

BIOAg Project Report

Report Type: Final

Title: Identifying biocontrol agents for X-disease vectors to allow integrated pest management in cherries

Principal Investigator(s) and Cooperator(s):

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Abstract:

X-disease, often colloquially referred to as “little cherry disease” is the critical threat to Pacific Northwest cherry production and the only management options are tree removal and control of leafhoppers that vector the phytoplasma pathogen. With little natural enemy knowledge, vector control focuses only on chemical controls, suspending integrated management principles. Here, we evaluate primers we developed for 3 potential leafhopper biocontrol agents identified in Central Washington cherry orchards: a fungal pathogen or symbiont, a parasitic fly, and a parasitic wasp to better understand the abundance of each and identify potential for control. By using molecular techniques to evaluate each, we are quickly screening each and leveraging USDA funding to conduct molecular gut content analysis and phytoplasma presence. This knowledge will help bring X-disease management into an integrated pest management framework, where we also promote biological control, when possible.

Project Description:

X-disease is the key threat to Pacific Northwest production of cherries and stone fruits. It is caused by the X-disease phytoplasma that cannot be treated within a tree, so the only management option is reducing transmission through the removal of diseased trees and controlling leafhoppers that vector the pathogen. There is a multi-year lag between the time a tree is infected and the emergence of symptoms, making it difficult to quickly respond to infections within blocks and allowing undetected pathogen spread. While the current epidemic has only recently re-emerged in the Pacific Northwest, a previous outbreak devastated the region in the 1940s and 1950, and more recently had a severe impact on California cherry production in the 1980s. During the California outbreak in the 1980s, a group of Californian researchers led by Bob Van Steenwyk found that the most important management technique was the removal of diseased trees, with vector control measures available at the time providing secondary benefits. In the Pacific Northwest, research focused on X-disease vector management techniques only started in 2020 after a >60-year hiatus. The leafhoppers that vector X-disease are highly mobile and have wide host range, including a diversity of broadleaf hosts that can also host X-disease phytoplasma. Recent surveys suggest that the key vector is *Colladonus montanus reductus*, with *C. geminatus* also being important but lower in abundance.

Unfortunately, very little is known about the natural enemies of X-disease vectors, limiting our ability to develop an integrated management program. In addition to reducing vector numbers within cherry and stone fruit blocks, natural enemies may help reduce leafhopper abundance in nearby apple and pear blocks and in natural vegetation thereby reducing the number of vectors migrating into cherries. In May

2020, we collected the two key vectors, *Colladonus geminatus* and *C. m. reductus* from a commercial Wapato cherry block and in the process of developing primers for the leafhopper nymphs, identified high rates of parasitism (four out of the five *C. geminatus* and two out of the five *C. reductus*) by a fly (family Pipunculidae) that parasitizes late instar nymphs and develops within the adults. This fly was mentioned (no data were presented) as attacking *C. geminatus* in Utah and Oregon in the 1950s, but little research has been conducted on it since then, and we are not aware of any previous reports of it occurring in Washington or attacking *C. m. reductus*. The parasitic fly is only known to emerge from adults, but may attack late instar nymphs, and then develop along with the adult. Recently, we have sequenced a *Hirsutella* spp. fungus from *C. m. reductus* and reared a wasp (family Dryinidae) from X-disease vectors, but it is unknown how common parasitism by these biological control agents is. *Hirsutella* fungi have been used to control hemipteran vectors of citrus greening and Pierce's disease, and Dryinid wasps are known to parasitize white apple leafhoppers in Washington, suggesting that each might be valuable in this context as well. However, more information is needed on these biological control agents before they can be integrated into management programs.

Outputs

- Overview of results:

We have validated the primers for natural enemies of X-disease vectors, with the *Tomosvaryella lepidipes* primer validated to species level, and *Hirsutella* sp. fungus validated to genus level. A third primer, used to identify a Dryinid wasp, *Gonatopus* sp. has been validated for some, but not all Dryinid wasps. Experts on Dryinid wasps are exceedingly rare, and therefore we have not been able to definitively morphologically identify the wasp. After initially waiting on species confirmation to determine that the variability in our primer amplification is driven by species differences, we have now developed a more general primer to focus broadly on Dryinid wasps to ensure that we do not miss parasitism by a different species. We have also developed primers to identify the two key vector species (*Colladonus geminatus* and *C. m. reductus*), which are helpful in identifying nymph species that are morphologically indistinguishable from non-vectors. We have found that these primers can even be used to detect *Colladonus* spp. eggs within plant tissue and identify the egg to species. We have been able to develop a 5-target multiplex that simultaneously evaluates a leafhopper for the presence of 5 genes: *C. m. reductus*, *C. geminatus*, *T. lepidipes*, Dryinid wasps, and X-disease. The first two genes are used to distinguish immature vectors from other immature species, the third and fourth genes are to identify parasitism, and the final gene is to determine vector status.

From our 2021 samples across 3 sites, we have detected parasitism by the parasitic fly, but not the parasitic wasp. We detected the *Hirsutella* sp. fungus in >99% of the *Colladonus* spp. leafhoppers, but none of the non-*Colladonus* spp. leafhoppers. Further experimental and genetic analysis of *Hirsutella* sp. suggests it is symbiotic with *Colladonus* spp. leafhoppers and vertically transmitted. In addition, genetic sequencing suggests that *C. m. reductus* and *C. geminatus* may each be associated with different *Hirsutella* strains. During the 2022 field season, we sampled 85 times from May 5th through October 20th across 47 sites, 19 locations, and 4 tree fruits (apple, cherry, nectarine, pear). Samples were collected from each site's ground cover. Thus far, we have sorted 2152 leafhoppers (nymphs and adults). The two primary X-disease vector species appear to have slightly different patterns of abundance across the Washington cherry-growing region. For example, the abundance of *C. m. reductus* was negatively correlated with latitude, despite samples taken from northern sites with very little leafhopper management conducted. In contrast, *C. geminatus* was not correlated with latitude, but was positively correlated with elevation. These samples will be evaluated for X-disease phytoplasma and the two parasitoids (fly and wasp) using the multiplex once it is completed. We will also evaluate the

geographic prevalence of *Hirsutella*, but given the finding that *Hirsutella* is maternally transmitted, we will not test as extensively amongst these sites as for the parasitic fly and wasp.

Methods and Results:

Objective 1) Develop a multiplex qPCR bioassay to evaluate parasitism by a fungal pathogen, parasitic wasp, and parasitic fly in the same bioassay, using 3 parasite-specific primers.

Since the submission of the proposal, we have described the genetic sequences of the parasitic fly, and the fungus identified within X-disease vectors, and developed primers for each. Due to difficulties identifying the Dryinid wasp found parasitizing X-disease vectors leaving variability in DNA amplification unexplained, we have not yet developed a species-specific primer for the wasp and have developed a more general Dryinid primer. We describe the development of each of these primers in more detail below.

Primers for natural enemies

Tomosvaryella lepidipes (parasitic fly)

The primer set developed for the parasitic fly is highly sensitive to small traces of fly DNA in leafhopper samples, allowing accurate identification, even in early life stages of the fly within a leafhopper. Primer development, optimization, and molecular validation (cloning) for *Tomosvaryella lepidipes* DNA were conducted using fly specimens (3 life stages) reared from field-collected *C. m. reductus* (previously only recorded on *C. geminatus*). Taxonomic identification of this parasitic fly was further confirmed by Jeff Skevington (the world's *Tomosvaryella* taxonomy expert at the Canadian National Insect Collection) using the genomic data generated during primer validation and examining leafhopper-reared adult fly samples.

Hirsutella sp. (fungus)

Initial sequencing data were generated by amplifying the internal transcribed spacer (ITS) of 17 clones derived from 1 field-collected *C. geminatus*, and 4 field-collected *C. m. reductus*. In addition, we have now conducted genetic sequencing of the *Hirsutella* sp. collected from *C. geminatus* and *C. m. reductus*, and they appear genetically distinct (72.8% pairwise identity in the 117 base pair ITS region). This further supports vertical transmission, which would allow better divergence between host species than if fungi were frequently transmitted between individuals that would reduce isolation between species. Sequence results obtained from both leafhopper species are similar to NCBI records of multiple *Hirsutella* species. To better understand *Hirsutella* sp. prevalence amongst leafhoppers that are X-disease vectors and non-vectors, we decided to design a robust primer set for identification at the *Hirsutella* genus level by generating a consensus sequence that included 150 *Hirsutella* records (including species described as entomopathogens) as the template. Records matching initial *Hirsutella* species found in field-collected leafhoppers were included in the design. Primers have been validated via molecular cloning. Recently, we obtained several isolates of different *Hirsutella* species in the USDA ARS Collection of Entomopathogenic Fungal Cultures (ARSEF) from a range of insects around the world and compared our sequence data to those obtained from the isolates. This comparison identified relatively high percentages of bases/residues that are identical among the *Hirsutella* species found in *Colladonus* leafhoppers from Central Washington and the cultured isolates (3/5). Out of the 5 isolates we obtained from the USDA ARSEF, *H. kirchneri* [*Abacarus hystrix* (Acari: Eriophyidae) on *Lolium perenne*, 1981, United Kingdom, ≈ 66.8% pairwise nucleotide identity], *H. lecanicola* [*Parthenolecanium corni* (Hemiptera: Coccidae), 2007, USA: Vermont, ≈ 87.7% pairwise nucleotide identity], and *H. repens*

[*Nilaparvata lugens* (Hemiptera: Delphacidae), Aug 1986, Republic of Korea, ≈ 89.5% pairwise nucleotide identity] showed over 65% similarity in their sequences.

Given the high prevalence of *Hirsutella* spp. in *Colladonus* spp., we conducted a series of experiments to determine whether the *Hirsutella* is maternally transmitted to eggs. We first took two steps to evaluate the possibility that high *Hirsutella* sp. prevalence was not just due to contamination. We first tested its presence in adults and nymphs and detected it in nearly 100% of the samples. We evaluated two types of controls (non-*Colladonus* sp. leafhoppers and negative controls) and did not observe amplification. In addition, we extracted and tested leafhoppers in a separate lab (in USDA ARS Wapato, compared to WSU Wenatchee) and replicated the findings. Next, to see if *Hirsutella* is acquired through plant feeding, we put *C. m. reductus* on potted plant species (alfalfa, clover, and ryegrass). Plants were tested for the presence of *Hirsutella* before any leafhoppers were placed on the plants, no *Hirsutella* was found on the plants. Leafhoppers were placed on the plants and observed for 10 days to encourage feeding and oviposition events. We tried to identify any leafhopper eggs using a blue light, but none were found. After the 10-day period, plants and leafhoppers were collected, and DNA was extracted. *Hirsutella* was found in the leafhoppers but not on the leaves, suggesting that this strain of *Hirsutella* is not transmitted or acquired by leafhopper feeding. We were able to detect the *Hirsutella* sp. fungus in leaves with eggs present, but not in leaves without eggs. Finally, to isolate just the eggs, we dissected female *C. m. reductus* and removed eggs from ovarioles and tested the dissected eggs for the presence of *Hirsutella* sp. fungus. Again, we observed PCR amplification ITS of *Hirsutella* sp., suggesting that *Hirsutella* sp. is maternally inherited. We also tested heads and bodies and found *Hirsutella* in the bodies, and relatively low concentrations within the heads.

Gonatopus sp. (wasp)

We initially developed a series of possible working primers to identify the presence of *Gonatopus* sp. wasp parasitism on *Colladonus* leafhoppers. These primers were developed using field-collected adult Dryinid wasps as well as excised thylacium (larval sac) of parasitized field-collected leafhoppers (nymphs and adults, not necessarily *Colladonus* sp.). After testing primers developed from genetic sequences from a subset of wasps, we noticed that the designed primers did not amplify for all samples, suggesting there may be more than one species of Dryinid wasp parasitizing *Colladonus* leafhoppers in our collection sites. Initially, we worked to identify the Dryinid wasps parasitizing the *Colladonus* spp. leafhoppers, as well as field-collected Dryinids, but despite efforts working with expert taxonomists, this has proven difficult. Therefore, we have recently generated reliable sequences for the development of working primers for the parasitic wasp that allow for future taxonomic identification. This was achieved by submitting *Colladonus*-reared adult Dryinid wasps to a non-destructive DNA extraction protocol. The protocol involves extracting hemolymph from the sample, leaving the specimen externally intact for morphological identification. Initially, we focused our analysis on the cytochrome c oxidase subunit 1 (CO1) gene of the samples, and molecular cloning to verify sequences, but did not obtain reliable reads, potentially due to the presence of host CO1 genes. Therefore, we developed analyses that focus on a relatively conserved region of the 28S genetic sequence. Due to the gene sequence's conserved nature, 28S sequences obtained from *Colladonus*-reared Dryinids matched records that correspond to diverse or unidentified *Gonatopus* species, based on divergence of other genes. Wasp specimens from which sequence data were originally obtained were identified to genera and potential species by experts at USDA ARS in Wapato. Preliminary taxonomic identification suggests that the *Colladonus*-reared Dryinid wasps key out to *Gonatopus brooksi*, and the molecular data obtained from the samples were similar molecularly as database records for *G. ashmeadi* (although it is not a definitive match, it could potentially indicate a close relationship between *G. brooksi*, *G. clavipes*, and *G. ashmeadi*). Unfortunately, there is no identical sequence data available to cross-reference this

yet (NCBI/BOLD). There is a high possibility that molecular data for this Dryinid wasp found parasitizing *Colladonus* leafhoppers in WA has not yet been submitted to any genetic database.

Given that we have not been able to acquire a definitive identification from a taxonomist, we switched to developing a primer that more widely amplifies for Dryinid wasps. This way, we can quickly screen leafhoppers for parasitoids, and if necessary, we can sequence the DNA from the leafhoppers from which Dryinids were detected to get a specific genetic sequence for the parasitoid.

Multiplex assay

We initially waited to develop the multiplex to determine whether it is worthwhile to evaluate *Hirsutella* sp. presence in all samples, as well as get an identification of the *Gonatopus* sp. wasp we have documented parasitizing X-disease vectors. Now that *Hirsutella* sp. appears to be a symbiont rather than a pathogen, we did not screen it for presence on all samples and do not need to include it in the multiplex. Instead, we developed a multiplex using primers for *T. lepidipes*, Dryinid species, and X-disease phytoplasma. In addition, there is a need to quickly identify *Colladonus* nymphs and screen them for parasitism. Because leafhopper nymph ID is challenging, we developed and validated a set of primers to differentiate between *C. m. reductus* and *C. geminatus* leafhopper nymphs using sequence data from field-collected leafhopper adult samples from 2020. Thus, the multiplex assay that can identify nymphs of the two *Colladonus* species and test for the X-disease phytoplasma, as well as screen for parasitoids.

Other findings: When evaluating the leafhopper eggs for *Hirsutella* sp. presence, we tested our leafhopper-specific primers on the eggs within host leaves and found that we can use the species-specific primers to correctly identify leafhopper eggs within host plants. This may prove useful in future experiments to better document X-disease vector host plant use in future lab and field experiments.

Objective 2) Evaluate seasonal trends in X-disease vector parasitism at two stone fruit orchards (one cherry, one nectarine), where each of the parasites have been identified.

We have tested the detection efficacy of the parasitic fly primer set using a sample set composed of 33 *C. geminatus* and 6 *C. m. montanus* collected in 2020 (May, June, August, and October) from various weeds in Washington tree fruit orchards. Of those collected 12.12% (4/33) *C. geminatus* and 0/6 *C. m. reductus* tested positive for the parasitic fly, *Tomosvaryella lepidipes*.

During the 2021 field season, we sampled 76 times from May 13th through November 5th across 3 sites in Wapato and Wenatchee, and 3 cultivars (apple, cherry, nectarine). Samples were collected from each site's ground cover using a modified leaf blower as an insect vacuum. We collected >2,000 leafhoppers during that season, which included 540 adult *C. m. reductus* and 23 *C. geminatus*. In addition, our multiplex revealed 122 *C. m. reductus* nymphs and 23 *C. geminatus* nymphs. In preliminary evaluation of sample dates in May, June and September 197 out of 199 adults were positive for *Hirsutella* sp., and therefore, *Hirsutella* was not included in the multiplex. Across the dates and sites, the multiplex revealed 0.5% and 0% Dryinid parasitism of *C. m. reductus* adults and nymphs, respectively and 4.6% and 2.5% *T. lepidipes* parasitism of *C. m. reductus* adults and nymphs, respectively. Across the dates and sites, the multiplex revealed no Dryinid parasitism of *C. geminatus* adults or nymphs and 0% and 8.3% *T. lepidipes* parasitism of *C. geminatus* adults and nymphs, respectively. We only found one leafhopper that tested positive for X-disease: a *C. m. reductus* adult from Wapato.

Objective 3) Conduct a geographical survey of the stone fruit growing region in Washington to evaluate the geographical distribution of three X-disease vector natural enemies: fungal pathogen, a parasitic wasp, and a parasitic fly.

During the 2022 field season, we sampled 85 times from May 5th through October 20th across 47 sites, 19 locations, and 4 cultivars (apple, cherry, nectarine, pear). Samples were collected from each

site's ground cover using a modified leaf blower/vac modified to be used as an insect vacuum. We have sorted out 1,118 *C. m. reductus* adults, and 143 *C. geminatus* adults, in addition to 1,128 unidentified nymphs. Analysis of the abundances of these key X-disease vectors suggests that *C. m. reductus* counts were most abundant in the southern growing regions of Washington, with a statistically significantly negative relationship between counts and latitude (GLM: $z = -2.648$, $P = 0.0081$). This finding was despite collections taking place in northern orchards with very little X-disease vector management being conducted. In contrast, abundance of *C. geminatus*, which appears to prefer more natural vegetation, was not associated with latitude, but was significantly positively increased with elevation across the Washington growing region (GLM: $z = 2.26$, $P = 0.0238$).

We have conducted molecular analysis on the leafhoppers from the 2022 season, including 788 *C. m. reductus* adults, and 199 verified *C. m. reductus* nymphs, along with 86 *C. geminatus* adults and 49 *C. geminatus* verified nymphs. We are still working on analyzing the data, and have taken a conservative approach for what we classified a "positive" test result. Using this approach, across all sites and dates we observed 1.3% and 2.5% Dryinid parasitism of *C. m. reductus* adults and nymphs, respectively, and 4.1% and 1.5% *T. lepidipes* parasitism of *C. m. reductus* adults and nymphs, respectively. For *C. geminatus*, we observed 1.1% and 0% Dryinid parasitism of adults and nymphs, respectively, and 2% and 6% *T. lepidipes* parasitism of adults and nymphs, respectively. Given that many nymphs were not old enough to be parasitized and many parasitized adults likely died before being collected, these estimates are presumably underestimates of the parasitism rates. We observed parasitism by *T. lepidipes* most commonly in the Yakima and Tri Cities regions, and by Dryinids most commonly in the region between Royal City and Orondo. We observed 8 X-disease-positive *C. m. reductus* adults and 2 X-disease-positive *C. geminatus* (both adults), though two of the *C. m. reductus* had high Ct values (>37) suggesting low phytoplasma titers.

- Publications, Handouts, Other Text & Web Products:

We developed business card-sized laminated handouts of key X-disease vectors and non-vectors for comparisons. Stakeholders love these handouts because they fit nicely in their wallets. We have distributed over 50 to stakeholders at field days. In addition, we have written entries in English and Spanish describing this project for the BioAg blog: <https://csanr.wsu.edu/tracking-beneficial-parasites-cherry-production/>

- Outreach & Education Activities:

PI Northfield presented the preliminary research presented here to the Washington Tree Fruit Research Commission's Cherry Research Review in 2022 and 2023 as part of an update on Little Cherry Disease Taskforce research. PI Northfield co-organized a session at the annual meeting of the Washington State Tree Fruit Association in 2022, and the research described here was summarized in the session by Adrian Marshall, a postdoctoral research fellow at WSU. The graduate student funded by this project, Cesar Reyes Corral also presented this research in a Spanish-speaking session at WSU Tree Fruit Days, and Adrian Marshall presented included research described here in his English-speaking session at WSU Stone Fruit Day. PI Northfield presented the research described here at 6 grower meetings in 2023, and Cesar Reyes Corral presented at a WSU field day in the Yakima region in 2023.

Impacts

- Short-Term: Development of a key research tool to optimize research on biological control agents
- Intermediate-Term: Identification of key biological control agents to account for in integrated pest management of X-disease vectors.

- Long-Term: Development of a sustainable X-disease management program that incorporates natural enemies of X-disease vectors.

Additional funding applied for/secured:

\$2 million/year congressional appropriations for collaborative research between WSU and USDA ARS Wapato on X-disease and Little cherry disease pathosystems.

\$295,376 USDA NIFA Crop Protection Pest Management grant combining models and fieldwork to better predict X-disease prevalence in plants and vectors.

Graduate students funded:

Cesar Reyes Corral, PhD candidate, Entomology, Tree Fruit Research and Extension

Recommendations for future research:

In future studies, we recommend evaluating the role that *Hirsutella* has on *Colladonus* sp. biology and any potential effects on vector competency, as well as the impacts of the two parasitoids we have documented attacking X-disease vectors.