novel
Overall Natal/Novel			
Borrichia	326	0.564	0.436
Iva	45	0.533	0.467
Female Natal/Novel			
Borrichia	264	0.553	0.447
Iva	38	0.474	0.526
Male Natal/Novel			
Borrichia	61	0.623	0.377
Iva	7	0.857	0.143
Timucuan Borrichia Locality Test			
Timucuan vs Talbot	47	0.553	0.446
Timucuan vs Courtney-Campbell	27	0.593	0.407
Courtney-Campbell Borrichia Locality Test			
Courtney-Campbell vs Timucuan	34	0.471	0.529
Courtney-Campbell vs Talbot	34	0.441	0.559

Table 3: Summary table of proportional results of olfactometer trials. For locality tests the natal locality is used to name the overall locality tests. Not all locations produced a large enough test population so while Talbot is a test plant not enough Talbot midges emerged to be used in locality tests.


Borrichia
Iva
PERCENT OF MIDGES THAT CHOSE EACH PLANT IN OLFACTOMETER TRIALS
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
142
184
24
21
Borrichia Natal
NATAL HOST PLANT
Iva Natal


Borrichia
Iva
PERCENT OF MIDGES THAT CHOSE EACH PLANT IN OLFACTOMETER TRIALS
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
118
146
20
18
23
38
6
1
Female (Borrichia)
Female (Iva)
Male (Borrichia)
Male (Iva)
Sex and Natal Host Plant

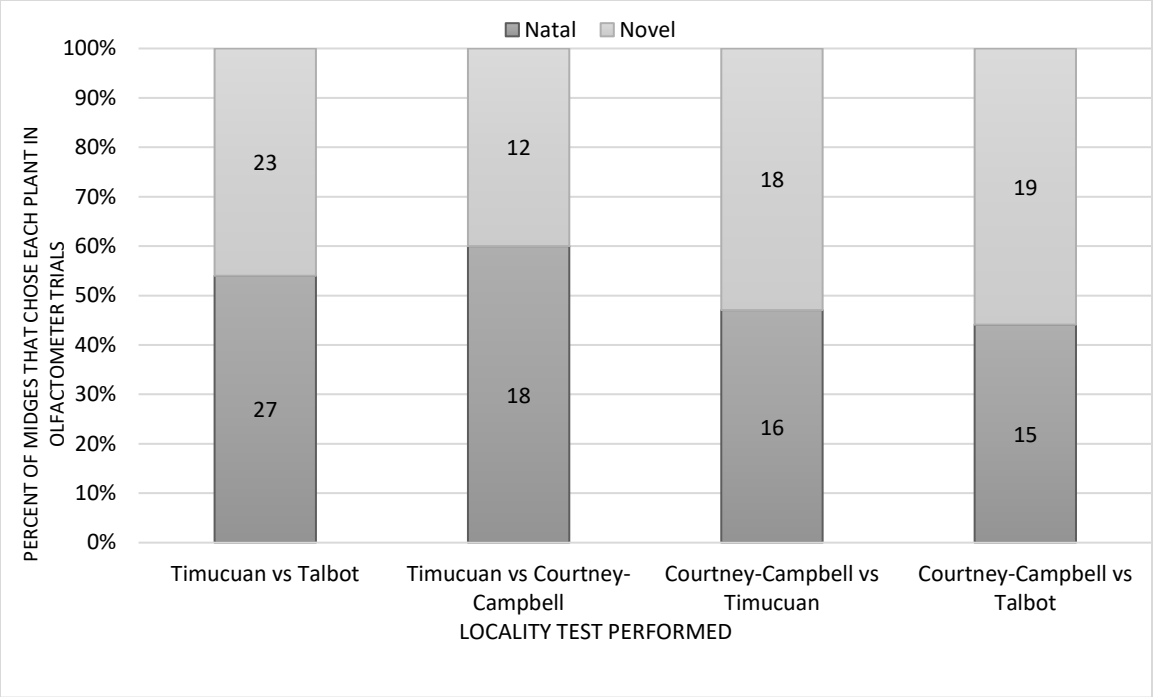


Figure 4 (a) (b) and (c): Frequency (percentage) of choice trials for *A. borrichiae* midges for their natal vs. alternative host plant.

Several significant, albeit weak, trends were detected from the olfactometry trials; for instance, overall *A. borrichiae* from both *B. frutescens* and *I. frutescens* populations exhibited slight, but significant ($\chi^2 = 5.25$; df = 1) preferences (56.4% and 53.3% respectively) for their natal host plant compared to its alternative. Interestingly, overall host recognition and/or preference by *A. borrichiae* appears slightly stronger in males (64.7%) than females (55.3%) at $\chi^2 = 7.3784$ at df = 1 in a χ^2 of independence. Limited trials (due to lack of midges obtained) using *A. borrichiae* collected from both north and central Florida , suggested that midges from the north Florida sites exhibit stronger but not significant ($\chi^2 = 1.013$ at df = 1 compared to Courtney Campbell's $\chi^2 = 0.294$ at df = 1) preference for their natal plant clone, compared to a novel one; 54% preferred Timucuan over Talbot, and 60% preferred Timucuan over Courtney Campbell (Table 2; Figure 4). However, Courtney Campbell midges displayed no preference for

their natal clone; in fact, chose the Timucuan *B. frutescens* clone 52.9% of the time and Talbot one 55.9% of the time compared to their natal plant clone.

Comparison	"Success" Proportion	Observed proportion	Sample Size	z-statistic	Significance level
Directionality (*Borrichia*)	*Borrichia* midge to the left	0.690	326	0.487	P = 0.6264
Directionality (*Iva*)	*Iva* midge to the left	0.689	45	0.180	P = 0.8574

Table 4: Results for Statistical Tests of One Proportion, where the expected proportion for all tests are 0.5. "Success" proportion does not indicate a goal in olfactometry but rather a proportion chosen for ease of use for the statistical test.

Further supporting the presence of a weak association for the natal host plant is the proportion of midges that went to their natal host plant are all <0.60 in all tests across all host plants. While there was some bias towards the left side of the olfactometer this was not found to be significant in tests of one proportion.

In time trials, midges took on average 2:20.64 minutes (0:02:20.64 total) (Table 5) to choose a host plant within the olfactometer. However, no significant differences were found overall or in *Borrichia*-derived midges in the average time for midges to make a choice within the olfactometer based on which plant they ultimately chose (natal/novel) (Table 6). This lack of significance extended into all categories including comparisons between male and female midges. There does appear to be some difference decision time made by Iva-natal midges (0:03:56.83 average to natal vs 0:02:24.01 average to novel)(Figure 5) however there was no significant difference as determined in the ANOVA, it should be noted that sample sizes are low for several trial combinations including the Iva natal group.

Group analyzed	N	Minimum	Maximum	Mean	Std. Deviation
Overall Data	179	0:00:01.86	0:20:13.75	0:02:20.64	0:03:24.710
Overall to Natal	95	0:00:01.86	0:20:13.75	0:02:28.02	0:03:43.455
Overall to Novel	84	0:00:01.86	0:13:14.16	0:02:12.30	0:03:02.177
Borrichia only (to Natal)	88	0:00:01.86	0:20:13.75	0:02:20.95	0:03:31.308
Borrichia only (to Novel)	78	0:00:01.86	0:13:14.16	0:02:11.40	0:03:00.065
Iva only (to Natal)	7	0:00:17.28	0:15:16.22	0:03:56.83	0:05:53.411
Iva only (to Novel)	6	0:00:18.09	0:10:02.58	0:02:24.01	0:03:46.785
Female to Natal	74	0:00:02.70	0:15:16.22	0:02:29.20	0:03:34.449
Female to Novel	67	0:00:01.86	0:10:58.04	0:02:11.12	0:02:50.465
Male to Natal	21	0:00:01.86	0:20:13.75	0:02:23.86	0:04:18.458
Male to Novel	16	0:00:02.14	0:13:14.16	0:02:24.03	0:03:54.198

Table 5: Descriptive Statistics of time trials. “Overall” data includes time trial data from both *Borrichia*-emerged midges and those from *Iva*. Time is formatted as hours:minutes:seconds.milliseconds.

Source of Variation	d.f.	Mean Square	*F*-Value	Significance
Natal Host Plant Choice (with host plant factored)	3	Between: 23838.230 Within: 42215.980	0.565	0.639
Natal Host Plant Choice (without host plant factored)	1	Between: 11018.881 Within: 42080.747	0.262	0.609
Natal Host Plant Choice (*Borrichia* only)	1	Between: 3777.262 Within: 6381243.395	0.097	0.756
Natal Host Plant Choice (*Iva* only)	1	Between: 27836.575 Within: 1006553.100	0.304	0.592
Male/Female (with host plant factored)	3	Between: 47990.511 Within: 41963.230	1.144	0.333
Male/Female (without host plant factored)	1	Between: 323.912 Within: 42302.555	0.008	0.930

Table 6: One-ways ANOVAs on the average time (in hh:mm:ss.ss) it took for *Asphondylia borrichiae* to make a choice within the olfactometer. Two separate tests were conducted factoring if the midge originated from *Borrichia* or *Iva* (df=3) and a test where the natal host plant was not considered (df=1). Statistics conducted in SPSS.

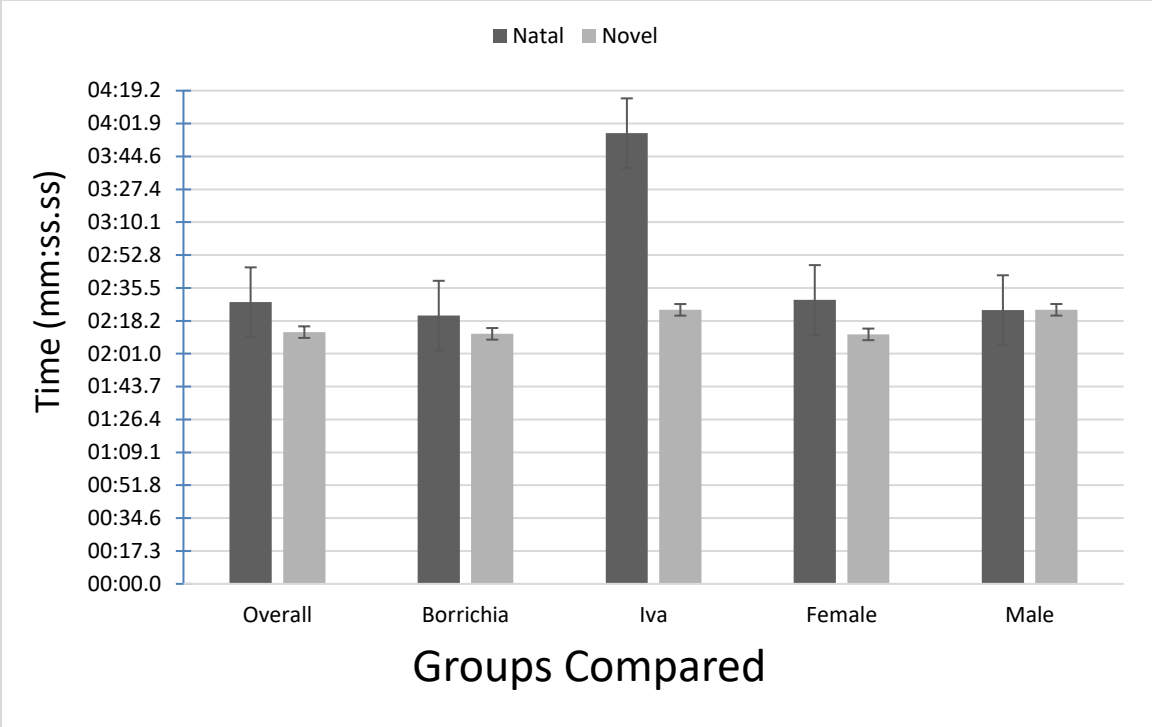


Figure 5: Time newly emerged midges took to select either their natal or novel host species (values are mean ± sem). X-axis gives the sample population in question for time comparisons with each bar representing the sample that chose the natal or novel host plant.

Fungal Analysis

BLAST searches on the sequenced ITS regions indicated the presence of two groups delineated by genus: *Cladosporium* and *Fusarium* (Table 7). Biogeographical dendrogram construction using known samples from Mycobank further supported the presence of these two distinct genera, reinforced by support values near 100 for each node (Figure 6). While there are not enough samples to test statistically, there is a weak association between fungal and plant genera. The majority of *Fusarium* specimens were identified on *Iva frutescens* galls (70% vs 30% found on *Borrichia*) while *Cladosporium* was mostly from *Borrichia frutescens* galls (62.5% vs 37.5%). Additionally, all *Cladosporium* samples originated from Jacksonville area plants

(Timucuan and Talbot) while the Tampa area plants only held *Fusarium*. However, given that there were only five of the eighteen samples were from Courtney Campbell plants this could be the result of low sample size.

Sample Location	Host Plant	Closest BLAST Taxon Identification	Total BLAST Score	Accession Number
Timucuan 1*	*B. frutescens*	*Cladosporium* sp. C61	1843	KY621330.1
Timucuan 2^	*B. frutescens*	*Cladosporium* sp. C61	1771	KY621330.1
Timucuan 3*	*B. frutescens*	*Cladosporium* sp. C61	1869	KY621330.1
Timucuan 4^	*B. frutescens*	*Cladosporium* sp. C61	1677	KY621330.1
Timucuan 5	*B. frutescens*	*Cladosporium* sp. C61	1640	KY621330.1
Timucuan 6	*B. frutescens*	*Fusarium* sp. G11	2120	LT719152.1
Timucuan 7	*B. frutescens*	*Fusarium* sp. G11	2109	LT719152.1
Timucuan 8	*I. frutescens*	*Cladosporium* sp. C61	1865	KY621330.1
Timucuan 9`	*I. frutescens*	*Fusarium decemcellulare* D096	1624	KU377441.1
Timucuan 10`	*I. frutescens*	*Fusarium decemcellulare* D096	1495	KU377441.1
Timucuan 11	*I. frutescens*	*Fusarium decemcellulare* D096	1626	KU377441.1
CCS 1'	*I. frutescens*	*Fusarium equiseti* SPF466	905	MH542620.1
CCS 2'	*I. frutescens*	*Fusarium* sp. G11	2150	LT719152.1
CCN 1¨	*I. frutescens*	*Fusarium* sp. G11	2238	LT719152.1
CCN 2¨	*I. frutescens*	*Fusarium* sp. G11	2214	LT719152.1
CCN 3	*B. frutescens*	*Fusarium* sp. JP39B-1X	2262	MG649271.1
Talbot 1~	*I. frutescens*	*Cladosporium* sp. C61	1843	KY621330.1
Talbot 2~	*I. frutescens*	*Cladosporium* sp. C61	1828	KY621330.1

Table 7: Table of samples identified down to species using the ITS region. Superscript symbols (^*`'~) indicate that these samples originated from the same isolate. Total BLAST scores are the sum of alignment scores of all segments from the same subject sequence, the most optimal alignment is given the highest score (https://www.ncbi.nlm.nih.gov/Web/Newsltr/V15N2/BLView.html).

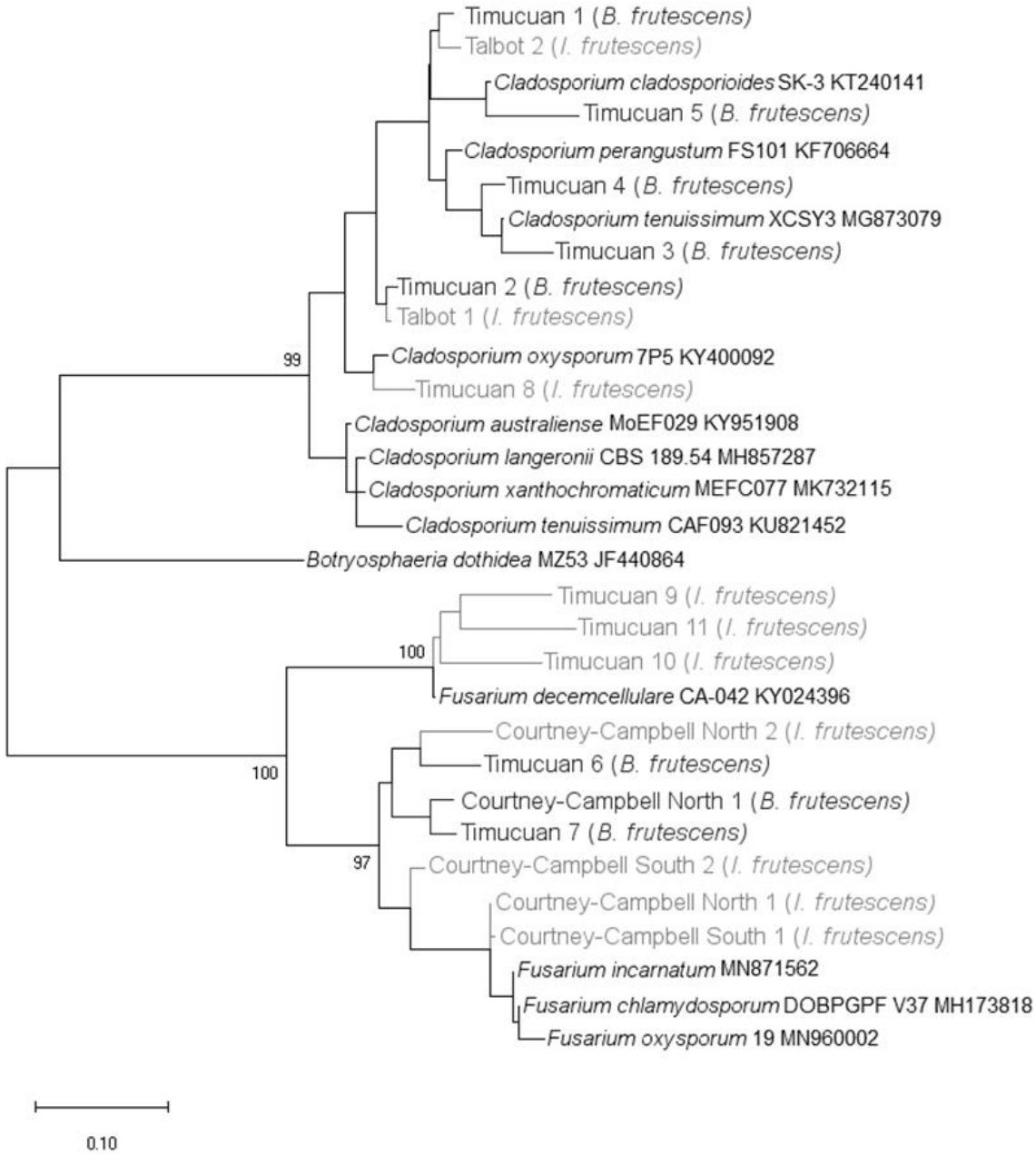


Figure 6: Biogeographical Dendrogram by Maximum Likelihood method. The genetic similarities were inferred by using the Maximum Likelihood method and General Time Reversible model (Nei and Kumar, 2000). The tree with the highest log likelihood (-10059.25) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 31 nucleotide sequences. There were a total of 1612 positions in the final dataset. Comparative taxa analyses were conducted in MEGA X (Kumar et al. 2018) Samples extracted from *Borrichia frutescens* are in darker grey and samples extracted from *Iva frutescens* are in light grey. *Borrichia* and *Iva* samples are combined contigs from both ITS1 and ITS4 fragments.

DISCUSSION

Olfactometery

Results from the current study suggest that *Asphondylia borrichiae* exhibits a weak preference for leaves of potential host plants, *Borrichia frutescens* or *Iva frutescens*, based on the natal host plant the midge emerged from. However, even this modest preference coupled with differential development periods of host-associated populations and the midges short adult lifespan, may help reduce gene flow between populations thereby driving divergence in sympatry. Moreover, this study is consistent with a previous field study (Rossi et al., 1999) that found a significant, but highly variable fidelity for the midge's natal host plant ranging from a high of 97% if *B. frutescens* is the natal host species to only 16% if *I. imbricata* was the original host. Rossi et al. (1999) noted a significant pattern of host fidelity *A. borrichiae* has for its natal host genus (i.e. either *Borrichia* or *Iva*); however, fidelity was imperfect and midges from the third less common host species (*I. imbricata*) actually showed a stronger preference for *I. frutescens* even when it was the natal host, which was further supported by a genetic study (Stokes et al., 2012). Thus, the current study suggests that olfactory cues for host location by *A. borrichiae*, while significant, are less important than phenological differences of the midges' host plant on reducing gene flow and driving divergence in sympatry. Additive effects of allochronic isolation, weak/imperfect host preference, interspecific differences in gall diameter and the resulting effects on parasitoid community composition have combined to promote divergence of host-associated populations of *A. borrichiae*, despite extensive overlap of the host plants.

Furthermore, the stronger associations observed in male *A. borrichiae* for their natal host plant compared to the female provides greater insight into what can further drive host fidelity and restrictions of gene flow. Adult male *A. borrichiae* are on average smaller than their female counterparts (Stiling et al., 1992), as female *A. borrichiae* not only carry the eggs but fat bodies to nurture them. While the primary purpose of these fat bodies is to nourish the egg, the female midge can also draw on them for energy which is an advantage that can allow her to find a new patch of host plants with lower crowding or risk of attack by parasitoids. Lacking these fat bodies, male *A. borrichiae* do not have the energy to expend in order to travel extensively to find a mate. In a situation where the male fly cannot detect a female fly, orienting towards the natal host plant would be a strategy in locating newly emergent mates. This behavioral pattern by male *A. borrichiae* could further drive restriction of gene flow between the two host-associated populations, especially if they exhibit the same olfactory preferences and behaviors in the wild.

While no studies have been conducted that directly compared the volatile profiles of leaves of *B. frutescens* and *I. frutescens*, studies have examined them separately. For example, Adams et al. (2012) reported that while leaf volatile oil composition could differ on an individual basis in *B. frutescens*, the monoterpenes sabinene and *β*-phellandrene, and the sesquiterpene germacrene D were the dominant volatile in certain individual plants. Additionally, Degenhardt and Lincoln (2006) found that the volatile profile of *Iva frutescens* had many different constituents but unwounded leaves routinely emitted a combination of eight different volatiles that were dominated by monoterpenes and sesquiterpenes including sabinene and germacrene D. The monoterpenes *α*-pinene, *β*-pinene, limonene, and the sesquiterpene α-humulene were

also similarly present in both *B. frutescens* and unwounded *I. frutescens* at similar concentrations, although unwounded *I. frutescens* also contained the sesquiterpenes β-caryophyllene and *cis*-β-guaiene. Similar, but distinct volatile profiles for *Borrichia frutescens* and *Iva frutescens* may partially account for the weak association of *Asphondylia borrichiae* for the leaves of their natal host species. Moreover, the weaker association of *A. borrichiae* for *I. frutescens* compared to *B. frutescens* is also consistent with previous studies (e.g. Rossi et al. 1999; Stokes et al. 2012 etc.) suggesting further that *B. frutescens* is the midge's ancestral host plant, though it is unknown how fungal endophytes may interact with these olfactory associations.

However, such a weak association implies that olfactory recognition is not the primary factor in reducing gene flow and driving divergence of host-associated populations of *A. borrichiae*, but likely reinforces it along with the host phenology. Additionally, other factors including leaf shape, leaf density, etc. may also influence midge orientation and host selection.

Nevertheless, while olfaction is believed to be important in cecidomyiids for both mate and host plant location (Hall et al. 2012), it is not the sole factor determining whether a female midge oviposits on a plant. For instance, although olfactory cues were the most significant in influencing egg-laying behavior for female Hessian flies, *Mayetiola destructor* (Diptera: Cecidomyiidae), differences in color hue and tactile cues still produced significant differences in egg-laying responses (Harris and Rose 1990). While the color *B. frutescens* and *I. furtescens* are similar (though *B. frutescens* may at times take a more yellow hue), venation and shape differ substantially (Figure 7). Additionally, in at some sites *I. frutescens* is heavily attacked by galling

mites, *Acalitus sp.* (Acari; Eriophyidae) which can substantially change the tactile profile of the leaves of *I. frutescens* (Figure 8).

Figure 7(a) and (b): Leaves of *B. frutescens* (a)(Fraser-Smith, S retrieved from Wikimedia Commons) vs those of *I frutescens* (b)(Allain, L retrieved from US Geological Survey). Note that the leaves of *B. frutescens* tend to be thicker and fleshier than *I frutescens* and while some serrations may be present on *B frutescens* they are overall less numerous than *I frutescens.*

Figure 8: Mite infestation on *I. frutescens* (Frances S Nagle, 2020)

As it is a weak association, these olfactory preferences are also not likely a stronger driver of speciation than allochrony influenced by host phenology and differences in gall microenvironment by host. Because the adult stage of the midge is extremely short lived (approximately 48 hours), the window for mating is brief. Lengthier development times of larval *A. borrichiae* within *I. furtescens* results in asynchronous emergence between *Borrichia* and *Iva* midge populations (Rossi et al., 1999). Additionally, gall midges must synchronize with their own host plant's phenology (Yukawa 2000; Spirko and Rossi 2015) in order to properly exploit the host plant for gall formation. Differences in flowering and development between *Borrichia* and *Iva* can further drive asynchronous emergence and mating. A recent study by Orta and Rossi (in review) found a negative association between galled and flowering terminals. Thus, along with

refuge from predators, *Iva* midges may persist due to an advantage of host space availability when *Borrichia* availability is restricted due to flowering (Spirko and Rossi 2015).

Although a previous reciprocal transplant experiment (Stiling and Rossi 1997; Rossi and Stiling 1998) suggested that midge populations attacking *B. frutescens* either preferred or performed better on their natal clone, the current study did not find a host fidelity association based on plant site or clone, though this may be in part due to a small sample size. However, Adam et al. (2015) noted that there were individual differences in which compound was the most dominant volatile. Although clones of *B. frutescens* exhibited significant phenotypic differences in leaf morphology, this variation was not apparent to the midges in the olfactometer and any orientation by leaf size or shape could not be assessed by the current study.

Fungus

Fungal taxonomy and molecular phylogenetics still faces difficulties in making definitive species identifications due to the complex and diverse evolutionary history of the taxa (Grube et al., 2017; Steencamp et al., 2018; Inderbitzin et al., 2020). Still, the ITS region provides a useful first diagnosis when approaching fungal species identification (Lücking et al., 2020) with ITS1 as a forward primer useful for ecological metabarcoding due to its ability to sequence across the entire region (Op de Beeck et al., 2014).

Compared to the Te Strake et al. (2006) paper, the current study found a much lower diversity of fungal genera. Only two genera were identified from BLAST, *Cladosporium* and *Fusarium,* compared to the eight identified by Te Strake. Notably missing from our findings that was present in the examination of newly emergent midges and external surfaces of the gall (as

well as galled stem and leaf tissues) reported by Te Strake et al. (2006) was evidence of *Alternaria sp.* which has hypothesized by Shaw (1992) to be part of the nutritional system of *A. borrichiae*. Also hypothesized to be part of the midge's nutritional system, *Bipolaris sp.* was also absent in our findings but found in galled stem tissue in Te Strake et al., though the absence of these two genera could be in part due to the differences in our methods as well as the specific nutritional requirements that were not provided by the culturing techniques used in the current study.

Te Strake et al. (2006) examined the apex, stems, and leaves of both galled and non-galled *B. frutescens,* while this investigation examined only the galls, but of both *B. frutescens* and *Iva frutescens*. Additionally, protocol for preparing tissues for incubation differed greatly especially in the use of agar and fungal extraction. Te Strake et al. (2006) employed potato dextrose agar, which is widely used for general cultivation of bacteria and fungi (Griffith et al., 2007). We employed SDA, which is widely for cultivating and isolating fungi, though is more widely used for medical diagnosis of fungal infections (Scognamiglio et al., 2010). Use of a single agar as well as agar choice from that used by Te Strake et al. could in part explain the different and more narrow range of genera identified. Even more importantly, Te Strake et al. (2006) used microscopy for identification rather than sequencing, which may have been less discerning than using DNA especially when the complex reproductive cycle of Ascomycetes and their multiple forms.

For tissue preparation, this study used sterile swabs to directly collect fungal conidia that lined the internal chambers of *A borrichiae* galls. Te Strake et al. took slices of plant tissue and directly applied it to the agar medium for fungal cultivation. As a result, there may have been

fungal endophytes that exist deeper within plant tissues that were present within the Te Strake et al. study and did not appear in ours. The presence of both fungal endophytes in this study was unsurprising given their ecology. *Cladosporium* are common outdoor molds and *Fusarium* are common soil fungi that are most often harmless saprobes. However, both genera do contain plant pathogens (De Hoog et al., 2000; Nelson et al., 1994) that could influence the development of an insect that depends on the plant for nutrition and shelter.

While no *Cladosporium* isolates were able to be definitively identified to species, a single *Cladosporium* strain was found to be the best match across localities and host plant species. This strain has been isolated in a study of fungal bioaerosols in Antioquia, Columbia, (Suarez-Roldan et al., 2017) indicating that this *Cladosporium* may be a globally distributed and ubiquitous fungus that has not been well described in its ecology and taxonomy. Interestingly, members of *Cladosporium,* including the widely distributed *C. cladosporioides* (G.A. de Vries, 1952), produce the anti-fungal metabolite cladosporin that had been found to inhibit the germination of *Penicillium* and *Aspergillus* spores and restricted growth of several other species on several different agar mediums including potato dextrose agar and Sabouraud dextrose agar (Scott et al., 1971). Implications of this could indicate that other fungal species' growth was inhibited giving us a narrower view of the endophytic diversity present within the galls.

Fusarium was the other genera identified through BLAST. Similar to *Cladosporium*, *Fusarium* is common genera found throughout soil communities. However certain species are important plant pathogens (Roncero et al., 2003). Two species were identified through BLAST: *Fusarium decemcellulare* (Brick, 1908) and *Fusarium equiseti* (Sacc. 1886). *Fusarium decemcellulare,* the anamorph or asexual reproductive form of *Albonectria rigidiuscula*

(Rossman and Samuels, 1999), is of particular interest because it is a known plant pathogen. *F. decemcellulare* has been known to attack important food crops such as avocados (*Persea americana,* Mill.), mangos (*Mangifera indica* L.), and cocoa (*Theobroma cacao* L.) causing diebacks, branch cankers, and galls (Ploetz, 2003). In rambutan (*Nephelium lappaceum* L), longan (*Dimocarpus longan* Lour.), and mangos, *F. decemcellulare* can additionally cause inflorescence wilt, vascular necrosis, and flower necrosis (Serrato-Diaz et al., 2015). Much like *F. decemcellulare*, *Fusarium equiseti* is has been found to be pathogenic to several species of plants including agriculturally important species of cucurbits, avocado (Joffe and Palti, 1967), wheat, and barley (Gale, 2003). *Fusarium equiseti* is a cosmopolitan species that found globally and can tolerate saline environments (Palmero et al., 2011) making it plausible to find it this study's locations.

The isolation of two pathogenic species of *Fusarium* within *Iva frutescens* may indicate how the nature of galls can weaken the host plant due to stress and the redirection of resources to the gall. Conversely, this plant pathogen may be antagonistic to the developing midge, redirecting nutrients to itself. While some associations between *Fusarium* and insects are mutualistic, these beneficial relationships exist mostly between the *F. solani* (Sacc. 1881) species complex and members of Coleoptera, especially flour beetles and wood-inhabiting beetles (Teetor-Barsch and Roberts, 1983). Many more interactions between *Fusarium* and insects are as entomopathogens (Sharma and Marques, 2018), that hinder the development of or even kill the insect. The production of the antibiotic and insecticidal peptide Beauvericin by numerous *Fusarium* species, especially *F. equiseti* (Logrieco et al., 1998) further supports the plausibility that a gall infested by endophytic *Fusarium* could be detrimental to the developing midge. If

Fusarium is in fact more dominant in *Iva frutescens* than it is in *Borrichia frutescens*, this could in part explain the lengthier developing times and lower fecundity of *A. borrichiae* midges on *Iva*. However, the ability of certain *Fusarium oxysporum* (Schltdl., 1824) volatiles to enhance root and shoot growth in *Arabidopsis thaliana* and tobacco plants (Bitas et al., 2015) demonstrates the importance in having a more accurate understanding of the endophytic community of *A. borrichiae* galls as some unidentified members of *Fusarium* could instead potentially be an important component for gall formation.

Summary

While there was a signficant association for their natal host plant species for *A. borrichiae*; the effect was weak. Moreover, the slightly greater fidelity for midges from *B. frutescens* compared to *I. frutescens* was consistent with previous studies suggesting *B. frutescens* is the ancestral host. Interestingly, males exhibited a stronger fidelity for their natal host than females. This result suggests that reduced gene flow, which promotes sympatric divergence, is reduced more though male host plant fidelity, than females. Males may use the host plant for mate location. Our molecular investigations of the fungal communities within *A. borrichiae* galls of *Borrichia* and *Iva* using the ITS region returned sequences usable for identification down to the genus level, and in some specimens down to species. Of the 18 samples identified, there were two genera identified: *Cladosporium*, which was more prevalent on *Borrichia* galls, and *Fusarium*, which was more prevalent on *Iva* galls. The two species identified, *F. decemcellulare* and *F equiseti* are both plant pathogens, and both of these species

were found on *Iva* galls which could be a component of why *A borrichiae* midges on *I. frutescens* are on average smaller and take longer to develop. However, further investigation into the endophytic communities is needed, although molecular methods provide an effective approach for identification.

Further Investigations

While olfaction is integral to the location of mates and host plants by *Asphondylia borrichiae,* we do not know the exact volatile or blend of volatiles the midge is most attracted to. Such a test could be performed either though an olfactometer test or antennal responses such as in Boddum (2013). Such an investigation could further elucidate why the preference for the natal host plant in *Borrichia* and *Iva* born midges was only a weak association- the volatiles that elicit the strongest response from the midges may be volatiles in high concentrations in both host plants. Adam et al. (2015) noted in their study of the volatile profiles of several *Borrichia frutescens* individuals along the Texas gulf coast that the dominant compound varied by individual. While our investigation did not find any definitive preference for locality by the midge, it may be because of the low sample size we were able to test. Additionally, analysis of leaf measurements did reinforce phenotypic differences by locality in regards to *B frutescens*. It may be possible that differences in the dominant volatile compound also varies by locality to a significant degree.

WORKS CITED

Adams, R., S. Carver, and M. Arnold. 2015. Preliminary study of variation in leaf volatile oil of *Borrichia frutescens* (L.) DC along the Texas gulf coast. Phytologia. 97: 164-169.

Alexander, C., and G. Hadley. 1985. Carbon Movement between Host and Mycorrhizal Endophyte During the Development of the Orchid *Goodyera repens* Br. New Phytologist. 101(4): 657-665.

Ali Niazee, M. T., and R. L. Penrose. 1981. Apple Maggot in Oregon: A Possible New Threat to the Northwest Apple Industry. Bulletin of the Entomological Society of America. 27: 245–246.

Anselmo-Moreira, F., L. Teixeira-Costa, G. Ceccantini, and C. M. Furlan. 2019. Mistletoe effects on the host tree *Tapirira guianensis*: insights from primary and secondary metabolites. Chemoecology. 29: 11–24.

Antlfinger, A. E. 1982. Genetic neighborhood structure of the salt marsh composite, *Borrichia frutescens.* Journal of Heredity. 73: 128–132.

Azcón-Aguilar, C., and J. M. Barea. 1997. Arbuscular mycorrhizas and biological control of soil-borne plant pathogens - an overview of the mechanisms involved. Mycorrhiza. 6: 457-464.

Barnewall, E. C., and R. A. De Clerck-Floate. 2012. A Preliminary Histological Investigation of Gall Induction in an Unconventional Galling System. Arthropod-Plant Interactions. 6: 449–459.

Batra, L., and R. Lichtwardt. 1963. Association of Fungi with Some Insect Galls. Journal of the Kansas Entomological Society. 36: 262-278.

Bellemain, E., T. Carlsen, C. Brochmann, E. Coissac, P. Taberlet, and H. Kauserud. 2010. ITS as an environmental DNA barcode for fungi: an *in silico* approach reveals potential PCR biases. BMC Microbiology. 10: 189.

Bensch K., J. Z. Groenewald, J. Dijksterhuis, M. Starink-Willemse, B. Andersen, B. A. Summerell, H. D. Shin, F. M. Dugan, H. J. Schroers, U. Braun, and P. W. Crous. 2010. Species and ecological diversity within the Cladosporium cladosporioides complex (Davidiellaceae, Capnodiales). Studies in Mycology. 67:1-94.

Berlocher, S. H., and J. L. Feder. 2002. Sympatric Speciation in Phytophagous Insects: Moving Beyond Controversy? Annual Review of Entomology. 47: 773-815.

Bernays, E. 2001. Neural limitations in phytophagous insects: Implications for diet breadth and evolution of host affiliation. Annual Review of Entomology. 46: 703-727.

Bernays, E. A., and R. F. Chapman. 1994. Host-plant selection by phytophagous insects. Chapman & Hall. Springer, Boston, MA.

Bernays, E., and D Funk. 1999. Specialists make faster decisions than generalists: experiments with aphids. Proceedings of the Royal Society B-Biological Sciences. 266: 151-156.

Bitas, V., N. McCartney, N. Li, J. Demers, J. E. Kim, H. S. Kim, K. M. Brown, S. Kang, 2015. *Fusarium oxysprum* Volatiles Enhance Plant Growth via Affecting Auxin Transport and Signaling. Frontiers in Microbiology. 6: 1248.

Boddum T. 2013. Gall midge olfaction and its role in speciation. PhD diss., Swedish University of Agricultural Sciences.

Borkent, A., and J. Bisset. 1985. Gall midges (Diptera: Cecidomyiidae) are vectors for their fungal symbionts. Symbiosis. 1: 185-194

Bush, G. L. 1966. The taxonomy, cytology, and evaluation of the genus *Rhagolelis* in North America (Diptera: Tephritidae). Bulletin of The Museum of Comparative Zoology, Harvard University. 134: 431-562.

Bush, G. L., and R. K. Butlin. 2004. Sympatric Speciation in Insects. Adaptive Speciation, Cambridge University Press, 229-248.

Carroll, G. 1988. Fungal Endophytes in Stems and Leaves: From Latent Pathogen to Mutualistic Symbiont. Ecology. 69: 2-9.

Chao, J., and G. Liao 2013. Histocytological Aspects of Four Types of Ambrosia Galls on *Machilus Zuihoensis Hayata* (Lauraceae). Flora - Morphology, Distribution. Functional Ecology of Plants. 208: 157–164.

Chen, B., K. Du, C. Sun, A. Vimalanathan, X. Liang, Y. Li, B. Wang, X. Lu, L. Li, and Y. Shao. 2018. Gut bacterial and fungal communities of the domesticated silkworm (*Bombyx mori*) and wild mulberry-feeding relatives. The ISME Journal. 12**:** 2252–2262.

Davis, S. D., and P. J. Landolt. 2013 A Survey of insect Assemblages Responding to Volatiles from a Upiquitous Fungus in an Agricultural Landscape. Journal of Chemical Ecology. 39: 860-868.

G. De Hoog, J. Guarro, J. Gené, and M. J. Figueras. 2000. Atlas of clinical fungi. 2nd ed. American Society for Microbiology, USA.

Degenhardt, D. C., and D. E. Lincoln. 2006. Volatile Emissions from an Odorous Plant in Response to Herbivory and Methyl Jasmonate Exposure. Journal of Chemical Ecology. 32: 725-743.

Dethier, V.G. 1959 Egg-laying habits of lepidoptera in relation to available food. Canadian Entomologist. 91: 554 561.

Egan, S. P., G. J. Ragland, L. Assour, T. H. Q. Powell, G. R. Hood, S. Emrich, P. Nosil, and J. L. Feder. 2015. Experimental evidence of genome-wide impact of ecological selection during early stages of speciation-with-gene-flow. Ecology Letters. 18: 817– 825.

Feder J. L. 1995. The effects of parasitoids on sympatric host races of the apple maggot fly, *Rhagoletis pomonella* (Diptera: Tephritidae). Ecology 76: 801–13.

Feder, J. L., and K. Filchak. 1999. It's about time: the evidence for host plant-mediated selection in the apple maggot fly, *Rhagoletis pomonella*, and its implications for fitness trade-offs in phytophagous insects. Entomologia Experimentalis et Applicata. 91: 211–25.

Feder, J. L., K. Reynolds, W. Go, and E. C. Wang. 1995. Intra- and interspecific competition and host race formation in the apple maggot fly, *Rhagoletis pomonella.* Oecologia 101: 416–25

Gagne, R.J. 1989. The plant-feeding gall midges of North America. Comstock Publisher Associates. Ithaca, NY.

Gale L. R. 2003. Population Biology of Fusarium species causing head blight of grain crops. In: *Fusarium* head blight of wheat and barley. In: Leonard K.J., Bushnell W.R., editors. American Phytopathological Society. St. Paul, MN. 120–143.

Gange, A. C. and H. E. Nice. 1997. Performance of the thistle gall fly, *Urophora cardui*, in relation to host plant nitrogen and mycorrhizal colonization. New Phytologist. 137: 335-343.

Gates, K., C. A. Stewart, M. James, and S. Kidder. 2020 An Assessment of the Differences between the Fungal Endophyte Communities in Insect-Affected and Unaffected Spruce Trees. Claro Undergraduate Interdisciplinary Journal. 8: 35–47.

Glass, E. H., and S. E. Lienk. 1971. Apple insect and mite populations developing after discontinuance of insecticides: 10-year record. Journal of Economic Entomology. 64: 23-26.

Gratton, C., and S. C. Welter. 1999, Does "enemy-free space" exist? Experimental host shifts of an herbivorous fly. Ecology, 80: 773-785.

Grauvogel-Stamm, L. 1978. La flore du grès à Voltzia (Bundsandstein supérieur) des Vosges du Nord (France,) Morphologie, anatomie, interprétotions phylogénetique et paléogéographique. Doctorat d'Etat thesis, Louis Pasteur University, Strasbourg, France.

Griffith, G. W., G. L. Easton, A. Detheridge, K. Roderick, A. Edwards, H. J, Worgan, J. Nicholson, and W. T. Perkins. 2007. Copper deficiency in potato dextrose agar causes reduced pigmentation in cultures of various fungi. FEMS Microbiology Letters. 276: 165–171.

Grube, M., E. Gaya, H. Kauserud, A. M. Smith, S. V. Avery, S. J. Fernstad, L. Muggia, M. D. Martin, T. Eivindsen, U. Koljalg, and M. Bendiksby. 2017. The next generation fungal diversity researcher. Fungal Biology Reviews. 31: 124-130.

Hall, D. R., L. Amarawardana, J. V. Cross, W. Francke, T. Boddum, and Y. Hillbur. 2012. The Chemical Ecology of Cecidomyiid Midges (Diptera: Cecidomyiidae). Journal of Chemical Ecology. 38: 2-22.

Hardoim, P. R., L. S. van Overbeek, G. Berg, A. M. Pirttilä, S. Compant, A. Campisano, M. Döring, and A. Sessitsch. 2015. The Hidden World within Plants: Ecological and Evolutionary Considerations for Defining Functioning of Microbial Endophytes. Microbiology and Molecular Biology Reviews. 79: 293–320.

Harris, M O., and S. Rose. 1990. Chemical, Color, and Tactile Cues Influencing Oviposition Behavior of the Hessian Fly (Diptera: Cecidomyiidae). Environmental Entomology. Volume 19: 303–308.

Hartley, S., and J. Lawton. 1992. Host-Plant Manipulation by Gall-Insects: A Test of the Nutrition Hypothesis. Journal of Animal Ecology. *61*: 113-119.

Hawkins, B. A., and R. D. Goeden. 1984. Organization of a parasitoid community associated with a complex of galls on *Atriplex* spp. in southern California. Ecological Entomology. 9: 271-292.

Hawksworth, D. 2011. A new dawn for the naming of fungi: impacts of decisions made in Melbourne in July 2011 on the future publication and regulation of fungal names. MycoKeys. 1: 7-20.

Heard, S. B., J.O. Stireman, J. D. Nason, G. H. Cox, C. R. Kolacz, and J. M. Brown. 2006. On the elusiveness of enemy-free space: spatial, temporal, and host-plant-related variation in parasitoid attack rates on three gallmakers of goldenrods. Oecologia. 150, 421.

Heath, J. J., and J. O. Stireman III. 2010. Dissecting the association between a gall midge, *Asteromyia carbonifera*, and its symbiotic fungus, *Botryosphaeria dothidea*. Entomologia Experimentalis et Applicata, 137: 36-49.

Inderbitzin, P., B. Robbertse, and C. L. Schoch. 2020. Species identification in plant-associated prokaryotes and fungi using DNA. Phytobiomes Journal.

Hood, G. R., T. H. Q. Powell, M. M. Doellman, S. B. Sim, M. Glover, W. L. Yee, R. B. Goughnour, M. Mattsson, D. Schwarz, and J. L. Feder. 2019. Rapid and repeatable host plant shifts drive reproductive isolation following a recent human-mediated introduction of the apple maggot fly, *Rhagoletis pomonella.* Evolution. vol. 74: 156–168.

Irwin, J. T., and R. E. Lee Jr. 2003. Cold winter microenvironments conserve energy and improve overwintering survival and potential fecundity of the goldenrod gall fly, *Eurosta solidaginis*. Oikos, 100: 71-78.

Joffe A. Z., and J. Palti. 1967. *Fusarium equiseti* (Cda) Sacc*.* Israel Journal of Botany. 16: 1–18.

Kasson, M. T., K. L. Wickert, C. M. Stauder, A. M. Macias, M. C. Berger, D. R. Simmons, D. P. Short, D. B. DeVallance, and J. Hulcr. 2016. Mutualism with aggressive wood-degrading *Flavodon ambrosius* (Polyporales) facilitates niche expansion and communal social structure in *Ambrosiophilus* ambrosia beetles. Fungal Ecology. 23: 86-96.

Kato, K., and N. Hijii. 1997. Effects of Gall Formation by *Dryocosmus kuriphilus Yasumatsu* (Hym, Cynipidae) on the Growth of Chestnut Trees. Journal of Applied Entomology 121: 9-15

Keil, T. 1999. Morphology and development of the peripheral olfactory organs. In: Hansson, B. (Ed.) Insect Olfaction. Springer, Berlin, Germany.

Kumar, S., G. Stecher, M. Li, C. Knyaz, and K. Tamura. 2018. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Molecular Biology and Evolution*.* 35: 1547-1549.

Larson, K. C., and T. G. Whitham. 1991. Manipulation of food resources by a gall-forming aphid: the physiology of sink-source interactions. Oecologia. 88: 15–21.

Logrieco, A., A. Moretti, G. Castella, M. Kostecki, P. Golinski, A. Ritieni, and J. Chelkowski. 1998. Beauvericin production by *Fusarium* species. Applied Environmental Microbiology. 64: 3084-8.

Lücking, R., M. C. Aime, B. Robbertse, A. N. Miller, H. A. Ariyawansa, T. Aoki, G. Cardinali, P. W. Crous, I. S. Druzhinina, D. M. Geiser, D. L. Hawksworth, K. D. Hyde, L. Irinyi, R. Jeewon, P. R. Johnston, P. M. Kirk, E. Malosso, T. W. May, W. Meyer, M. Öpik, V. Robert, M. Stadler, M. Thines, D. Vu, A. M. Yurkov, N. Zhang, and C. L. Schoch. 2020. Unambiguous identification of fungi: where do we stand and how accurate and precise is fungal DNA barcoding?. IMA Fungus. 11**:** 14.

Mani, M. S. 2013. *Ecology of Plant Galls*. Springer, Netherlands

Mapes, C. C., and P. Davies. 2001a. Indole-3-acetic acid and ball gall development on *Solidago altissima*. New Phytologist. 151: 195–202.

Martin, K. J., and P. T. Rygiewicz. 2005. Fungal-specific PCR primers developed for analysis of the ITS region of environmental DNA extracts. BMC Microbiology. 5: 28.

Moriya, S., M. Shiga, and I. Adachi. 2003. Classical Biological Control of the Chestnut Gall Wasp in Japan In: VanDriesche RG (ed) Proceedings of the 1st International Symposium on Biological Control of Arthropods. USDA Forest Service, Washington. pp 407-417

Nei, M., and S. Kumar. 2000. Molecular Evolution and Phylogenetics. Oxford University Press, New York.

Nelson, P. E., M. C. Dignani, and E. J, Anaissie. 1994. Taxonomy, biology, and clinical aspects of *Fusarium* species. Clinical Microbiology. Rev. 7: 479-504.

Op De Beeck, M., B. Lievens, P. Busschaert, S. Declerck, J. Vangronsveld, and J. V. Colpaert. 2014. Comparison and Validation of Some ITS Primer Pairs Useful for Fungal Metabarcoding Studies. PLoS ONE. 9.

Ou, S.H. 1980. Pathogen variability and host resistance in rice blast disease. Annual Review of Phytopathology. 18: 167–187.

Palmero, D., M. de Cara, C. Iglesias, L. Gálvez, and J. C. Tello. 2011. Comparative study of the pathogenicity of seabed isolates of *Fusarium equiseti* and the effect of the composition of the mineral salt medium and temperature on mycelial growth. Brazilian journal of microbiology : [publication of the Brazilian Society for Microbiology]. 42: 948–953.

Payne, J. A., R. A. Jaynes, and S. J. Kays. 1983. Chinese chestnut production in the United States: Practice, Problems, and Possible Solutions. Economic Botany 37: 187-200

Pinto-Tomás, A. A., M. A. Anderson, G. Suen, D. M. Stevenson, F. S. T. Chu, W. W. Cleland, P. J. Weimer, and C. R. Currie. 2009. Symbiotic Nitrogen Fixation in the Fungus Gardens of Leaf-Cutter Ants. *Science*. 326: 1120–1123.

Ploetz, R. C. 2003. Diseases of mango. *Diseases of Tropical Fruit Crops*. Page 327 CABI Publishing, Wallingford, UK.

Porter, T. M., and G. Brian Golding. 2011. Are similarity-or phylogeny-based methods more appropriate for classifying internal transcribed spacer (ITS) metagenomic amplicons?. New Phytologist. *192*: 775-782.

Rajendran, V. and T. Sandle. 2011. A review of fungal contamination in pharmaceutical products and phenotypic identification of contaminants by conventional methods. European Journal of Parenteral and Pharmaceutical Sciences. 17: 4-19.

Raman, A. 2012. Gall Induction by Hemipteroid Insects. Journal of Plant Interactions. 7: 29–44.

Raman, A., C. W. Schaefer, and T. M. Withers. 2005. Biology, Ecology, and Evolution of Gall-Inducing Arthropods. Science Publishers. 1.

Richardson, M. D. 2000. Alkaloids of endophyte-infected grasses: defense chemicals or biological anomalies?. Microbial Endophytes (Bacon, C.W.; White, J.F., eds.). New York: Marcel Dekker. pp. 323-340.

Rodriguez, R. J., J. F. White, A. E. Arnold, and R. S. Redman. 2009. Fungal endophytes: diversity and functional roles. The New Phytologist. 182: 314–330.

Roncero, M. I. G., C. Hera, M. Ruiz-Rubio, M. P. Madrid, Z. Caracuel, F. Calero, J. Delgado-Jarana, C. Velasco, J. Roa, D. Cordoba, and A. Di Pietro. 2003. *Fusarium* as a Model for Studying Virulence in Soilborne Plant Pathogens. Physiological and Molecular Plant Pathology. 62: 87–98.

Rossi, A. M., and P. Stiling. 1995. Intraspecific Variation in Growth Rate, Size, and Parasitism of Galls Induced by *Asphondylia borrichiae* (Diptera: Cecidomyiidae) on Three Host Species. Annals of the Entomological Society of America. 88: 39–44.

Rossi, A. M., and P. Stiling. 1998. The interactions of plant clone and abiotic factors on a gall-making midge. Oecologia. 116: 170–176.

Rossi, A. M., M. Murray, K. Hughes, M. Kotowski, D. C. Moon, and P. Stiling. 2006. Non-random distribution among a guild of parasitoids: implications for community structure and host survival. Ecological Entomology, 31: 557-563.

Rossi, A. M., P. Stiling, M. V. Cattell, and T. I. Bowdish. 1999. Evidence for host-associated races in a gall-forming midge: trade-offs in potential fecundity. Ecological Entomology. 24: 95-102.

Saveer, A., S. Kromann, G. Birgersson, M. Bengtsson, T. Lindblom, A. Balkenius, B. S. Hansson, P. Witzgall, P. Becher, and R. Ignell. 2012. Floral to green: mating switches moth olfactory coding and preference. Proceedings of the Royal Society B-Biological Sciences. 279: 2314-2322.

Schoch, C. L., K. A. Seifert, S. Huhndorf, V. Robert, J. L. Spouge, C. A. Levesque, W. Chen, and Fungal Barcoding Consortium. 2012. Nuclear Ribosomal Internal Transcribed Spacer (ITS) Region as a Universal DNA Barcode Marker for Fungi. Proceedings of the National Academy of Sciences. 109: 6241–6246.

Scognamiglio, T., R. Zinchuk, P. Gumpeni, and D. H. Larone. 2010. Comparison of Inhibitory Mold Agar to Sabouraud Dextrose Agar as a Primary Medium for Isolation of Fungi. Journal of Clinical Microbiology. 48: 1924–1925.

Scott, P. M., W. van Walbeek, and W. M. MacLean. 1971. Cladosporin, a new antifungal metabolite from *Cladosporium cladosporioides*. The Journal of Antibiotics. 24: 747–755.

Serrato-Diaz, L. M., M. Perez-Cuevas, L. I. Rivera-Vargas, R. Goenaga, and R. D. French-Monar. 2015. First Report of *Fusarium decemcellulare* Causing Inflorescence Wilt and Vascular and Flower Necrosis of Rambutan (*Nephelium lappaceum*), Longan (*Dimocarpus longan*), and Mango (*Mangifera indica*). Plant Disease. 99: 1187.

Sharma, L., and G. Marques. 2018. Fusarium, an Entomopathogen-A Myth or Reality?. Pathogens. 7: 93.

Shaw, P. 1992. Fungi, fungivores, and fungal food webs. In: G.C. Carroll and D. T. Wicklow, eds. The fungal community: its organization and role in the ecosystem. M. Dekker, Inc., New York. Pp. 295-3

Steenkamp, E. T., M. J. Wingfield, A. R. McTaggart, and B. D. Wingfield. 2018. Fungal species and their boundaries matter – Definitions, mechanisms and practical implications. Fungal Biology Reviews. 32: 104–116.

Stielow, J. B., C. A. Lévesque, K. A. Seifert, W. Meyer, L Iriny , D Smits, R Renfurm, G. J. Verkley, M. Groenewald, D. Chaduli, A. Lomascolo, S. Welti, L. Lesage-Meessen, A. Favel, A. M. Al-Hatmi, U. Damm, N. Yilmaz, J. Houbraken, L. Lombard, W. Quaedvlieg, M. Binder, L. A. Vaas, D. Vu, A. Yurkov, D. Begerow, O. Roehl, M. Guerreiro, A. Fonseca, K. Samerpitak, A. D. van Diepeningen, S. Dolatabadi, L. F. Moreno, S. Casaregola, S. Mallet, N. Jacques, L. Roscini, E. Egidi, C. Bizet, D. Garcia-Hermoso, M. P. Martín, S. Deng, J. Z. Groenewald, T. Boekhout, Z. W. de Beer, I. Barnes, T. A. Duong, M. J. Wingfield, G. S. de Hoog, P. W. Crous, C. T. Lewis, S. Hambleton, T. A. Moussa, H. S. Al-Zahrani, O. A. Almaghrabi, G. Louis-Seize, R. Assabgui, W. McCormick, G. Omer, K. Dukik, G. Cardinali, U. Eberhardt, M. de Vries, and V. Robert. 2015. One fungus, which genes? Development and assessment of universal primers for potential secondary fungal DNA barcodes. Persoonia. 35:242-63

Stiling, P., A. M. Rossi, D. R. Strong, and D. M. Johnson. 1992. Life History and Parasites of *Asphondylia borrichiae* (Diptera: Cecidomyiidae), a Gall Maker on Borrichia frutescens. The Florida Entomologist. 75: 130

Stokes, K., P. Stiling, M. R. Gilg, and A. M. Rossi. 2012. The Gall Midge *Asphondylia borrichiae* (Diptera: Cecidomyiidae): An Indigenous Example of Host-Associated Genetic Divergence in Sympatry. *Environmental Entomology*. 41: 1246-1254.

Stone, J. K., J. D. Polishook, and J. R. J. White. 2004. Endophytic fungi. In: Mueller G, Bills GF, Foster MS, eds. Biodiversity of fungi: inventory and monitoring methods. Burlington, MA, USA: Elsevier, 241–270.

Suarez-Roldan, M., L. M. Silva-Bedoya, C. X. Moreno-Herrera, O. I. Montoya, and C. E. Zapata. 2017. Study of fungal bioaerosols from filters of Particulate Material of 10 microns or less in Antioquia (Colombia). Unpublished manuscript submitted to *Geociencias y Medio Ambiente*.

Suman, A., A. N. Yadav, and P. Verma. 2016. Endophytic Microbes in Crops: Diversity and Beneficial Impact for Sustainable Agriculture. 10.

Taper, M. L., E. M. Zimmerman, and T. J. Case. 1986. Sources of mortality for a cynipid gall-wasp (*Dryocosmus dubiosus* (Hymenoptera: Cynipidae)): The importance of the Tannin/Fungus interaction. Oecologia. 68: 437–445.

Tasin, M., G. K. Knudsen, I. Pertot. 2011. Smelling a diseased host: grapevine moth responses to healthy and fungus-infected grapes. Animal Behaviour. 83: 555-562.

D. Te Strake, A. H. Keagy, and P. D. Stiling. 2006. Fungi associated with Borrichia frutescens (Asteraceae): Insect galls and endophytes. SIDA, Contributions to Botany, vol. 22, no. 1, pp. 755–763.

Teetor-Barsch, G. H., and D. W. Roberts. 1983. Entomogenous Fusarium species. Mycopathologia. 84: 3–16.

Tsopmbeng, G. R., D. A. Fontem, and K. F. Yamde. 2012. Evaluation of Culture Media for Growth and Sporulation of *Phytophthora Colocasiae* Racib., Causal Agent of Taro Leaf Blight. International Journal of Biological and Chemical Sciences. 6: 4.

Vega, F. E., F. Posada, M. C. Aime, M. Pava-Ripoll, F. Infante, and S. A. Rehner. 2008. Entomopathogenic fungal endophytes. Biological Control. 46: 72-82.

Wainright, P., G. Hinkle, M. L. Sogin, and S. K. Stickel. 1993. Monophyletic Origins of the Metazoa: an Evolutionary Link with Fungi. Science. 260: 340–342.

Walsh BD. 1864. On phytophagic varieties and phytophagic species. Proceedings of the Entomological Society of Philadelphia. 3: 403-30

Weis, A. E., and A. Kapelinski. 1994. Variable selection on *Eurosta*'s gall size. II. A path analysis of the ecological factors behind selection. Evolution. 48:734-745.

Wurzbacher, C., E. Larsson, J. Bengtsson-Palme, S. Van den Wyngaert, S. Svantesson, E. Kristiansson, M. Kagami, and R. H. Nilsson. 2019. Introducing ribosomal tandem repeat barcoding for fungi. Molecular Ecology Resources. 19: 118– 127.

Wyss, G. S., R. Charudattan, and J. T. DeValerio. 2001. Evaluation of Agar and Grain Media for Mass Production of Conidia of *Dactylaria higginsii*. Plant Disease. 85: 1165–1170.

Yee, W. L. and R. B. Goughnour. 2008. Host plant use by and new host records of apple maggot, western cherry fruit fly, and other *Rhagoletis* species (Diptera: Tephritidae) in western Washington state. The Pan-Pacific Entomologist. 84: 179-193.

Yukawa, J. 2000. Synchronization of gallers with host plant phenology. Population Ecology. 42:105–113

Zhang, D. X., P. Nagabhyru, and C. L. Schardl. 2009. Regulation of a chemical defense against herbivory produced by symbiotic fungi in grass plants. Plant Physiology. 150: 1072–1082.