"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less."

- Marie Skłodowska-Curie

Surveillance for Red Pine Scale, *Matsucoccus matsumurae* (Kuwana, 1905) (Hemiptera: Matsucoccidae), Using Environmental DNA

by

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Abstract

The red pine scale (RPS; *Matsucoccus matsumurae*) (Kuwana, 1905) is a highly invasive insect native to Asia that is rapidly killing red pine (*Pinus resinosa*) trees across the northeastern United States. As of 2025, it has never been detected in Canada. However, infestations have alarmingly been detected within 50 miles of southern New Brunswick. To support timely surveillance, I tested three trap types for visual detection and specimen collection. I then developed and validated a species-specific qPCR assay to detect RPS DNA from targeted environmental DNA (eDNA) samples collected from conventional insect traps. The canopy pitfall trap collected the highest number of RPS individuals, making it the most effective option. I developed the assay primers and probe *in silico* to target a 242-base pair region of the mitochondrial cytochrome oxidase I (COI) gene. *In vitro* testing showed that the assay reliably differentiated RPS from its main sympatric congener in the study area, the white pine bast scale (WPS; *Matsucoccus macrocicatrices*), demonstrating high specificity. Using a gBlock-based standard curve, I determined the limit of detection to be one copy per reaction. I validated the assay by molecular testing of eDNA samples collected from two RPS-positive sites in the United States and one RPS-negative control site in Canada. Known RPS infestations consistently corresponded with positive eDNA detections, confirming assay accuracy. Additional testing is required to broaden the assay's applicability in other geographic regions. The assay successfully detects RPS populations at the tested sites, and the sequenced COI barcodes for both RPS and WPS provide valuable genetic reference data. Overall, this study provides the foundation for an effective and reliable approach for RPS detection and establishes a baseline for future surveillance and management efforts.

Dedication

This thesis is dedicated to my husband, Dylan T. Stone, my parents, Bandana Jha and Dheeraj Mishra, and my younger brother, Harsh Mishra. Each of you have supported me in various ways, whether by encouraging me through difficult times, reminding me to keep moving forward, or listening to my ideas and concerns with patience and understanding.

Ever since my childhood in Nepal, my parents have prioritized my education and made countless sacrifices to provide me with opportunities and a comfortable life, a privilege I will never take for granted. My brother, though seven years younger than me, has grown into one of my closest friends and shares my deep love for animals, especially our beloved dogs, Rex and Peanut. I will forever be thankful to Dylan for being a loving partner who shares my passion for science and the pursuit of knowledge. His strong work ethic and unwavering principles continue to inspire me.

This thesis is a reminder that no accomplishment is ever achieved alone. It reflects the love, patience, sacrifices, and unwavering belief of those closest to me. For that, I will be forever grateful.

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negative. This dual visualization approach illustrates the sensitivity of molecular detection patterns to replicate variability while retaining biologically meaningful amplification signals. 56

Chapter 1: General Introduction

General Overview

Invasive species pose some of the most severe and persistent threats to forest ecosystems worldwide, altering ecological processes, reducing biodiversity, and causing significant economic losses (Aukema *et al*., 2011; Bradshaw *et al*., 2016; Liebhold *et al*., 2012). The increased globalization of trade and transport has accelerated the unintentional movement of non-native insects, pathogens, and plants across biogeographical boundaries (Hulme, 2009). In North America, invasive forest insects alone are estimated to cost over US $2 billion annually in tree mortality, management, and loss of ecosystem services (Aukema *et al.*, 2011). These introductions are often irreversible, and their impacts can persist for decades after establishment (Lovett *et al.*, 2016). Consequently, early detection and rapid response are critical components of modern forest biosecurity programs (Blackburn *et al.*, 2011; Lodge *et al.*, 2016).

The red pine scale (RPS; *Matsucoccus matsumurae*) (Kuwana, 1905) is one such invasive forest pest currently threatening the ecological integrity of northeastern North America. Native to East Asia, RPS was first reported in Connecticut in the mid-20th century (Bean & Godwin, 1955) and has since spread across several U.S. states including Massachusetts, New Hampshire, and Maine (Whitcomb, 2014; Choi *et al*., 2019). The insect infests red pine (*Pinus resinosa*), a species of high ecological and economic value in Canadian forests, contributing to timber production, habitat structure, and ecosystem resilience (Anand *et al*., 2013; Franklin *et al*., 2002). Infestation by RPS results in chlorotic needle discoloration due to physiological disruption of the tree's vascular and photosynthetic systems, canopy thinning, and ultimately widespread mortality (McClure, 1977; Plumb, 1950).

Conventional methods for detecting scale insects rely primarily on visual identification and morphological examination under microscopy (Doane, 1959). However, these approaches are labour-intensive, require specialized expertise, and often fail to detect low-density infestations due to the insects' cryptic habits and minute size (Brockerhoff *et al.*, 2006; Liebhold & Tobin, 2008). By the time visible symptoms appear, infestations are typically well established, limiting the effectiveness of management interventions (Haack *et al.*, 2014). Therefore, developing sensitive, species-specific detection tools capable of identifying incipient populations during early colonization is essential for minimizing spread and ecological impact.

Recent advances in molecular ecology have introduced environmental DNA (eDNA) as a powerful tool for species detection and biodiversity monitoring (Ruppert *et al.*, 2019; Taberlet *et al.*, 2018). eDNA refers to genetic material shed into the environment through biological processes such as excretion, molting, or decomposition, which can then be recovered from soil, water, or air samples (Thomsen & Willerslev, 2015; Barnes & Turner, 2016). Quantitative polymerase chain reaction (qPCR)-based eDNA assays offer exceptional sensitivity and specificity, enabling the detection of minute quantities of target DNA without the need for direct organism capture (Bohmann *et al.*, 2014; Bustin *et al.*, 2009). In terrestrial and forested systems, eDNA has successfully detected invasive and cryptic insects including the emerald ash borer (EAB; *Agrilus planipennis*) (Kyle *et al*., 2024) and hemlock woolly adelgid (HWA; *Adelges tsugae*) (Kirtane *et al.*, 2022). These applications demonstrate eDNA's potential for transforming biosecurity surveillance in forestry.

In addition to its sensitivity, eDNA sampling provides several logistical and ethical advantages. It is non-destructive, scalable, and compatible with conventional

trapping programs (Harper *et al.*, 2019). Furthermore, eDNA analysis can detect species that are rare, elusive, minute in size, or present only as eggs or larvae, making it particularly useful for timely detection of invasive pests, potentially even before visible symptoms occur (Deiner *et al.*, 2017). However, successful application depends on careful assay design, validation against closely related species, and an understanding of environmental variables influencing DNA persistence and detection probability (Goldberg *et al.*, 2016; Mächler *et al.*, 2019).

With recent detections of RPS reported only 50 miles south of the New Brunswick border (Marino, 2023), there is growing concern that it will soon establish in Canadian forests. Monitoring and surveillance alone will not stop the establishment of RPS, but it will serve as a timely warning for mitigating its impacts. To address the need for an effective surveillance tool for RPS, I developed a qPCR assay targeting a unique region of the mitochondrial cytochrome oxidase I (COI) gene. This locus is widely used for DNA barcoding due to its interspecific variability and availability in public databases (Hebert *et al.*, 2003). By comparing RPS and its sympatric congener, the white pine bast scale (WPS, *Matsucoccus macrocicatrices*), I designed a primer-probe set specific to RPS to prevent cross-amplification. The assay was optimized and validated both *in vitro* and in field samples collected from two infested sites in New Hampshire and Maine, USA and an uninfested site in NB, Canada.

Beyond assay development, I also evaluated the relative efficacy of three trap types for collecting RPS individuals and recoverable eDNA from canopy pitfall traps, 3D-printed traps, and green sticky traps. Green sticky traps were only used for visual assessment because they were used successfully to collect hemlock woolly adelgid, another minute hemipteran (Fidgen *et al.*, 2020), but are not compatible with molecular

eDNA extraction methods. Trap performance was assessed based on capture rates, temporal variation, and compatibility with molecular processing. These experiments allowed me to identify the most effective trap type to deploy for eDNA sampling for pest surveillance.

The overarching goal of my research was to establish a sensitive, specific, and field-validated molecular detection tool for timely monitoring of RPS in North American forests. Specifically, my objectives were to:

1. Determine the most efficient trap design for collecting eDNA and RPS individuals in the field;
2. Develop and validate a qPCR assay specific to RPS, ensuring high sensitivity and species discrimination; and
3. Evaluate the assay in field-collected eDNA samples to confirm its reliability under natural conditions.

Bibliography

Anand, M., Leithead, M., Silva, L. C. R., Wagner, C., Ashiq, M. W., Cecile, J., Drobyshev, I., Bergeron, Y., Das, A., & Bulger, C. (2013). The scientific value of the largest remaining old-growth red pine forests in North America. *Biodiversity and Conservation, 22*(8), 1847-1861. https://doi.org/10.1007/s10531-013-0497-1

Aukema, J. E., Leung, B., Kovacs, K., Chivers, C., Britton, K. O., Englin, J., Frankel, S. J., Haight, R. G., Holmes, T. P., Liebhold, A. M., McCullough, D. G., & Von Holle, B. (2011). Economic impacts of non-native forest insects in the continental United States. *PLoS ONE*, *6*(9), e24587. https://doi.org/10.1371/journal.pone.0024587

Barnes, M. A., & Turner, C. R. (2016). The ecology of environmental DNA and implications for conservation genetics. *Conservation Genetics, 17*(1), 1-17. https://doi.org/10.1007/s10592-015-0775-4

Bean, J. L., & Godwin, P. A. (1955). Description and bionomics of a new red pine scale, *Matsucoccus matsumurae*. *Forest Science, 1*(2), 164-176. https://doi.org/10.1093/forestscience/1.2.164

Blackburn, T. M., Pyšek, P., Bacher, S., Carlton, J. T., Duncan, R. P., Jarošík, V., Wilson, J. R. U., & Richardson, D. M. (2011). A proposed unified framework for biological invasions. *Trends in Ecology & Evolution*, *26*(7), 333-339. https://doi.org/10.1016/j.tree.2011.03.023

Bohmann, K., Evans, A., Gilbert, M. T. P., Carvalho, G. R., Creer, S., Knapp, M., Yu, D. W., & de Bruyn, M. (2014). Environmental DNA for wildlife biology and biodiversity monitoring. *Trends in Ecology & Evolution*, *29*(6), 358-367. https://doi.org/10.1016/j.tree.2014.04.003

Bradshaw, C. J. A., Leroy, B., Bellard, C., Roiz, D., Albert, C., Fournier, A., Barbet-Massin, M., Salles, J.-M., Simard, F., & Courchamp, F. (2016). Massive yet grossly underestimated global costs of invasive insects. *Nature Communications*, *7*(1). https://doi.org/10.1038/ncomms12986

Brockerhoff, E. G., Liebhold, A. M., & Haack, R. A. (2006). The ecology of forest insect invasions and advances in their management. *Canadian Journal of Forest Research, 40*(2), 258-272. https://doi.org/10.1139/x06-013

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., & Wittwer, C. T. (2009). The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry*, *55*(4), 611-622. https://doi.org/10.1373/clinchem.2008.112797

Canadian Food Inspection Agency. (2026). *Plant health survey report for 2025-2026*. https://inspection.canada.ca/en/plant-health/invasive-pests-and-plants/plant-pest-surveillance/2025-2026-report#a1b

Choi, J., Cha, D., Kim, D.S., & Lee, S. (2019). Review of Japanese pine bast scale, *Matsucoccus matsumurae* (Kuwana) (Coccomorpha: Matsucoccidae), occurring on japanese black pine (*Pinus thunbergii* Parl.) and Japanese red pine (*P. densiflora* Siebold & Zucc.) from Korea. *Forests*, *10*(8), 639. https://doi.org/10.3390/f10080639

Deiner, K., Bik, H. M., Mächler, E., Seymour, M., Lacoursière-Roussel, A., Altermatt, F., Creer, S., Bista, I., Lodge, D. M., Vere, N., Pfrender, M. E., & Bernatchez, L. (2017). Environmental DNA metabarcoding: Transforming how we survey

animal and plant communities. *Molecular Ecology*, *26*(21), 5872-5895. https://doi.org/10.1111/mec.14350

Doane, C. C. (1959) The Red pine scale. *Circular 207, The Connecticut - Agricultural Experiment Station, New Haven*. https://portal.ct.gov/-/media/CAES/DOCUMENTS/Publications/Circulars/C207pdf.pdf

Fidgen, J. G., Whitmore, M. C., Studens, K. D., Macquarrie, C. J. K., & Turgeon, J. J. (2020). Sticky traps as an early detection tool for crawlers of *Adelges tsugae* (Hemiptera: Adelgidae). *Journal of Economic Entomology*, *113*(1), 496-503. https://doi.org/10.1093/jee/toz257

Franklin, J. F., Spies, T. A., Pelt, R. V., Carey, A. B., Thornburgh, D. A., Berg, D. R., Lindenmayer, D. B., Harmon, M. E., Keeton, W. S., Shaw, D. C., Bible, K., & Chen, J. (2002). Disturbances and structural development of natural forest ecosystems with silvicultural implications, using Douglas-fir forests as an example. *Forest Ecology and Management*, *155*(1-3), 399-423. https://doi.org/10.1016/s0378-1127(01)00575-8

Goldberg, C. S., Turner, C. R., Deiner, K., Klymus, K. E., Thomsen, P. F., Murphy, M. A., Spear, S. F., McKee, A., Oyler-McCance, S. J., Cornman, R. S., Laramie, M. B., Mahon, A. R., Lance, R. F., Pilliod, D. S., Strickler, K. M., Waits, L. P., Fremier, A. K., Takahara, T., Herder, J. E., & Taberlet, P. (2016). Critical considerations for the application of environmental DNA methods to detect aquatic species. *Methods in Ecology and Evolution*, *7*(11), 1299-1307. https://doi.org/10.1111/2041-210x.12595

Haack, R. A., Britton, K. O., Brockerhoff, E. G., Cavey, J. F., Garrett, L. J., Kimberley, M., Lowenstein, F., Nuding, A., Olson, L. J., Turner, J., & Vasilaky, K. N. (2014).

Effectiveness of the international phytosanitary standard ISPM no. 15 on reducing wood borer infestation rates in wood packaging material entering the United States. *PLoS ONE*, *9*(5), e96611. https://doi.org/10.1371/journal.pone.0096611

Harper, L. R., Buxton, A. S., Rees, H. C., Bruce, K., Brys, R., Halfmaerten, D., Read, D. S., Watson, H. V., Sayer, C. D., Jones, E. P., Priestley, V., Mächler, E., Múrria, C., Garcés-Pastor, S., Medupin, C., Burgess, K., Benson, G., Boonham, N., Griffiths, R. A., & Lawson Handley, L. (2019). Prospects and challenges of environmental DNA (eDNA) monitoring in freshwater ponds. *Hydrobiologia*, *826*(1), 25-41. https://doi.org/10.1007/s10750-018-3750-5

Hebert, P. D. N., Cywinska, A., Ball, S. L., & deWaard, J. R. (2003). Biological identifications through DNA barcodes. *Proceedings. Biological Sciences*, *270*(1512), 313-321. https://doi.org/10.1098/rspb.2002.2218

Hulme, P. E. (2009). Trade, transport and trouble: Managing invasive species pathways in an era of globalization. *Journal of Applied Ecology, 46*(1), 10-18. https://doi.org/10.1111/j.1365-2664.2008.01600.x

Kirtane, A., Dietschler, N. J., Bittner, T. D., Lefebvre, M. B., Celis, S., O'Connor, K., Havill, N., & Whitmore, M. C. (2022). Sensitive environmental DNA (eDNA) methods to detect hemlock woolly adelgid and its biological control predators Leucotaraxis silver flies and a Laricobius beetle. *Environmental DNA*, *4*(5), 1136-1149. https://doi.org/10.1002/edn3.317

Kyle, K. E., Allen, M. C., Siegert, N. W., Grabosky, J., & Lockwood, J. L. (2024). Design of an eDNA sampling method for detection of an endophagous forest pest. *NeoBiota, 95,* 149-164. https://doi.org/10.3897/neobiota.95.118267

Liebhold, A. M., & Tobin, P. C. (2008). Population ecology of insect invasions and their management. *Annual Review of Entomology*, *53*(1), 387-408. https://doi.org/10.1146/annurev.ento.52.110405.091401

Liebhold, A. M., Brockerhoff, E. G., Garrett, L. J., Parke, J. L., & Britton, K. O. (2012). Live plant imports: the major pathway for forest insect and pathogen invasions of the US. *Frontiers in Ecology and the Environment*, *10*(3), 135-143. https://doi.org/10.1890/110198

Lodge, D. M., Simonin, P. W., Burgiel, S. W., Keller, R. P., Bossenbroek, J. M., Jerde, C. L., Kramer, A. M., & Rutherford, E. S. (2016). Risk analysis and bioeconomics of invasive species to inform policy and management. *Annual Review of Environment and Resources*, *41*(1), 453-488. https://doi.org/10.1146/annurev-environ-110615-085532

Lovett, G. M., Weiss, M., Liebhold, A. M., Holmes, T. P., Leung, B., Lambert, K. F., Orwig, D. A., Campbell, F. T., Rosenthal, J., McCullough, D. G., Wildova, R., Ayres, M. P., Canham, C. D., Foster, D. R., LaDeau, S. L., & Weldy, T. (2016). Nonnative forest insects and pathogens in the United States: Impacts and policy options. *Ecological Applications*, *26*(5), 1437-1455. https://doi.org/10.1890/15-1176

Mächler, E., Little, C. J., Wüthrich, R., Alther, R., Fronhofer, E. A., Gounand, I., Harvey, E., Hürlemann, S., Walser, J., & Altermatt, F. (2019). Assessing different components of diversity across a river network using eDNA. *Environmental DNA*, *1*(3), 290-301. https://doi.org/10.1002/edn3.33

Marino, P. (2023). A tree-destroying insect is in N.H. and Canada fears we'll accidentally send it north. *Concord Monitor*. https://www.concordmonitor.com/2023/12/26/pine-invasive-insect-52938046/

McClure, M. S. (1977). Population dynamics of the red pine scale, *Matsucoccus resinosae* (Homoptera: Margarodidae): the influence of resinosis. *Environmental Entomology*, *6*(6), 789-795. https://doi.org/10.1093/ee/6.6.789

Plumb, George H. 1950. A new and serious insect pest of red pine. *Connecticut Agricultural Experiment Station*. https://portal.ct.gov/-/media/caes/documents/publications/special_circulars/scfebruary1950pdf.pdf?rev=ffebcf5277a946bcb2623ca5e726b571&hash=CD2FFB31B2CC2DEB956D195323862232

Ruppert, K. M., Kline, R. J., & Rahman, M. S. (2019). Past, present, and future perspectives of environmental DNA (eDNA) metabarcoding: A systematic review in methods, monitoring, and applications of global eDNA. *Global Ecology and Conservation*, *17*. https://doi.org/10.1016/j.gecco.2019.e00547

Taberlet, P., Bonin, A., Zinger, L., & Coissac, E. (2018). Environmental DNA. In *Oxford Scholarship Online*. Oxford University Press. https://doi.org/10.1093/oso/9780198767220.001.0001

Thomsen, P. F., & Willerslev, E. (2015). Environmental DNA - An emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation*, *183*, 4-18. https://doi.org/10.1016/j.biocon.2014.11.019

Whitcomb, W. E. 2014. "Pest alert: red pine scale". *Maine Department of Agriculture Conservation and Forestry*, Maine Forest Service.

https://www.maine.gov/dacf/mfs/forest_health/documents/condition_reports/2014/cond_2014_5.pdf

Chapter 2: Surveillance Tactics for Red Pine Scale Using Conventional Traps and Environmental DNA

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Abstract

The monitoring and management of invasive insect pests are critical for forest biosecurity. This study targeted the red pine scale (RPS; *Matsucoccus matsumurae*), an invasive pest threatening red pine (*Pinus resinosa*) in northeastern North America, using an environmental DNA (eDNA)-based surveillance approach. I first evaluated three trap types for RPS surveillance through specimen collection and targeted eDNA recovery. Trap performance was assessed by visually identifying and enumerating captured RPS individuals to determine capture efficiency and suitability for eDNA sampling. Then, I developed a species-specific TaqMan quantitative PCR (qPCR) assay for RPS detection. DNA extracted from RPS specimens was used to amplify and sequence a portion of the mitochondrial cytochrome oxidase I (COI) gene. To ensure specificity, the same region was sequenced from several congener white pine bast scales (WPS; *Matsucoccus macrocicatrices*) from multiple locations and genetic clusters. COI barcode comparisons from RPS populations in New Hampshire and Maine, and WPS from diverse geographic origins informed the design of an assay with a primer-probe set that amplified RPS DNA while excluding WPS. The third objective was to validate the assay using field-collected eDNA samples. The canopy pitfall trap yielded higher RPS captures and eDNA recovery. The assay showed high specificity to RPS, an amplification efficiency of 85% ($R^2 = 0.999$), and a detection sensitivity of one copy per reaction. Field testing across Maine, New Hampshire, and New Brunswick showed overall agreement between eDNA detections and visual observations of tree decline, with detection most reliable from early June to mid-July. The assay needs to be tested further with RPS and congeners from other regions to broaden its geographic applicability. Overall, this study establishes a molecular foundation for RPS surveillance and monitoring.

Introduction

History of RPS

RPS was first identified in North America in 1946 by the Division of Insect Identification (Plumb, 1950) as *Matsucoccus resinosae* (Bean & Godwin, 1955). It was discovered when red pine (*Pinus resinosa*) mortality was observed in western Connecticut (Duda, 1961). As the infestation was extensive, the pest would have existed on the host undetected for several years. RPS is originally from Japan, where the native species is named *M. matsumurae* or the Japanese pine bast scale (McClure, 1977). It is speculated that the insects arrived on non-native pines planted on the grounds of the World's Fair in New York in 1939 and were subsequently moved to Connecticut.

The same insect was also identified in the 1920s in Korea as *M. thunbergianae*, or the black pine bast scale. *Matsucoccus matsumurae, M. resinosae* and *M. thunbergianae* were synonymised due to lack of differences in morphology, genetics and sex pheromone cross-attractiveness (Choi *et al*., 2019; Park *et al*., 1986). According to Bean & Godwin (1955), RPS had begun spreading throughout New England after already being discovered on pine trees south of its native range. Anderson *et al*., (1976) estimated that the pest was moving up north at a rate of 1-3 km/year in the 1970s. More recently (2012), infestations have been reported in New Hampshire (Marino, 2023) and in Maine in 2014 (Whitcomb, 2014).

RPS Life Cycle and Morphology

RPS undergoes two generations per year (Figure 1). All its life stages occur on the branches and stems of the tree. Adult females can lay around 250 eggs in woolly ovisacs, and these eggs take about 15 days to hatch (Bragard *et al.,* 2024). The summer generation begins with eggs laid around May, reaching the adult stage in August. The

fall generation's eggs are laid around late August with adults emerging the following spring (McClure, 1977).

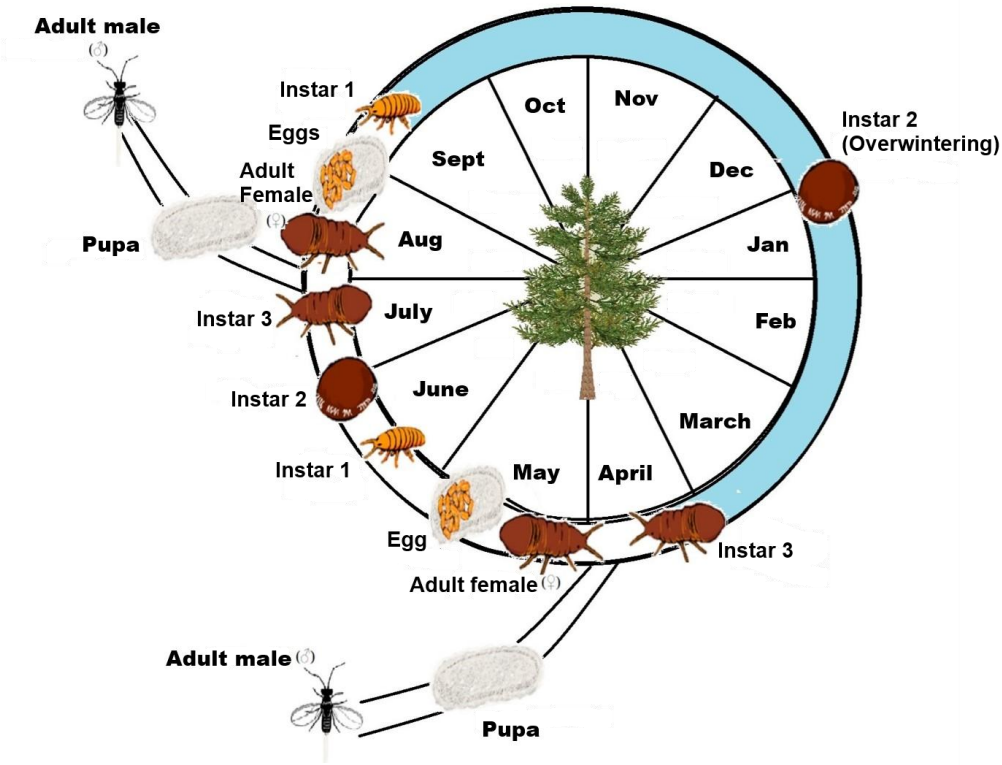


Figure 1. Red pine scale (RPS; *Matsucoccus matsumurae*) life cycle with timeline. Life stages of RPS are illustrated, with sexual dimorphism apparent after the second instar phase. This figure shows the two generations of RPS in one calendar year. (Adapted from Choi *et al*., 2019; https://creativecommons.org/licenses/by/4.0/)

After hatching, first instar individuals are mobile crawlers that move to bark crevices and become 'settled' cysts after starting to feed on the tree phloem with their stylets (Bragard *et al.,* 2024; Doane, 1959) (Figure 2A). The second instar, or intermediate stage cysts, are sessile. According to Anderson *et al*. (1976) and Liu *et al*. (2014), the overwintering generation range occurs during late April to early July, whereas the summer generation occurs between mid-July to October.

After the second instar phase, the males and females display sexual dimorphism (Choi *et al.*, 2019; Bragard *et al.*, 2022). The male pre-adult prepupa spins a loose

spindle-like cocoon (Anderson *et al.*, 1976). The pre-adult females on the other hand do not pupate and resemble a larger version of the second instar (Guinn, 2014). Males emerge from the cocoons as true winged adults, although there is no evidence suggesting they can fly (Cain, 2014). Adult females do not develop wings but do regain their legs, allowing them to move to an appropriate oviposition site (Plumb, 1950). Males locate females with the help of a sex pheromone (Doane, 1966). The females can ultimately lay up to 250 small yellow eggs in woolly ovisacs (Doane, 1959).

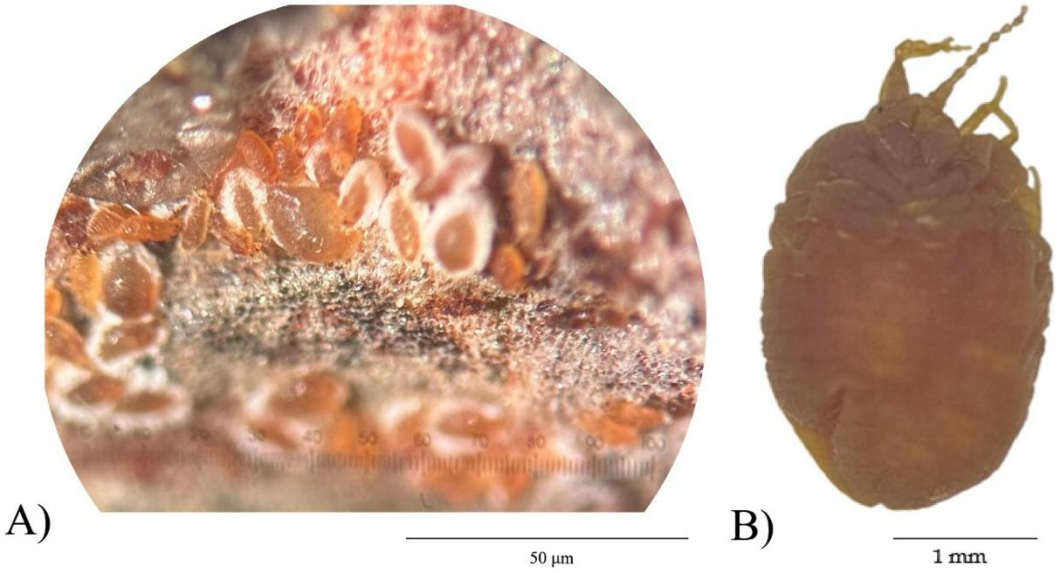


Figure 2. Images taken through microscope lens of red pine scale (RPS, *Matsucoccus matsumurae*) specimens collected from Strafford, New Hampshire. A) Several first instar 'crawlers' and second instar sessile cysts present on branch sample. Scale bar: 50 µm B) Dorsal view of adult female specimen with antennae, legs and dorsal folds visible. Scale bar: 1 mm.

The visual identification of insects in the family *Matsucoccidae* is typically based on the morphology of adult females due to their distinct features (Mech *et al.*, 2013; Kondo & Watson, 2022) (Figure 2B). As described by Bean & Godwin (1955), the insect's first stage larvae are long ovoids, measuring about 410µm long and 197µm wide. The intermediate larval stage is 2.0 mm long, elliptical in shape, and amber yellow

in colour. If male, the intermediate larva is smaller and less spherical than females, with the derm turning darker with age (Duda, 1961). The wingless, pear-shaped female third instars are about 2.5 to 4.0 mm in length and appear brownish red in colour with coarse wrinkles. At the male pupa stage, the primordia of most organs of an adult male are recognizable, including antennae, compound eyes, legs, wings, and the stylet (Bean & Godwin, 1955).

The adult female can have an average length of 4.25 mm and width of 2.5 mm. It is brownish red in colour and lacks wings. Its mouthparts are now vestigial, and its small eyes are located at the centre of a flattened cone. The antennae have nine segments, with two sensory setae on each of the four terminal segments. They are described as having a distinct imbricate-reticulate pattern on the dorsal side (Bean & Godwin 1955). The genitals are usually hidden by dermal folds and located at the base of a shallow vertical cleft at the apex of the abdomen (Duda, 1961).

Host importance and risks

Red pine trees are important ecologically, culturally and economically due to their wood, ability to provide shelter and their use in traditional medicine (Anand *et al*., 2013). Apart from the production of wood, the red pine is considered an attractive tree due to its colourful bark and is therefore sold commercially (Moore, 2002). Prior to European settlement, 7 million acres of red pine forests existed in the Lake States of the United States alone. Now, there are only 1 million acres due to high timber harvesting, land conversion and fire suppression (Gilmore & Palik, 2006). The infestation of red pine by RPS in Canada would mean further threat to local red pine populations and the trees being replaced by other more resilient species (Solomon & Bartlein, 1992). Red

pine stands in Atlantic Canada located near the United States border (Little Jr., 1971) are likely to be at the highest risk as RPS range expands northward.

Although there are some rules in place regulating the import of non-processed wood into Canada (Canadian Food Inspection Agency, 2024), the lack of management tools specifically for RPS puts the host population at risk. As the infested area continues to expand and spread within approximately 50 miles of the New Brunswick border, it poses an increasing threat to red pine in Canada (Marino, 2023). Given the importance of the host, development of RPS monitoring methods is important for raising awareness and notifying associated organizations.

Effect of RPS on red pine

In most cases of RPS infestation, host tree necrosis occurs (Bean & Godwin, 1955). Upon hatching, the first instar crawlers of RPS insert their feeding stylets beneath the bark flakes into the vascular tissue. These insects remain immobile and fixed in place throughout the feeding period (McClure, 1976). Phloem necrosis occurs from a combination of the insects consuming cell contents and potentially injecting toxins during feeding (Plumb, 1950; McClure, 1977).



Figure 3. Natural yellowing of pine needles compared to brick red colour of red pine (*Pinus resinosa*) needles from a tree infested with red pine scale (RPS, *Matsucoccus matsumurae*). A) Yellow colour of naturally falling red pine needles from Boston, MA in October. (Image from Hoey, 2023; http://creativecommons.org/licenses/by-sa/4.0/) B) Needles of a fallen red pine tree in August that had RPS in a plantation in Strafford, NH, exhibiting the brick-red colour.

According to Plumb (1950), the impact of RPS on the tree is devastating, with new growth foliage initially turning yellow to olive-green, followed by the same effect on old-growth needles. Ultimately, the affected tree's foliage becomes brick-red upon necrosis (Figure 3B) as opposed to the straw-like yellow colour of tree needles dying naturally (Figure 3A). Reddish-brown lesions can also be seen below the surface of infested bark, potentially due to toxins from RPS causing chlorosis of the foliage (Doane, 1959). Due to damage to the phloem (McClure, 1977), food transport disruption could also cause these lesions. Over time, the infestation can result in the devastating loss of entire red pine stands.

There are currently no chemical sprays available that are effective at eliminating RPS from plantations. Avoiding tree fertilization is recommended to potentially reduce worsening of infestation and applying a 2% horticultural oil once in early June and again in early September can provide some protection (Maine Forest Service, 2023). The most

efficient solution is to harvest the affected trees in the winter months, and the branches and bark should be burned (Bean & Godwin, 1971).

Traditional Surveying

When invasive insects are monitored, the usual method is setting up traps such as pitfall traps, malaise traps, sticky traps or beating sheets on or around the host tree (Montgomery *et al*., 2021; Ryall, 2015). Other traditional methods include branch sampling, crown surveys and tree bark peeling to visually identify the infestation (Griess *et al*., 2013; Yemshanov *et al*., 2019). These traditional surveying methods rely on accurate morphological identification of specimens, which can be time-consuming, difficult when applied to immature life stages, and often require specialized taxonomic training to ensure reliability (Hajibabaei *et al*., 2011), especially when look-alike congeners inhabit the same environment. While traditional surveying techniques have worked for years, alternative methods can be explored to improve detection timing and monitoring effectiveness. In this project, traditional surveying traps serve not only as tools for specimen collection and visual identification, but also as a means of collecting environmental genetic material.

Molecular Method for Timely Diagnosis and Monitoring

An alternative to traditional surveying is the use of molecular methods. Environmental DNA (eDNA), which is genetic material from organisms that can be found in field sites, where insect pests are present, can be used to mitigate limitations of traditional methods (Rishan *et al*., 2023; Ruppert *et al.*, 2019). This material can include both cellular and extracellular components of the insect (Taberlet *et al.*, 2018). Unlike conventional surveying methods, eDNA is less prone to identification error and requires only a small environmental sample (e.g., water, soil, branches) to identify pests (Odero

et al., 2018). This approach is particularly useful for elusive species and may be more cost effective than some traditional survey methods (Abbott *et al.*, 2021; Thomsen & Willerslev, 2015). As scale insects like RPS can be challenging to visually identify because of their size, morphology and the presence of congeners, eDNA is an appropriate tool for detection and monitoring (Ruppert *et al.*, 2019; Kestel *et al.*, 2022).

This molecular approach has been used for detecting other pests. For example, the hemlock woolly adelgid (HWA; *Adelges tsugae*) is a species with many similarities to RPS, as both are elusive invasive insect species that are host-specific, suck tree sap and lead to decline in host health causing mortality (Childs, 2016). They also have two mobile crawler stages that increases the probability that they will fall into the traps. A study by Kirtane *et al.* (2022) shows that when compared to conventional methods that involve visually identifying the insects, eDNA methods show 2.8 to 4.5 times higher positive detection rates with regards to HWA. Given the similarities between RPS and HWA, the same molecular approach can be applicable. While the use of eDNA is a powerful approach, its effectiveness depends on assay sensitivity and specificity and is best complemented by traditional survey methods (Ruppert *et al.*, 2019).

Despite its advantages, the molecular approach is subject to several important limitations. Surveys involving eDNA face some challenges: samples often contain DNA from multiple species, making species-level identification difficult when congeners are present; detection sensitivity decreases when target species are rare, as insufficient DNA may be shed for reliable amplification (Porco *et al.*, 2022). Additionally, eDNA can be diluted or degraded under field conditions, and extraction protocols vary in their ability to remove PCR inhibitors (Taberlet *et al.*, 2018). To improve specificity and reduce false positives, quantitative polymerase chain reaction (qPCR) assays can be designed using

species-specific single nucleotide polymorphisms (SNPs) after sequencing congeners (Nagy *et al.*, 2017).

Barcodes from various genes can be used for species specific detection. However, not all provide enough genetic distance to design an assay to distinguish between close congeners. This is often the case for barcodes originating in the nuclear genome. Nuclear markers such as the 18S and 28S rRNA genes could be used for species identification in this project. However, studies of East Asian RPS populations have specifically found low genetic distance in these markers between the target species and closely related species called congeners in the *Matsucoccus* genus, limiting their utility for species discrimination (Ahmed *et al.,* 2020). One option to overcome this lack of interspecific variation is the use of barcodes originating from the mitochondrial genome which evolves significantly faster due to a higher mutation rate. For this reason, barcodes such as the mitochondrial cytochrome oxidase I (COI) gene can provide more interspecific variation for assay design. This gene is commonly used for assay development because it allows for species-level discrimination, supported by extensive taxonomically verified sequence databases (Deagle *et al*., 2014). A potential downside of using this gene is that it is highly conserved across species (Simpson *et al*., 2023). This can cause an issue with cross-amplification if there is not enough variance between the congeners for primer and probe design. Since the mitochondrial genome is inherited maternally (Kriezis *et al*., 2024), the assay could fail to identify some hybrids depending on the direction of the cross.

Congeners may co-occur with the target species, potentially leading to false-positive identifications through both visual methods and molecular assays. To avoid amplification of non-target DNA, primers and probes must be designed with

multiple base-pair mismatches relative to all non-target species (Adcock *et al*., 2023). One such congener for RPS is the white pine bast scale (WPS, *Matsucoccus macrocicatrices*). WPS exists in southeastern Canada and throughout the USA, including in Maine and New Hampshire (Whitney *et al*., 2019). Prior to this project, COI barcode sequences for WPS were not available in GenBank; generating this barcode provides critical information for RPS assay design. Although not found within our area of interest, there are also other congeners in the genus *Matsucoccus* that occur throughout southern North America, including *M. gallicolis* and *M. alabamae* (Morrison, 1939). In order for the assay to be applicable to these broader geographic regions, sequences for these other congeners would have to be barcoded and tested for specificity.

It is important to note that the molecular methods used in this project do not follow a conventional eDNA approach where solely the genetic material found in the environment is collected rather than the animal itself (Çevik & Çevik, 2025). Due to the minute size of the insect and my objective in this project of capturing the pest itself for additional confirmation, I collected targeted eDNA instead. The definition for targeted eDNA is the selective detection of DNA from a single species or taxon within environmental samples (Abbott *et al*., 2021). In this study, the approach is modified using traps designed to capture whole organisms, thereby increasing the likelihood that collected eDNA contains RPS DNA. The trapping and molecular methods that I employed were intended to recover both indirect sources of RPS DNA (e.g., cells, eggs, and other non-taxonomically identifiable propagules) and direct DNA from multiple RPS life stages. This also enables direct comparison between visual and molecular detection methods.

Assay Evaluation

Once a qPCR assay is designed, its performance is evaluated to ensure specificity, sensitivity and amplification efficiency. Specificity is evaluated by confirming that the assay selectively amplifies the target species without cross-amplification of closely related non-target taxa (MacDonald & Sarre, 2016).

Assay sensitivity can be assessed using a standard curve generated from serial dilutions of target DNA to determine the limit of detection (LoD) and limit of quantification (LoQ) (Little, 2015). The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines define LoD as a concentration that can be detected with reasonable certainty (Bustin *et al*., 2009). The LoQ is the lowest concentration that can be reliably detected and quantified while meeting predefined accuracy and precision criteria and may be equal to or higher than the LoD (Armbruster & Pry, 2008).

When generating the standard curve, rather than relying on DNA extracted from sequenced specimens, synthetic gBlocks can be used as quantified standards (Schmidt et al., 2021; Zhu et al., 2024). Synthetic gBlock Gene Fragments are chemically synthesized, double-stranded DNA fragments manufactured to match a user-defined nucleotide sequence (Integrated DNA Technologies). These fragments provide a precise and stable source of target DNA with known sequence composition and copy number, making them well suited for use as quantitative standards in molecular assays.

Finally, amplification efficiency is assessed across the dilution range to ensure consistent and reliable quantification. A conventional range for assay efficiency is 90-110% (Rogers-Broadway & Karteris, 2015). Together, these performance metrics

establish the analytical reliability of the assay prior to its application to environmental or field-derived samples.

Project Objectives

The first objective of this project involves using traditional insect traps to gather RPS specimens and eDNA. The second objective is to develop and validate an RPS-specific qPCR assay with sensitivity and species-level discrimination. Finally, the third objective is to test the assay using eDNA collected from sites in RPS-positive sites in Maine and New Hampshire (USA) and a negative control site in New Brunswick (Canada).

Methods

Field Sample Collection

To capture different life stages of RPS in 2024, I deployed three existing trap types at a field site in Strafford, New Hampshire. I replaced the traps every 2 weeks for a total of 12 timepoints. A total of 30 traps were installed: 10 replicates of each trap type, arranged in a completely randomized design in a private forest stand (Figure 4A). This site was selected because RPS was positively identified visually and my goals were to see if the traps would collect specimens for use in barcoding and subsequent assay design, while also assessing phenology trends and determining best time for trap deployment. Each replicate was placed under a different tree, selected randomly within the lot. Traps were installed in May, positioned approximately 1.5 meters above ground level. I ensured that all traps were placed roughly 1 meter from the bole of the tree, within the southern quadrant of the tree's understory, and spaced at least 10 meters apart to minimize interference.

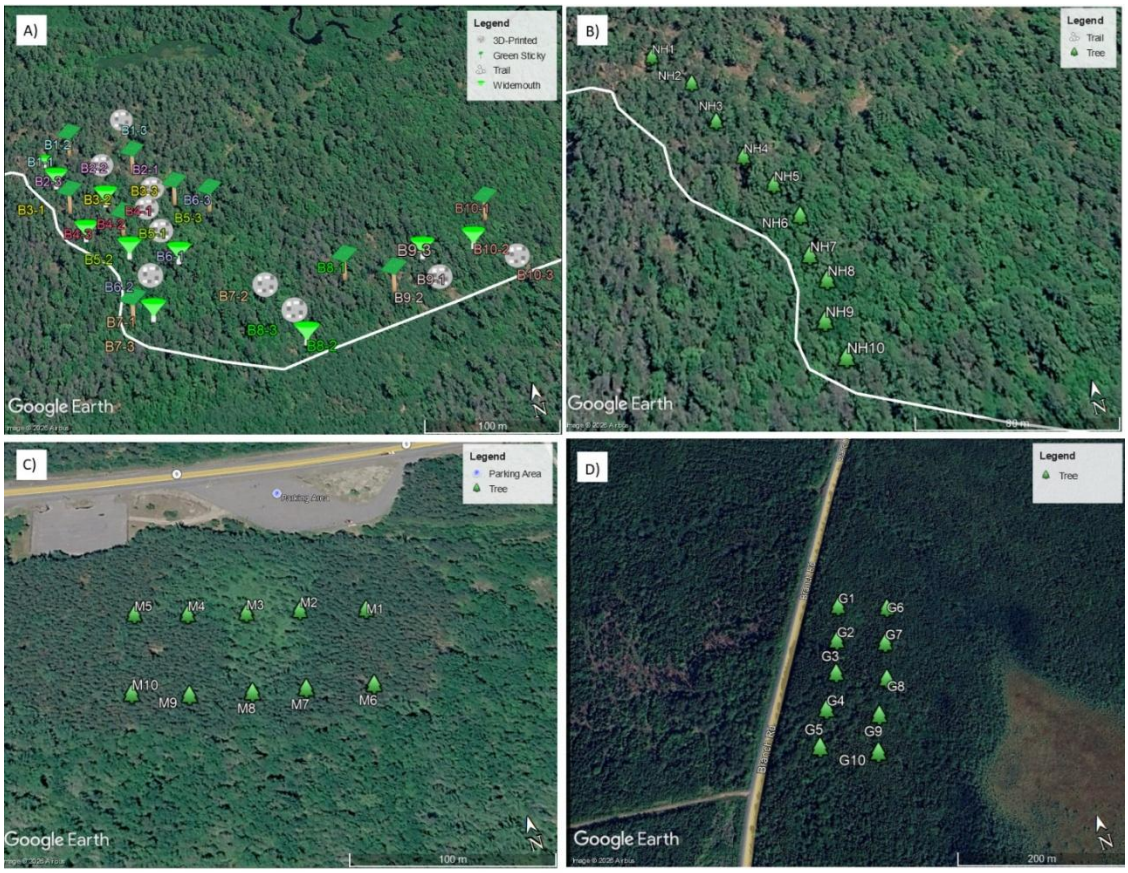


Figure 4. Spatial arrangement of traps at each study site; maps generated using Google Earth Pro. A) Strafford, New Hampshire site in 2024, showing trap placement in a randomized complete design. (B) Strafford, New Hampshire site in 2025, where traps were positioned in random cardinal directions around individual trees. (C) Cherryfield, Maine site in 2025, with traps placed in random cardinal directions surrounding each tree. (D) Gagetown, New Brunswick site in 2025, where traps were similarly deployed beneath trees in random cardinal directions.

In 2025, I deployed the three trap types in three separate sites: Strafford, NH, Cherryfield, ME, and Gagetown, NB. This time, I arranged the three trap types in a random cardinal direction around the trunk of each selected tree. The traps were placed approximately 1 meter from the bole of the tree, like in the 2024 deployment. I selected a total of 10 trees for trap deployment at each site and spaced them approximately evenly across the study area to reduce spatial clustering (Figure 4B, C, D). Exact distances between trees were not uniform, as tree placement was constrained by natural

variation in forest structure and growth patterns. Each tree served as a single deployment unit, with all three trap types installed concurrently to allow direct comparison under similar environmental conditions.

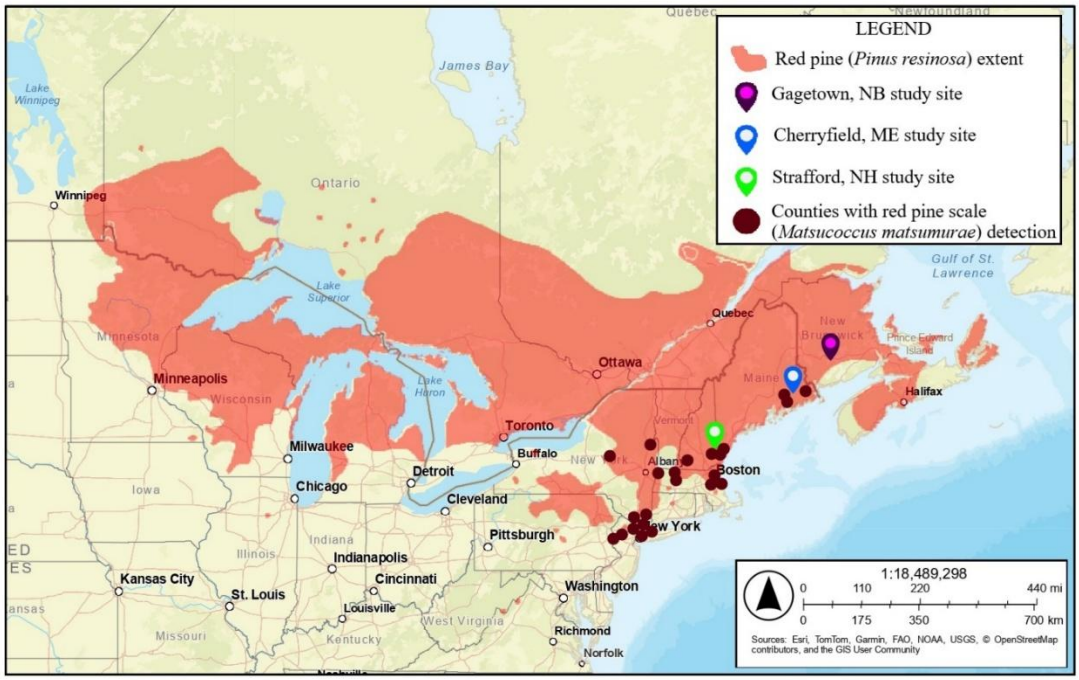


Figure 5. Geographic distribution of red pine scale (RPS; *Matsucoccus matsumurae*) detections and study sites for this project in eastern North America. Dark red circles indicate counties with confirmed RPS presence. Colored markers represent the three study sites: Gagetown, New Brunswick (purple), Cherryfield, Maine (blue) and Strafford, New Hampshire (green). The shaded red area shows the approximate natural range of red pine. (Map created in ArcGIS Online using data from Little (1971) and National Forest Damage Agent Range Maps).

The sites selected were chosen to represent areas within and near the known distribution of RPS across the natural range of red pine (Figure 5). County-level records of confirmed RPS presence were used to contextualize study locations relative to known detections in eastern North America.

The Strafford, NH site was pre-determined to be infested with RPS with the help of Dr. Jeff Garnas from University of New Hampshire, and the property owners, the

Lovejoy family from the Blue Hills Foundation. They granted permission to set up the traps and monitoring efforts for this project. This site consists of diverse tree species including the red pine, eastern white pine, spruce, fir as well as some hardwood trees. In 2024, many of the red pine trees in this site appeared to show symptoms of RPS infestation including reddening of needles and crown dieback. In 2025, red pine decline seemed to have progressed drastically with most trees appearing to have extensive crown thinning and needle loss.

The landowner of the Cherryfield, ME site, BBC Land LLC, granted access to their land in 2025 to collect samples for RPS research. The red pines in this site appeared to also show signs of decline. Although not as drastic as in the Strafford, NH site, discolouration of needles was evident in many of the trees. This site was mostly occupied by red pine trees, along with some hardwood and eastern white pine trees. There were no previous accounts of infestation levels or images of the site available for comparison.

The Gagetown, NB site owned by the Department of National Defence had no signs of RPS infestation in 2025. There were mainly red pines at the site and the trees appeared healthy with no signs of discolouration or crown dieback. As this area is part of an active military base, I ensured to obtain authorization from range control prior to visiting the site.

Preparation and Processing of Traps

To determine the most effective sampling approach and collect specimens of RPS, I assessed the performance of three trap designs: the canopy pitfall trap (funnel trap with an Allison collar by Synergy Semiochemicals; Allison *et al*., 2014), the

3D-printed trap (designed by the Partridge Lab; Dunham *et al*., 2025), and the green sticky trap with Tree Tanglefoot™ (Figure 6).

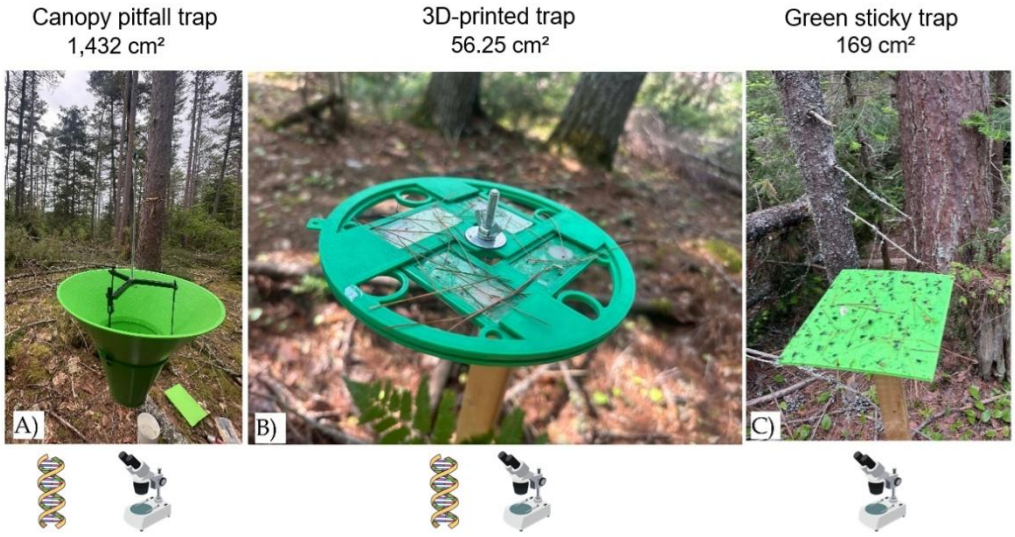


Figure 6. Trap types used for sampling red pine scale (RPS; *Matsucoccus matsumurae*) and their respective capture surface areas. A) Canopy pitfall trap (surface area: 1,432 cm²) with a collection cup positioned on the ground. B) Custom 3D-printed trap (surface area: 56.25 cm²) equipped with four petroleum jelly-coated microscope slides, mounted on a stake. C) Green sticky trap with Tree Tanglefoot™ (surface area: 169 cm²) affixed to a wooden stake adjacent to a red pine (*Pinus resinosa*) tree. Canopy pitfall and 3D-printed traps were processed for visual and molecular identification while the green sticky trap was processed only for visual identification.

The canopy pitfall trap (Figure 6A) has a surface area of 1432 cm². I filled its collection cup with a propylene glycol (PG) solution to trap insects and suspended the trap from a rope stretched between two red pine trees. I used clean, airtight bottles to store the PG solution before field deployment. Prior to deployment, I prepared 250 mL of a 50% PG solution and added a single drop of unscented dish soap to reduce surface tension and facilitate insect submersion. In 2024, brand new traps were deployed. In 2025, I washed and scrubbed the bottles and a combination of used and new traps with dish soap, washed them and soaked them in a 10% bleach solution for at least 10 minutes and dried them under a laminar flow hood. I stored the clean traps and filled

bottles in airtight bags prior to deployment. I also wore nitrile gloves when handling traps at the lab and the site in 2025. I saved a bottle from the preparation stage at the lab in the airtight container and processed the solution to check for eDNA (negative control). I transported the remaining PG solution bottles to the field site in the bags, only opened them at the site and poured the liquid into the trap collection cups. Every two weeks, I transferred the contents of each trap's collection cup back into the initial labelled bottle, transported the samples back to the lab unrefrigerated, and then stored them at approximately 4 °C until processing, within 48 hours.

To process samples from 2024 where eDNA was not tested, I started by rinsing empty mason jars and their lids thoroughly and washing 425 µm and 105 µm sieves. I stacked the larger 425 µm sieve on top of the 105 µm sieve and poured the trap contents through, rinsing any remaining debris from the collection cup and lid onto the sieves. I returned material retained on the 425 µm sieve to the collection cup for later examination, while material captured on the 105 µm sieve was gently rinsed through a clean funnel into a mason jar labeled with the corresponding canopy trap ID. After re-washing the sieves, I prepared a second mason jar labeled with the same ID plus "blank," ran water through the sieves again, and collected any material from the 105 µm sieve into the blank jar. The blank sample was confirmed blank before a new sample was processed. This procedure was repeated for all traps and was done to ensure that there was no cross-contamination between samples. For filtration, I used a thoroughly washed Buchner funnel fitted with wetted filter paper, poured the contents of each mason jar onto the filter, and lightly rinsed the jar and lid before recycling the jar. I rinsed the Buchner funnel with a fine-tip Nalgene bottle during spinning to remove remaining material, then transferred the filter paper to a labeled tray using clean tweezers. I washed

both the funnel and tweezers between processing each blank and subsequent sample to prevent RPS carryover from one collection cup to the next. The contents that I collected from the 425 µm sieve were later examined to look for any larger RPS specimens.

To minimize cross-contamination during the 2025 field season where eDNA was collected, I implemented contamination-control precautions including the use of nitrile gloves and bleaching of work surfaces and tools prior to sample processing. Once collected, trap samples were stored in a -2°C walk-in fridge and were filtered within 48 hours of retrieval. I processed each trap by pouring the contents of the collection cup into a Pall Gelman Magnetic Filter funnel 47 mm filtration apparatus. I placed an 850 µm sieve (2", ERCER Montana, St Paul, USA) at the top of the apparatus to remove large debris, with a 100 µm filter (47mm, Great Lakes Filters, Bloomfield Hills, USA) lined on the filtration base. I examined the contents of the 850 µm sieve under a dissecting microscope, looking for any RPS individuals. I also gently rinsed the collection cup and lid through the top sieve to ensure that all remaining material was transferred to the filtration system. After filtration was complete, I used treated bleached tweezers to remove the nylon filter paper from the filtration base and placed it into a clean, labelled Falcon tube for further molecular processing. I cleaned and decontaminated all the tools between samples to prevent cross-contamination. I also included filtration controls, running only water through the filtration apparatus and saving the resulting filter paper for further processing. This was done at the start before processing the first sample, after half of the samples were processed, and at the end of filtering the samples. I filtered the PG negative controls using the same procedure.

The 3D-printed trap (Figure 6B) consisted of four microscope slides arranged within a 3D printed lightweight frame and each slide was coated with a thin

layer of heated petroleum jelly (Dunham *et al.,*2025; Sanders *et al.,* 2022). Combined, the exposed top surface of these slides had a surface area of 56.25 cm^2 and I mounted each trap on a vertical wooden stake and positioned it beneath red pine trees. The petroleum jelly served as an adhesive surface for intercepting RPS. I prepared the slides by heating up petroleum jelly, dipping the slides approximately 80% into the heated mixture and storing them in clean Falcon tubes prior to field deployment. I used nitrile gloves when preparing these in 2025 to ensure no DNA contamination occurred. I also saved a preparation control, where a set of 4 slides were prepared, sealed in a Falcon tube, and processed to check for eDNA. After field deployment, I placed the slides back into the same labelled Falcon tubes they were originally stored in. In 2024, I examined each slide under a microscope to record RPS presence and life stages. In 2025, I used nitrile gloves and carefully counted RPS on the slides. For each sample, I removed petroleum jelly from each set of four microscope slides using a sterilized metal spatula and transferred the material into a single bead-beating tube containing 0.1- and 2.0-mm beads (Zymo Research BashingBead™ Lysis Tubes (0.1 & 0.2 mm)). I repeated this for all sets of 4 slides from each 3D-printed trap and stored the tubes at -80 °C until eDNA extraction.

The green sticky traps (Figure 6C) had a sticky surface area of 169 cm^2 on one side and were constructed from adhesive-coated green cards mounted on stakes approximately one meter from the bole of red pine trees. This trap has been successful at catching HWA for visual assessment (Fidgen *et al*., 2020). I coated each card with Tree Tanglefoot™, a non-drying adhesive effective for retaining small arthropods. I examined these traps under a dissecting microscope to record RPS presence and developmental stages. As shown in Figure 6, both the canopy pitfall trap and 3D-printed trap are

compatible with eDNA extraction methods, but I did not process the green sticky traps for eDNA extraction due to challenges associated with adhesive removal and DNA recovery.

DNA Extraction from Specimens

To develop the assay, I extracted genomic DNA from multiple adult RPS specimens collected at the Strafford, New Hampshire site using the DNeasy Blood and Tissue Kit (QIAGEN), following the manufacturer's protocol with minor modifications to improve insect tissue lysis. The modifications included freezing the specimens at -80 °C for at least 24 hours to facilitate tissue disruption, then homogenizing in 180 µL of ATL buffer. I added 20 µL of the supplied 600 mAU/mL Proteinase K and incubated the samples at 56 °C overnight with constant mixing (at 30 RPM) on a thermal shaker. After lysis, I followed the kit's instructions, eluting DNA in 100 µL of AE buffer. DNA concentration and purity were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and a Qubit 4 fluorometer (Thermo Fisher Scientific). The congener WPS DNA was extracted by Dr. Thomas Whitney's team and provided to me with measured concentrations. Twenty-three WPS samples were chosen to represent populations from various genetic clusters collected from 17 different geographic locations in the United States (Whitney *et al*., 2019). This allows me to confirm that there were no genetic variants in the primer and probe regions that could potentially cause false positives from WPS amplification.

COI Barcode Amplification and Sanger Sequencing

Once extracted, my goal was to generate a portion of the COI gene barcode for both RPS and WPS. I tested a primer set designed by Ann Bryant (University of Maine Extension Applied Genetics and Genomics Laboratory) and it was successful at

concurrently amplifying a portion of the COI region for both species. The forward primer sequence was 5′-GAATATTTTCTGTTAATCATAAAAATATTGG-3′ and the reverse primer sequence was 5′-TAAACTTCTGGATGACCAAAAAATCA-3′.

Each 25 µL reaction contained 14.15 µL of nuclease-free water, 2.5 µL of 10× PCR reaction buffer, 2.5 µL each of forward and reverse primers (10 µM), 0.75 µL of $MgCl_2$ (25 mM), 0.5 µL of dNTP mix (10 mM), 0.1 µL of Taq polymerase (Thermo Fisher Scientific), and 2 µL of DNA template. The PCR thermal profile consisted of an initial denaturation at 94 °C for 1 min; five cycles of 94 °C for 30 s, 45 °C for 90 s, and 72 °C for 1 min; followed by 45 cycles of 94 °C for 30 s, 55 °C for 90 s, and 72 °C for 1 min; and a final extension at 72 °C for 5 min. The same primer set and parameters were used for both RPS and WPS. I visualized the amplified products using 1% agarose gel electrophoresis and submitted them to Génome Québec (Montréal, QC, Canada) for Sanger sequencing.

I thoroughly checked the resulting chromatograms for each RPS and WPS sample submitted. I visualized, trimmed and edited the sequences using MEGA X (Kumar *et al*., 2018), BioEdit v7.2 (Hall, 1999), and UGENE (Okonechnikov *et al*., 2012). I submitted the edited reads of RPS from New Hampshire and WPS from various locations to the NCBI GenBank database (accession numbers PZ172457-PZ172474, PZ178828-PZ178831 and PZ171127-PZ171145). Four RPS samples from Maine were sequenced by Dr. Thomas Rounsville's team (University of Maine) (accession numbers PZ653566-PZ653569) and I compared them to the samples from New Hampshire. Altogether, there were 22 RPS and 23 WPS specimens analyzed (Table A1).

qPCR Assay Design and Optimization

For qPCR assay development, I aligned COI sequences from RPS and WPS to identify SNP variants between species that are fixed within each species. For assay sensitivity, I used BLAST searches against the NCBI GenBank database and checked for the absence of *in silico* cross-amplification with WPS sequences. I then selected a region with a high-density SNPs for probe design and ensured that both the forward and reverse primer binding sites also contained multiple mismatches, while also taking into consideration the recommendation that the amplicon be as short as possible to maximize efficiency. Using aligned consensus sequences, I designed RPS-specific qPCR primers producing a 242-bp amplicon, along with a hydrolysis probe labeled with fluorescein amidite (FAM) and a non-fluorescent quencher-minor groove binder (NFQ-MGB). The forward primer contained three species-specific SNPs, the reverse primer contained two, and the probe contained five. I also used the COI barcode information to design a 250-base pair long synthetic gBlock (Table 1). The melting temperature (TM), which dictates the correct annealing temperature during the qPCR were 56.6 °C for the forward and reverse primers, and 60.3 °C for the probe.

Table 1. Red pine scale (RPS; *Matsucoccus matsumurae*)-specific qPCR primers and probe sequences and melting temperature (TM), and gBlock sequence. The single nucleotide polymorphisms (SNPs) specific to RPS are underlined within the forward primer, reverse primer and probe and the primers and probe sequences are italicized within the gBlock sequence.

Name	Sequence (5'to 3')	TM
RPS qPCR Forward Primer	CATTATATCCTCCTTTAACACTT	56.6
RPS qPCR Reverse Primer	AGCTAGAAGAATTACTATAATTTTAC	56.6
RPS qPCR Probe	TGGATTATCTTCTTTATTAAGTTCATTAAATA	60.3
RPS gBlock	GCA*CATTATATCCTCCTTTAACACTT*GC CGACAGCAGCCCTAGGGACACAGCA GTTACCGCACTCCATGGCAA*TGGATT ATCTTCTTTATTAAGTTCATTAAATA*CGG GAGTCACCACGACGAATACTACCTT AGAGTTGCGTTGTCTGGGCCTTTTAC CATCATCTACGTGAGCATGGCGTGTG ACTCCCCTCCTATTCATTGCAGCAAG CCCAGCTGT*AGCTAGAAGAATTACTAT AATTTTACTGGG*	N/A

I generated the qPCR standard curve using a gBlock containing the target amplicon sequence. I calculated the gBlock copy number using the molecular weight provided by the manufacturer (Integrated DNA Technologies) and the reported stock concentration. The molecular weight of the gBlock fragment (154,340 g mol^{-1}), derived from fragment length, was used to convert DNA mass to molar quantity. The calculation yielded an estimated concentration of 3,902,000 copies μL^{-1} for the resuspended gBlock stock. To prepare working stocks, I diluted the manufacturer-provided gBlock solution (10 ng μL^{-1}; 1,000,000 fg μL^{-1}) stepwise in nuclease-free water. I generated a 1,000 fg μL^{-1} intermediate stock through sequential ten-fold dilutions by combining 5 µL of gBlock solution with 45 µL of water at each step. From this material, I prepared a 500,000 copies μL^{-1} standard using the $C_1V_1 = C_2V_2$ dilution equation, with a final

volume of 50 µL. I used this concentration so that addition of 2 µL per qPCR reaction corresponded to an input of 1,000,000 copies per reaction. I then prepared a ten-fold serial dilution series ranging from 500,000 to 0.5 copies μL^{-1} by combining 5 µL of each dilution with 45 µL of nuclease-free water. I amplified each standard in triplicate using the following qPCR protocol: 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s to plot cycle threshold (Ct) values against $\log_{10}$ copy number to construct the standard curves. I used the QuantStudio Design and Analysis 2.8.0 software to generate this standard curve automatically from raw data after correcting the maximum fluorescence to 5% (Thermo Fisher Scientific, 2024).

I calculated efficiency (E) from the slope of the standard curve ($E = 10^{(-1/slope)} - 1$) (Ma *et al*., 2020). I determined the LoD based on the lowest dilution where 3/3 technical replicates amplified. To determine the LoQ, I first calculated the coefficient of variation (CV). I set the threshold CV value to 35% (Forootan *et al*., 2017). I therefore defined the LoQ as the lowest copy number per reaction for which the CV among technical replicates was less than 35%. To optimize assay performance, I evaluated multiple qPCR parameters to achieve acceptable amplification efficiency. I tested a range of annealing temperatures and primer-probe volumes to identify conditions that maximized assay efficiency. I also aliquoted primers and probe and stored them at -20 °C to minimize contamination and the repeat of freeze-thaw cycles.

Extraction of eDNA

In 2025, I extracted eDNA from the canopy pitfall traps and 3D-printed traps, deployed in Maine (USA), New Hampshire (USA) and New Brunswick (Canada).

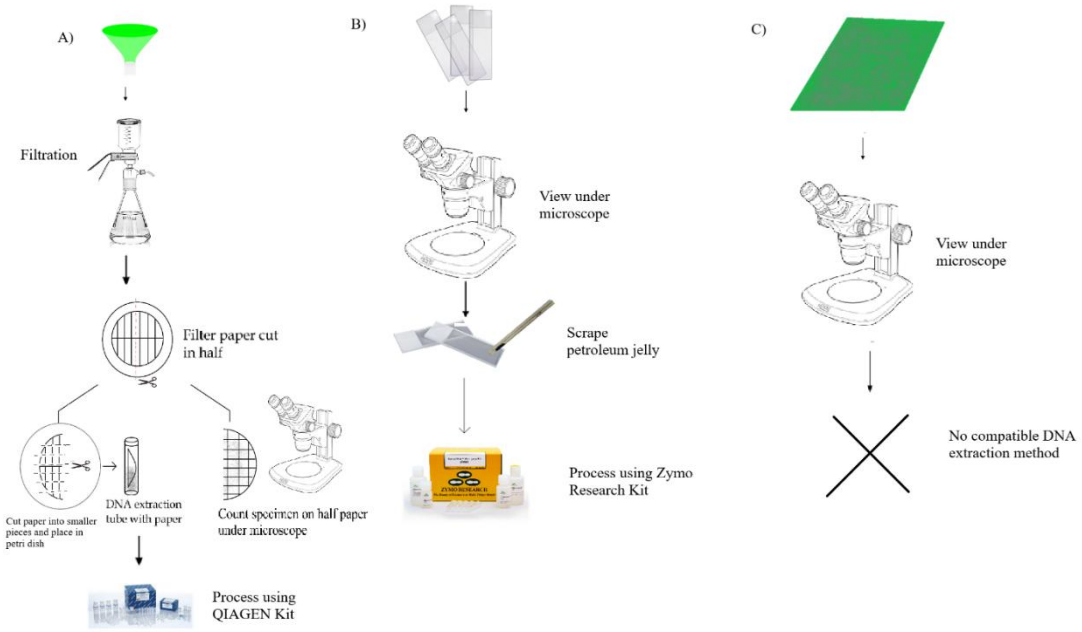


Figure 7. Trap processing flowchart for 2025 showing how the three trap types were processed. A) Contents of canopy pitfall trap is filtered, then filter paper is cut in half, and one half is examined under a dissecting microscope while other half is processed for molecular analysis. B) Four slides of each 3D-printed trap were examined under the microscope and then the petroleum jelly was scraped to be used for eDNA extraction. C) Green sticky trap was viewed under the microscope.

For the canopy pitfall samples (Figure 7A), after filtration, I cut the 100 µm nylon filter paper in half using a sterile scalpel and viewed half the paper under a dissecting microscope to count the number of RPS and make note of life stages. I further cut the other half of the filter paper into smaller sections and stored them in a PowerBead Pro Tube from a DNeasy PowerSoil Pro Kit (QIAGEN). I bead milled the samples using a TissueLyser II bead mill (QIAGEN) at 24 Hz for two 5-minute intervals with intermediate vortexing to maximize cell lysis. I followed the standard PowerSoil Pro Kit protocol, including elution in 100 µL of CD6 buffer. I stored the resulting eDNA at -20 °C until qPCR analysis.

For the 3D-printed traps (Figure 7B), I extracted eDNA using the Quick-DNA™ Tissue/ Insect Microprep Kit (Zymo Research) with bead-beating and column-based purification. After visual assessment, I had stored the scraped petroleum jelly and specimens from the slides in bead-beating tubes. To these tubes, I added 750 µL of Bead-Bashing Buffer and incubated samples at 65 °C with intermittent vortexing at to liquefy the petroleum jelly. I then used a TissueLyser LT compact bead mill (QIAGEN) to bead-beat samples at 50 Hz for 2 min and repeated the heat-and-beating cycle five times to ensure adequate homogenization. Following centrifugation, I transferred the aqueous phase to a Zymo-Spin III-F filter, combined the filtrate with Genomic Lysis Buffer, and bound DNA to a Zymo-Spin 1C column. I washed the column with DNA prewash and g-DNA wash buffers, dried it by centrifugation, and eluted purified DNA in 20 µL of preheated elution buffer. I stored extracted DNA at -80 °C for downstream qPCR analysis. These methods were provided by Dr. Charlyn Partridge (Dunham *et al*., 2025).

I inspected the green sticky traps (Figure 7C) visually under a dissecting microscope, with no further DNA extraction performed.

qPCR Protocol and Detection of RPS from Field Samples

To prepare the reaction mixture, I added 5 µL of 2× QuantiTect PCR NoROX Master Mix (QIAGEN), 1 µL of primer-probe mix, and 3 µL of nuclease-free water to a sterile 1.5 mL conical microcentrifuge tube. I multiplied these amounts by the number of total reactions I was performing to make a master mix solution. I dispensed 9 µL of this mixture into each well of a sterile qPCR plate and added 1 µL of DNA template separately. I performed all the qPCR preparation procedures in a bleached, sterile environment with clean nitrile gloves. When loading the samples into the plate, I always

ensured to include a positive template control (gBlock) and a negative template control (nuclease-free water) in each plate. I vortexed the plate and centrifuged it, ensuring no bubbles were present afterwards. I conducted thermocycling on a QuantStudio3 Real-Time PCR System (Applied Biosystems by Thermo Fisher Scientific) with the same parameters used for the standard curve reactions. I visualized the qPCR results and read the Ct values on the QuantStudio Design and Analysis 2.8.0 software (Thermo Fisher Scientific, 2024).

I analyzed each extracted eDNA sample in triplicates using the developed qPCR assay under the optimized conditions described. I considered samples producing amplification curves with quantification cycle (Cq) values within the assay's LoQ as positive detections. I confirmed the presence of RPS by comparing Cq values to the gBlock-based standard curve. Only samples falling between the standard curve were identified as positive and used for further analysis. As technical replicates were used, I also calculated the standard deviation between the triplicates using the QuantStudio Design and Analysis 2.8.0 software (Thermo Fisher Scientific, 2024).

Variation among qPCR Technical Replicates

To ensure that I retained biologically meaningful data while still maintaining stringent quality control, I evaluated the standard deviation of all qPCR triplicates. Although I initially deemed results with a SD ≤ 0.5 to be positive, I found that some of the samples (20 of the 240) exceeded this threshold despite showing clear, sigmoidal amplification curves and consistent quantification across replicates. To mitigate this issue, I performed reprises: once with the same samples and primer-probe stock, once with fresh primer-probe stock, another time where the samples were diluted 10-fold to reduce potential inhibitors, and once with bovine serum albumin (BSA) (Kreader, 1996).

I also had another individual pipette the samples and still got a high standard deviation in the subset of samples tested. I tried a using TaqMan Environmental Master Mix 2.0 instead of QuantiTect PCR NoROX Master Mix as this was used successfully by Dunham *et al*., (2025) with their eDNA assay for HWA. However, I saw no amplification in the eDNA sample with this master mix, although the gBlock positive control did amplify. In order to minimize false negatives, I therefore determined samples with SD ≤ 1.0 are positives if all technical replicates amplified with clear amplification curves within the LOQ.

Statistical Analysis

Effect of Trap Type, Time Point and Trap Location on insect capture

To evaluate the effects of trap type, sampling timepoint, and trap location on the number of RPS captured per trap at each sampling timepoint, I analyzed data from each year separately using two different generalized linear mixed models (GLMMs) (R v4.3.1). I used negative binomial GLMMs because in count datasets, the variance often exceeds the mean due to excess zeros and heterogeneity among sampling units, resulting in overdispersion (Lee *et al*., 2011). The negative binomial distribution explicitly accommodates this overdispersion by allowing the conditional variance to exceed the mean (Cox, 1983), making it more appropriate for the zero-inflated and highly variable capture data expected in this study. I used the *DHARMa* package to confirm the model accounts for overdispersion, has normal distribution and visualize the zero-inflation (Hartig, 2016).

For both GLMMs, factors included trap type, timepoint, and trap placement location. I did not include site itself as a factor because traps were deployed at only a single site in 2024 (New Hampshire), and sites used in 2025 differed substantially in

infestation status or were not infested, precluding meaningful site-level comparisons. Therefore, I performed 2 separate GLMM analyses for the following: 2024 New Hampshire data and 2025 Maine data. I did not perform GLMM on 2025 New Brunswick and 2025 New Hampshire data as none of the traps captured any RPS. It is also important to mention that I multiplied the total capture by canopy pitfall traps in 2025 by 2 to account for only counting RPS from one half of the filter paper.

In 2024, I specified trap type as a fixed effect in the GLMM to directly test its influence on the abundance of RPS captured per trap sample. I then included trap location as a random factor as it was scattered around the site. For 2025, I included random intercepts in the GLMM for individual traps nested within the tree (location) where they were deployed to account for repeated measurements from the same trap and shared environmental conditions among traps at the same location. I also treated sampling timepoint (week) as a random effect for both data from both years to account for unexplained temporal variation in captures across the sampling period.

Following GLMM model fitting, I calculated estimated marginal means (EMMs) for each trap type using the *emmeans* package (Lenth, 2017) (R v4.3.1). The output represents predicted mean RPS counts caught by each trap type after already accounting for the random effects (sampling timepoint and location). I then performed post-hoc analysis using Tukey-adjusted pairwise comparisons to evaluate differences among trap types. Results were reported as incidence rate ratios (IRRs), which is the ratio of predicted mean RPS counts between trap types, along with 95% confidence intervals and p-values.

RPS Phenology

For each sampling date, I calculated the mean number of RPS for each life stage (first instar, second instar, third instar, adult female, adult male) caught in all ten replicates of the canopy pitfall traps. I chose to focus only on canopy pitfall traps for this analysis as it captured the most specimens and therefore can provide an adequate insight on phenology. I then plotted the mean number of each life stage in a time-series plot (R v4.3.1), visually comparing captures from all life stages in the different traps in 2024 and 2025. This information was important to determine the ideal trap deployment time ranges.

Trap Type Efficiency for eDNA Signal

To evaluate whether increasing count of total RPS abundance in the 3D-printed traps and the canopy pitfall traps predicted the likelihood of the presence of a molecular signal (Status = 1), I fitted a binomial logistic regression model with presence/absence of molecular signal as the response variable and count as the predictor. I performed logistic regression in R using the *glm* function with a binomial error distribution and logit link (Gilmour *et al*., 1985; Tang *et al*., 2012). I assumed a binomial error distribution for the logistic regression because the response variable was binary and used a logit link function to ensure that predicted probabilities remained bounded between 0 and 1 (Auger-Méthé *et al*., 2021). Model fit was visualized in *ggplot2* by plotting the presence/absence (0 or 1) observations with jitter function to reduce overplotting (Dagal *et al*., 2025).

To compare molecular detection success between trap types in the 2025 Maine dataset, I fitted a binomial GLMM with logit link, with detection outcome (0 = negative, 1 = positive) as the response variable and trap type as a fixed effect. Tree and sampling

week were included as random effects to account for repeated sampling at the same locations and temporal variation throughout the study period. I interpreted the model coefficients as odds ratios, which quantify differences in the odds of detecting RPS among the two trap types. The model was fitted in R using the *glmer* function in the *lme4* package with a binomial error distribution and logit link. Model assumptions were evaluated by testing for singularity and overdispersion using the *performance* and *DHARMa* packages.

In order to further visualize the data, I used heat maps and evaluated the agreement between molecular and visual detection methods for individual traps across sampling dates, locations, and trap types. For each trap at each sampling event, I classified detection outcomes into one of four categories: (1) both absent, when neither method detected RPS; (2) both detected, when both methods detected RPS; (3) molecular only, when RPS was detected only by molecular analysis; and (4) visual only, when RPS was detected only by visual inspection. I generated the heat maps in R (v. 4.5.1) using the *ggplot2* package, with sampling date represented on the x-axis and trap number represented on the y-axis. I faceted the data by trap type and location to visualize temporal patterns in agreement between detection methods.

Results

Influence of Trap Type, Sampling Time, and Trap Placement on Insect Capture

In 2024, I captured a total of 6,508 RPS in the New Hampshire site and in 2025, I captured 350 RPS in Maine. I confirmed that none of the traps deployed to the New Hampshire and New Brunswick sites in 2025 captured any RPS that could be identified positively. Therefore, I did not perform any further visual count analysis on these two datasets.

In 2024, trap type significantly influenced the number of RPS captured. The results showed that captures differed among trap types after accounting for variation associated with trap location and sampling date. Random effects estimates indicated substantial temporal variation in RPS captures across sampling weeks (variance = 1.442), whereas variation attributable to trap location was comparatively smaller (variance = 0.283), suggesting that week-to-week changes contributed more to unexplained variability than spatial differences among trap placements. EMMs revealed canopy pitfall traps had higher predicted RPS counts than the other two trap types in both 2024 and 2025 (Table A2). Post hoc pairwise comparisons of model EMMs revealed significant differences in RPS capture rates among all trap types in 2024. I examined the IRRs and corresponding p-values to compare the traps (Table A3). Canopy pitfall traps captured substantially more RPS than both green sticky traps (IRR = 55.31, $p < 0.001$) and 3D-printed traps (IRR = 135, $p < 0.001$). Green sticky traps also captured significantly more RPS than 3D-printed traps (IRR = 2.48, $p < 0.001$).

In the 2025 Maine dataset, I found that trap type significantly influenced RPS capture rates after accounting for repeated measurements from individual traps nested within trees and temporal variation among sampling weeks. Random-effects variance

estimates showed pronounced temporal variation among sampling weeks (variance = 0.997) and moderate variation among trees (variance = 0.276), whereas little additional variation was attributable to differences among traps nested within trees (variance = <0.0001). The predicted RPS capture by canopy pitfall trap was once again greater than the other two trap types. Post-hoc pairwise comparisons of EMMs confirmed that canopy pitfall traps captured more RPS than both 3D-printed traps (IRR = 2.48 $p<0.001$) and green sticky traps (IRR = 1.90, $p = 0.015$), while differences between green sticky traps and 3D-printed traps were not significant ($p = 0.47$), unlike in 2024.

Seasonal Phenology and Optimal Trap Deployment

In 2024, mean capture by canopy pitfall traps was dominated by first-instar individuals throughout the sampling season, with two pronounced peaks (Figure 8A). The first peak occurred in early July, when mean first-instar capture exceeded 200 individuals per trap, followed by a sharp decline by mid-July. A second, broader increase occurred from late August to late September, culminating in a peak of approximately 190 individuals per trap in late September before declining to near zero by late October. Mean capture of adult males, adult females, and later instars remained consistently low throughout the 2024 season, with only minor increases during periods of elevated first-instar capture.

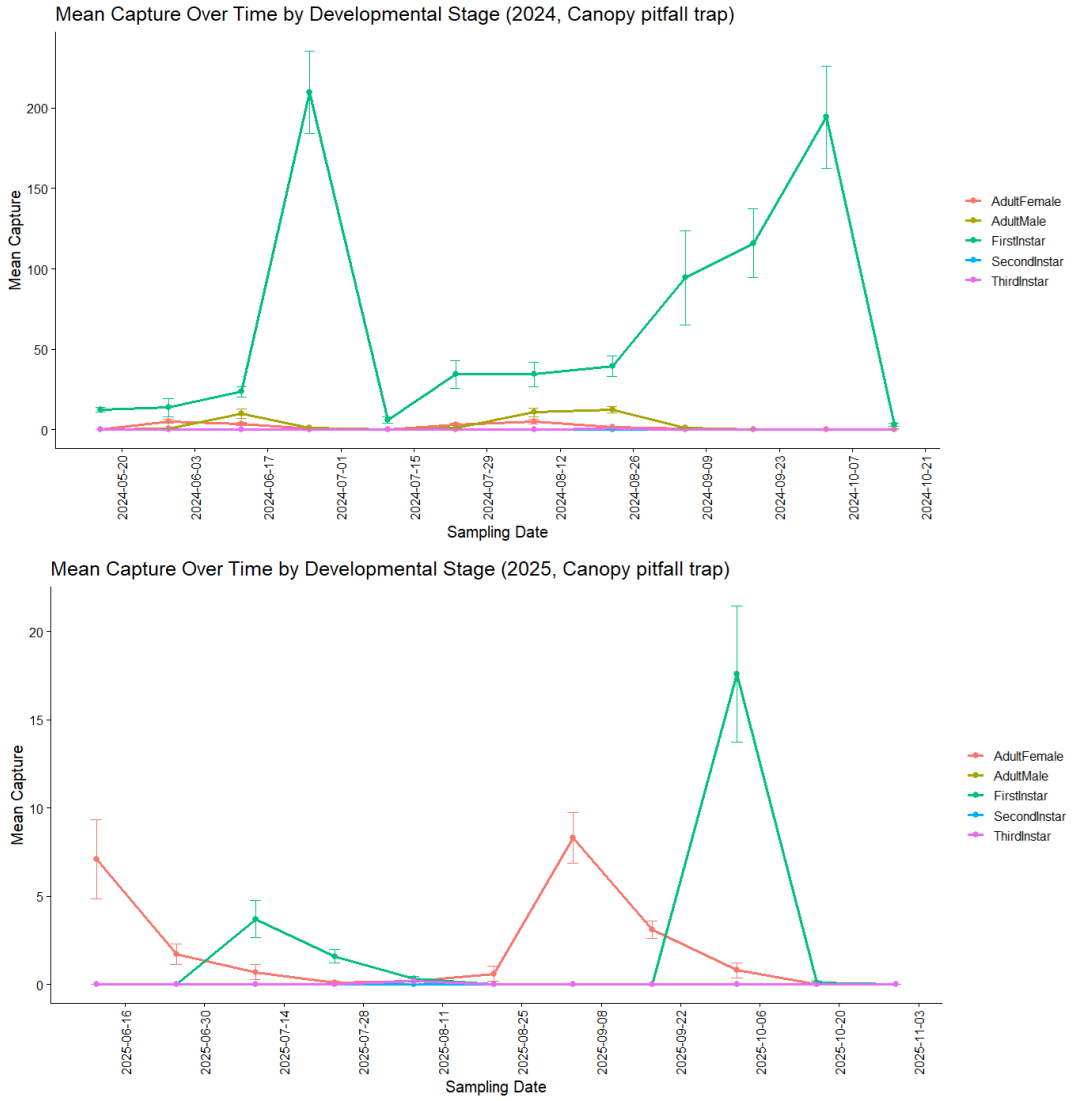


Figure 8. Mean capture of different life stages of red pine scale (RPS; *Matsucoccus matsumurae*) in ten canopy pitfall trap replicates at each sampling date (standard error bars shown) for A) 2024 and B) 2025. Mean captures are shown separately for adult females, adult males, first-instar, second-instar, and third-instar individuals. Sampling dates represent trap collection dates. The trap capture was done in New Hampshire in 2024 and in Maine in 2025.

In 2025, overall mean capture by canopy pitfall traps was substantially lower than in 2024 across all sampling dates (Figure 8B). First-instar individuals again represented most captures. A small early-season increase occurred in mid-July, followed by a single, distinct peak in early October, when mean first-instar capture reached

approximately 18 individuals per trap before declining to zero by mid-October. Adult females showed brief, intermittent increases early in the sampling period and again in late August, but mean captures remained low and short-lived. Adult males and second- and third-instar individuals were rarely captured and did not exhibit clear temporal trends.

qPCR Primer/ Probe Design

To develop a species-specific qPCR assay, I designed multiple candidate primers and probes within the COI barcode region generated from RPS specimens. I examined the sequence alignments of all available RPS barcodes to identify conserved regions suitable for primer binding while maximizing differentiation from non-target species (WPS). I tested several forward primer, reverse primer, and probe combinations experimentally to evaluate amplification efficiency, specificity, and signal strength. Candidate assays targeting different regions of the COI sequence produced variable amplification performance, with some combinations yielding weak or absent amplification.

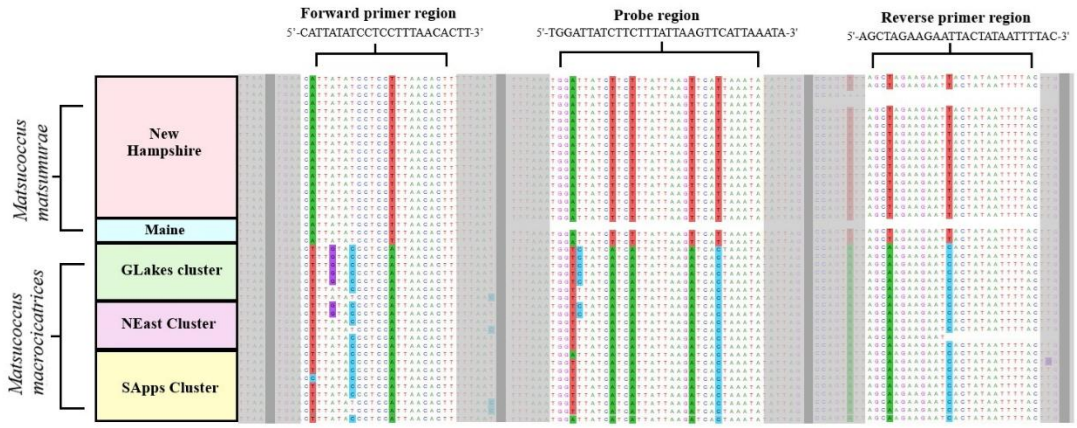


Figure 9. Alignment of COI barcode sequences from red pine scale (RPS; *Matsucoccus matsumurae*) and white pine bast scale (WPS; *Matsucoccus macrocicatrices*) specimens showing the location of the final qPCR assay target regions. The selected forward primer, probe, and reverse primer were designed within conserved regions of the mitochondrial cytochrome oxidase I (COI) gene that were shared across the analyzed specimens and contained several single nucleotide polymorphisms (SNPs) in the WPS barcodes. RPS from two locations – New Hampshire and Maine were analyzed whereas WPS from three genetic clusters – 'GLakes': Michigan and Wisconsin, 'NEast': Maine, New Hampshire, Pennsylvania, Virginia and West Virginia, and 'SApps': Georgia, North Carolina and Tennessee were analyzed.

One primer-probe set targeting a highly conserved region of the RPS COI barcode was identified and selected for subsequent assay development. There were several SNPs present in the assay target regions in WPS from three distinct genetic clusters (Figure 9). The final assay design incorporated primer and probe binding sites that were conserved across all analyzed RPS specimens, providing a robust target for downstream species detection.

eDNA Assay Performance Metrics

Only DNA from RPS samples showed amplification with specific qPCR primers. I further visualized assay specificity using gel electrophoresis, which showed amplification of the expected amplicon size (around 242 bp) for RPS from New Hampshire and the gBlock, no amplification for WPS samples from any of the genetic

clusters or the no-template control was observed (Figure 10A). I also sent these amplicons for Sanger sequencing to make sure the amplified products were what I was expecting. In addition, the BLAST searches I performed against the NCBI GenBank database revealed no evidence of *in silico* cross-amplification with any other congeners that were available on the database.

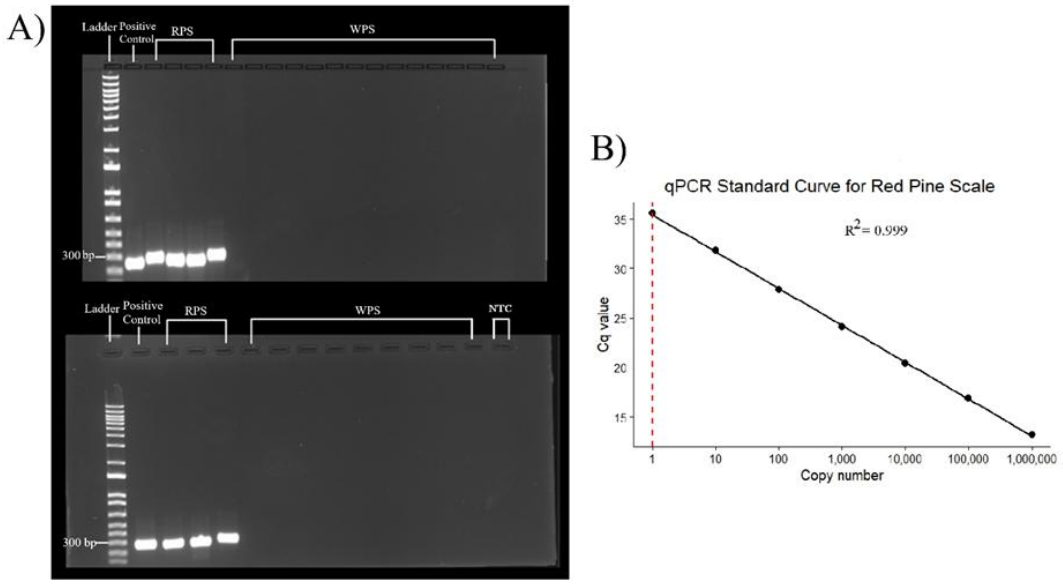


Figure 10. Gel electrophoresis image and standard curve. A) Gel electrophoresis result showing ladder, positive control (gBlock), red pine scale (RPS; *Matsucoccus matsumurae*), white pine scale (WPS; *Matsucoccus macrocicatrices*) and no-template control (NTC). B) Standard curve showing created from linear dilutions of a custom gBlock gene fragment (Integrated DNA Technologies). The red dotted line shows the limit of quantification (LoQ) and limit of detection (LoD). Samples were analyzed in triplicates with $R^2 = 0.999$.

I calculated the amplification efficiency from the slope of the standard curve to be 85% using the formula $E = 10^{(-1/slope)} - 1$, where the slope was -3.729. The standard curve showed a strong linear relationship between Cq values and log-transformed copy number, with an R^2 value of 0.999 (Figure 10B). The LoD was determined to be 1 copy per reaction as at this dilution, the assay consistently detected the gBlock. The LoQ was also identified as 1 copy per reaction based on variability among technical replicates.

The CV at this concentration was 28.9%, below the predefined threshold for CV of 35% (Forootan *et al*., 2017). The LoD and LoQ are indicated by the red dotted line on the standard curve (Figure 10B). Based on the standard curve results, I defined a cycle cutoff of 35.55 cycles, such that reactions with Cq values ≤ 35.55 were considered positive if all three technical replicates amplified.

Visual Counts and Molecular Detection Comparison

I used the assay to amplify target RPS DNA from eDNA samples collected in 2025 from Maine, New Hampshire and New Brunswick. The assay amplified RPS DNA in most environmental samples collected from sites with confirmed species presence, with a few exceptions. No amplification occurred in any of the negative controls and a few positives were detected in samples from the New Hampshire site.

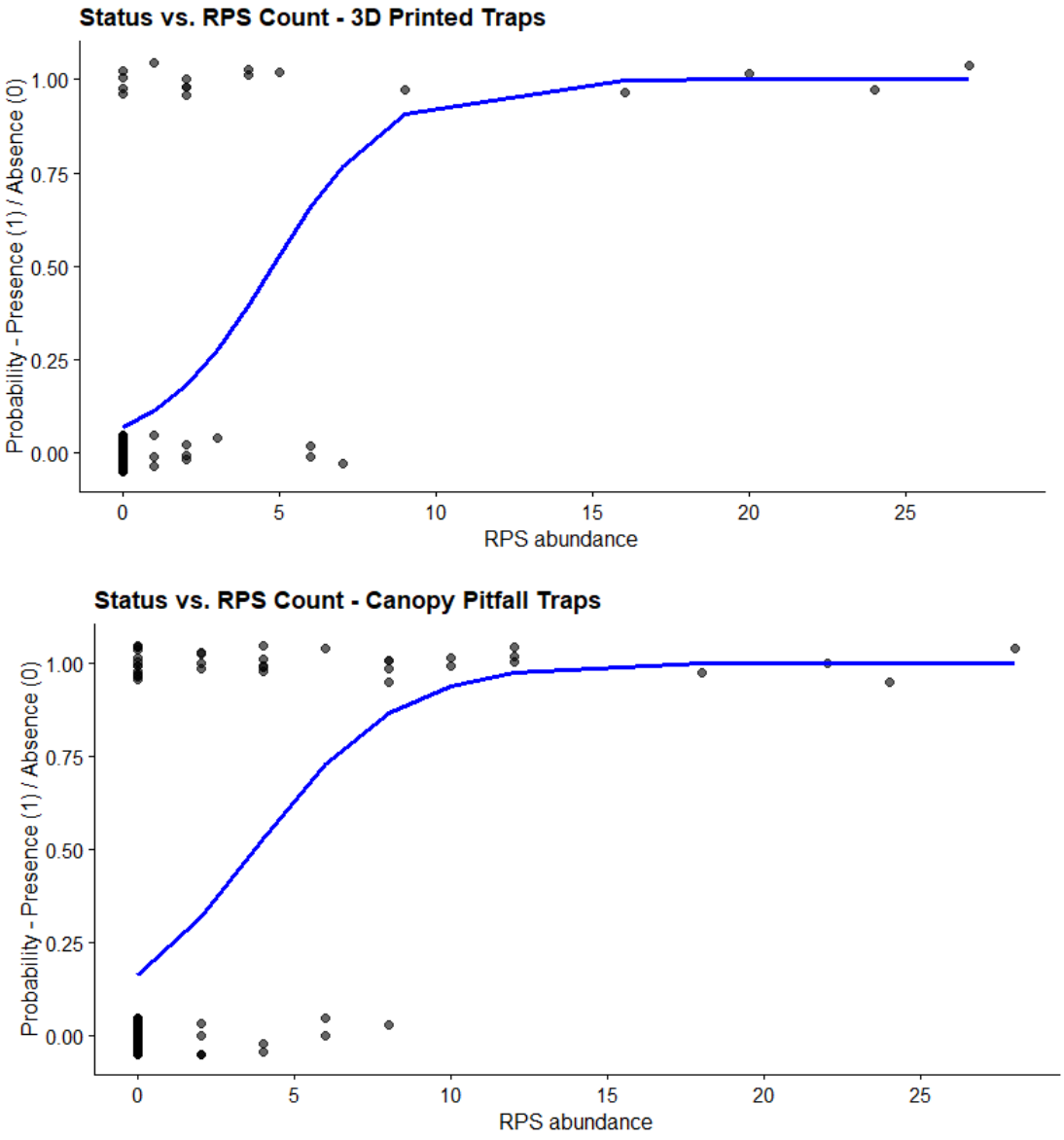


Figure 11. Logistic regression of detection status (probability of 1 = positive, 0 = negative) from eDNA as a function of visual count of red pine scale (RPS; *Matsucoccus matsumurae*) individuals collected in 3D-printed traps and canopy pitfall traps during 2025 in the Maine site. A) 3D-printed trap data from four sampling timepoints, showing a gradual increase in detection probability with higher counts. B) Canopy pitfall trap data from the same period, illustrating a steeper increase in comparison at detecting probability at low counts, followed by an asymptote. Blue curves represent fitted logistic regression models, and black points indicate observed data.

I compared visual counts of RPS across trap types with molecular detection outcomes and found that detection probability increased with increasing RPS abundance for both molecularly compatible trap types, indicating a positive relationship between observed abundance and molecular signal (Figure 11). In the 3D-printed traps, detection probability increased more gradually across the range of observed counts (Figure 11A), whereas canopy pitfall traps showed a slightly sharper increase in detection probability with increasing counts (Figure 11B). There was a greater proportion of positive molecular signals at zero visual count in the canopy pitfall trap data than the 3D-printed traps. Some samples where the variance among the triplicates was high (SD > 1.0) were removed from this analysis as they could not be classified as true positives or negatives.

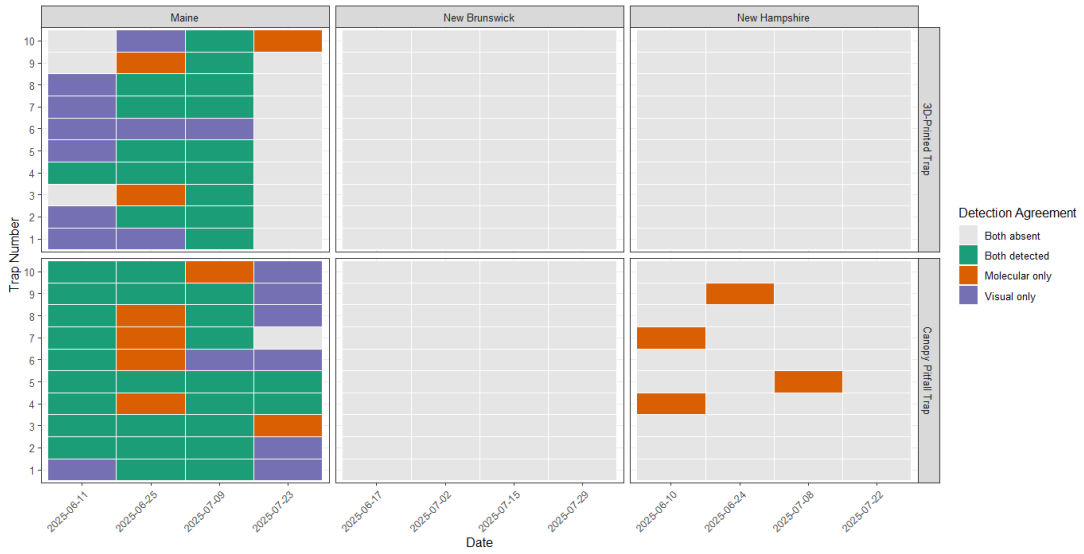


Figure 12. Spatial and temporal patterns of agreement between molecular and visual detection methods for red pine scale (RPS; *Matsucoccus matsumurae*) detection in 3D-printed traps and canopy pitfall traps across Maine, New Brunswick, and New Hampshire during four collection timepoints. The x-axis shows sampling dates, and the y-axis shows individual trap numbers. Grey tiles indicate agreement in absence, green tiles indicate agreement in detection, orange tiles indicate molecular-only detections, and purple tiles indicate visual-only detections. Each tile represents the detection outcome for a single trap at a single sampling event.

Agreement between molecular and visual detection methods varied among trap types and locations (Figure 12). No detections were recorded in New Brunswick for either trap type throughout the sampling period, resulting in complete agreement between methods and exclusively "both absent" observations. Similarly, 3D-printed traps in New Hampshire yielded only non-detections across all sampling dates. Detection activity was concentrated in Maine, where both trap types showed substantially greater agreement in positive detections. For canopy pitfall traps, most traps were classified as "both detected" during June and early July, indicating strong concordance between molecular and visual methods. However, several discrepancies occurred throughout the study period, including both molecular-only and visual-only detections.

For 3D-printed traps in Maine, detections were less consistent early in the season, with several visual-only detections observed on 11 June 2025. Agreement in positive detections increased during late June and early July, particularly on 9 July 2025 when most traps were classified as "both detected." Detection agreement subsequently declined on 23 July 2025, when most traps returned to a "both absent" classification and one molecular-only detection was recorded. In New Hampshire, canopy pitfall traps produced a small number of molecular-only detections across the sampling period, whereas visual detections were not observed. These isolated molecular detections occurred in different traps and sampling events, but the majority of observations remained classified as "both absent."

I also visualized patterns of molecular and visual trap positivity over time to examine how molecular signals reflected visually observed captures under different qPCR replicate-variance thresholds (Figure 13).

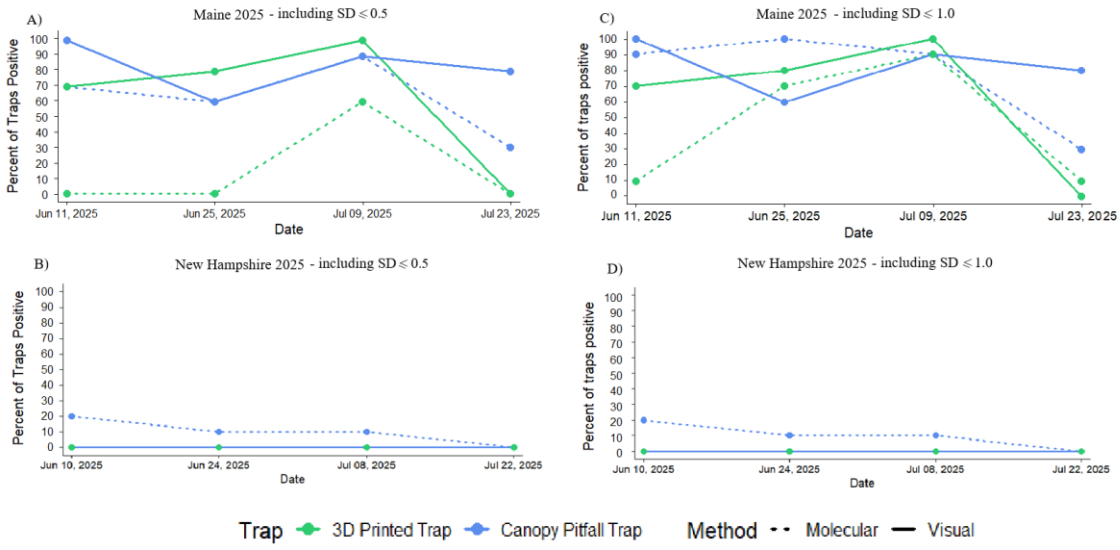


Figure 13. Comparison of visual and molecular trap positivity under differing qPCR replicate variance thresholds. Percent of traps positive over time for Maine (A and C) and New Hampshire (B and D), separated by trap type (3D-printed traps in green; canopy pitfall traps in blue) and detection method (molecular detection shown with dashed lines; visual detection shown with solid lines). Plots on the left (A and B) include only samples meeting a stringent quality control threshold of molecular presence or absence standard deviation (SD) ≤ 0.5 among qPCR technical triplicates. Plots on the right (C and D) include all molecular positives with SD ≤ 1.0, excluding eight samples that consistently exceeded SD > 1.0 and could not be confidently classified as positive or negative. This dual visualization approach illustrates the sensitivity of molecular detection patterns to replicate variability while retaining biologically meaningful amplification signals.

If the standard deviation threshold is set to samples being positive only if SD ≤ 0.5, fewer traps had a positive signal, and this was especially true when eDNA was collected using the 3D printed traps (Figure 13A). When I relaxed the replicate variance threshold to include molecular detections with SD ≤ 1.0, molecular and visual trends became more consistent while revealing some low-level detections that were not always reflected in visual counts (Figure 13C). In contrast, the New Hampshire dataset showed uniformly low positivity across both detection methods and trap types, with molecular detections remaining rare and closely aligned with minimal visual captures throughout the season (Figure 13B, D). Across both regions, canopy pitfall traps consistently

showed higher molecular positivity than 3D-printed traps, while visual detections remained sparse at low abundance levels. Molecular detection captured biologically meaningful signals that generally corresponded with visual abundance while also revealing low-density presence not consistently detected through visual inspection, particularly under less stringent replicate filtering.

When visualizing molecular signal and RPS capture in each tree location (M1-M10), some locations performed better (Figure A1). For example, tree M4 had more traps with a positive molecular signal than any of the other trees (Figure A1A). Some of the trees also had traps that captured over 20 RPS while others consistently captured less than 10 (Figure A1B). Overall, some trap locations were more successful at capturing RPS and producing a positive molecular signal.

Comparison of Molecular Detection Success Between Trap Types

For the 2025 Maine dataset, I found that molecular detection outcome differed significantly between trap types. The binomial GLMM with logit link showed that trap type had a strong effect on the probability of obtaining a positive eDNA detection after accounting for variation among trees and sampling weeks. The odds of detecting eDNA were approximately 18 times higher in canopy pitfall traps than in 3D-printed traps (odds ratio = 18, $z = 3.11$, $p = 0.0019$). Including trap type significantly improved model fit relative to models without trap type. The model showed no evidence of singularity or overdispersion, indicating that model assumptions were met. Detections of RPS were non-existent in the New Hampshire samples in 2025. As a result, there was insufficient data for the model to estimate temporal effects.

Discussion

This project had three objectives: 1) to determine the most effective trap design for simultaneously collecting eDNA and red pine scale individuals; 2) to develop and validate a sensitive and species-specific qPCR assay for red pine scale; and 3) to evaluate the assay using field-collected eDNA.

Trap Type Influences RPS Capture

Across both study years, I found that trap type strongly influenced RPS capture rates, although the degree of differentiation among trap types varied by year. In 2024, canopy pitfall traps captured substantially more RPS than either green sticky traps or 3D-printed traps, even after accounting for temporal and spatial variation. The large effect sizes observed among all trap types indicated that trap design played a major role in capture efficiency under high-abundance conditions. The larger surface area and funnel shape of the canopy pitfall trap may have contributed to its success when compared to the other two trap prototypes. Surface area has shown to influence insect capture probability when traps are deployed in a similar manner (Fidgen *et al.,* 2020; Sanders *et al.,* 2023). More RPS can fall into the canopy pitfall trap because it was larger, and the insects may have difficulties climbing out of the trap due to the funnel shape and the PG solution in the cup. This trap type was also shown to be more successful at trapping HWA compared to other traps with smaller surface areas (Fidgen *et al.*, 2025). The consistently low captures observed in 3D-printed traps suggested that their lower surface area may have limited interception efficiency, particularly during periods of high crawler activity.

In 2025, although canopy pitfall traps again captured more RPS than the other trap types, differences between green sticky traps and 3D-printed traps were not

statistically significant. I attributed this reduced differentiation primarily to the much lower overall population density observed in 2025. In the New Hampshire site, the RPS population dropped significantly from high capture to zero capture from 2024 to 2025. This population decline can be explained by host mortality, as well as overwintering mortality of the sessile nymphs (McClure, 1983). Focusing on the 2024 New Hampshire data and 2025 Maine data, the canopy pitfall trap was beneficial as it captured specimens more often than the other trap types. The lack of detection in New Hampshire in 2025 may suggest that RPS eDNA is not persistent across years when collected through these traps. If this is the case, the detection likely directly mirrors the current year's population levels. However, whether or not eDNA is truly persistent is complicated, and testing different methods of eDNA collection to capture different forms of eDNA (dissolved, intracellular, intraorganellar) can provide insight (Mauvisseau *et al*., 2022).

In 2025, I also noticed that there may have been a peak for crawler count before my first trap deployment timepoint. I planned trap deployment for 2025 based on 2024 phenology data. In 2025, it seems that the crawlers emerged earlier than in 2024. This could be due to the change in weather, precipitation and temperature (McClure, 1989). Missing this peak can affect the comparison between trap types because early-season differences in capture efficiency would not be detected, potentially underestimating total captures and obscuring true trap type performance differences.

Sampling Date Affects RPS Capture

In both study years, I found that sampling date explained more variability in RPS captures than spatial differences among trap locations or trees. Random effect estimates consistently showed that week-to-week variation was a dominant source of unexplained variability, whereas variation attributable to trap placement was comparatively small.

I expected sampling date to have an effect, because some stages of RPS are more likely to fall into the traps than others due to mobility (Doane, 1959) and abundance of RPS changes throughout the season, where different life stages emerge (Choi *et al.,* 2019). I caught more RPS in the traps when the mobile stages were abundant. This included the first instar crawler phase and the adult female phase. These life stages are abundant at certain timepoints and can fall into the traps when they walk around the branches and pine needles, unlike the sessile phases where RPS is latching onto the tree (Doane, 1959).

Variation among trap replicates is expected, as individual traps may capture different numbers of RPS depending on the infestation intensity of the tree or the adjacent surroundings under which they are deployed. Although I did not find that this variation was as drastic as that of the time of deployment, it is still important to deploy multiple replicates of the traps, if possible, for monitoring and surveillance purposes. A prior study on the use of sticky traps found substantial spatial and temporal variation in insect trap catches and concluded that multiple traps were required to obtain reliable estimates of population abundance, highlighting the value of trap replication in monitoring programs (Taylor *et al.,* 2013). Collecting multiple field sample replicates is also important for increasing DNA capture and improving the likelihood of detecting the target eDNA (Rishan *et al*., 2023).

Seasonal Phenology Impacts Monitoring

In 2024, I observed two peaks in first-instar captures: one in early July and a second, broader peak from late August through late September. First instar individuals dominated captures throughout the season, while adult male and other instar stages were consistently rare. The adult female was captured more than the second instar, third instar

and adult male stages, but not more than the first instar phase. I interpreted these bimodal peaks as reflecting distinct periods of crawler emergence, consistent with known bivoltine *Matsucoccus* life-history patterns (McClure, 1977).

In contrast, captures in Maine in 2025 were substantially lower across all sampling dates and exhibited a simplified phenological pattern, with only a modest early-season increase and a single autumn peak. This could be due to differences in infestation level at the Maine site compared to New Hampshire. A similar occurrence was seen in a past study looking at another scale insect, the oystershell scale (*Lepidosaphes ulmi*). A decrease in scale population was observed in the next sampling year, and it was hypothesized that this may be a result of climatic conditions or the timing of winter crawler emergence (Crouch *et al*., 2024). Climate data can be recorded and analyzed to test if RPS population can decline in different conditions. I also theorized that no RPS specimens were caught by the traps in New Hampshire in 2025 because of mass tree mortality. Resource depletion has been identified as a cause of infestation decline in another study about bark beetles (Washaya *et al*., 2024). As there was a large abundance of RPS at this site in the previous year and possibly even earlier, the sudden reduction in RPS population suggests host mortality could have led to pest decline through resource depletion.

As mentioned prior, I could have missed the first adult female peak due to earlier development which could have happened before the first trap deployment timepoint. I set up the traps in 2025 based on when adult females emerged in 2024. These results demonstrate that RPS monitoring based on a single year and site of data may fail to capture the full range of seasonal dynamics. This highlights the importance of multi-year and multi-site monitoring for invasive species surveillance (Hajdukiewicz *et al*., 2025).

Another factor that could affect RPS phenology is the climate. With changing climates, adult female emergence can be sooner if the warmer temperatures occur earlier (Frank, 2020). All in all, it can be beneficial to deploy traps for several timepoints to maximize the number of specimens and therefore targeted eDNA collected. However, this can be costly and time consuming. As first instar crawlers are most likely to fall into the traps in copious amounts, it is best to include timepoints with peak crawler activity when setting up traps. Therefore, phenology trends can be used for targeting the trap deployment timings.

qPCR Assay is Sensitive and Specific to RPS

The eDNA assay developed for this study was specific to RPS and discriminated well against the only known congener in the region. This assay was designed to detect the RPS populations based on COI barcodes sequenced from Maine and New Hampshire. The assay might have some lower detection capabilities if SNP variants from other regions (origin or introduced), which are currently unknown, exist in the primer probe regions. Other genetic clusters of RPS were not considered during assay design due to lack of adequate COI barcode sequences availability. Gel electrophoresis confirmed that the assay amplified only the target RPS DNA and showed no evidence of cross-amplification with WPS. To further evaluate assay specificity, the primer and probe sequences, as well as the complete target amplicon, were queried against the NCBI GenBank nucleotide database using BLAST in an *in silico* analysis. The search identified several non-target sequences with 100% query coverage across the target region; however, none exhibited 100% sequence identity to the assay target. Perfect matches were observed only for RPS sequences. These results support the specificity of the assay for RPS among all currently available GenBank records. However, complete

assay specificity cannot be confirmed because COI sequence data are not available for every closely related congeners, and some species remain untested or some may not yet be discovered.

The lack of sequence data for congeners is a known issue during the *in silico* validation phase when developing an eDNA assay (Mason *et al.,* 2025). While WPS COI barcodes were not available on online databases, I did sequence multiple specimens from various genetic clusters as WPS is a known congener in New Brunswick. This included specimens from New Hampshire and Maine, as well as other regions (Table A1). Since no WPS DNA amplified in our trials, this assay is unlikely to produce false positives if used in this geographical area unless other unknown congeners are present. For broader application in other areas, additional testing would be necessary, including evaluating local congeners to determine whether they produce false positives.

For sensitivity, I calculated the LoD and LoQ of the assay to be one copy per reaction, indicating high analytical sensitivity. I calculated the efficiency of this assay to be 85% using the gBlock-based standard curve. As the conventional range for assay efficiency is 90-110% (Rogers-Broadway & Karteris, 2015), an 85% efficiency falls below this range. A lower efficiency increases the risk of delayed amplification, leading to inaccurate quantification of low-copy targets (Bustin *et al*., 2026), but the assay does detect down to one copy per reaction. Since the purpose of this project was environmental application and precise absolute quantification was less critical than consistent detection across low and variable target concentrations, the assay remained sufficiently robust for reliable detection. Additionally, there have been other studies that have deemed an efficiency of at least 85% as acceptable (Gupta *et al*., 2021; Wu *et al*., 2017). Regardless, I attempted to improve assay efficiency with several optimization

strategies, including varying primer and probe concentrations and changes in qPCR parameters (Zhao *et al*., 2021), but was unable to improve it further without considering new primer design, which is unlikely due to the absence of enough SNP variants for congener discrimination in other COI-flanking regions. There is a possibility that an assay designed to work with other conserved gene regions, such as the 18S ribosomal RNA gene (Bininda-Emonds, 2021), could have greater efficiency. However, low genetic distance is a known issue within the *Matsucoccus* genus, and the barcodes for other regions are not vastly available in databases for RPS and its congeners. Generating these barcodes would require a separate study to develop and validate a reference database. Despite the efficiency, the assay exhibited excellent linearity and model fit (R^2 = 0.999) and success in serial dilutions and environmental samples from specimens from the specific locations.

Variability among technical replicates increased at low concentrations in some samples, highlighting the trade-off between sensitivity and precision near the LoD. Previous research on eDNA from preserved tissue suggests that this variability may result from a low abundance of template molecules with adequate DNA integrity (Dietrich *et al.,* 2013). Inconsistent PCR amplification has been a challenge for scale insects, potentially due to their small body size leading to smaller amounts and concentrations of DNA extracted (Park *et al*., 2011; Wang *et al*., 2019). Another explanation for the variability in a few samples may be the presence of inhibitors. Although I did dilute the eDNA and tried adding BSA to the reaction master mix (Kreader, 1996), the potential inhibitors may not have been removed as the issue persisted. This issue was more prevalent in eDNA samples extracted from the 3D-printed trap slides where I used the Zymo research kit, and in three canopy pitfall trap

collection. If inhibitors are the issue, changes with eDNA precipitation during the extraction protocol can help, like multi-filter isolation (Hunter *et al*., 2019) or the use of a different extraction kit.

Visual Counts Positively Correlates with Molecular Signal

When I compared visual RPS counts with molecular detection outcomes, I observed a positive relationship between abundance and detection probability for both trap types. There were some outliers, which can be explained by samples that amplified within the Cq value, but did not meet the standard deviation threshold. As visual counts increased, the likelihood of molecular detection also increased, confirming that the eDNA signal broadly reflected organismal abundance. Quantifying the amount of DNA in the sample has also been used for estimating infestation levels in the HWA study (Dunham *et al.,* 2025). This suggests that eDNA can provide information on relative abundance, although the strength and form of the relationship varied among trap types. I found that the eDNA samples collected using 3D-printed traps required more RPS individuals to be caught by the traps in order to get a positive signal when compared to the canopy pitfall trap. The substantial number of zero counts and the overlap between presence and absence across low to moderate abundance values also indicate considerable variability. These differences can be attributed to several factors such as variation in trap design, the differences in eDNA extraction protocols and the infestation level around where the traps are deployed (Dunham *et al.,* 2025). Together, these results suggest that while RPS abundance provides useful information about the likelihood of occurrence, molecular presence-absence patterns inferred from trap data should be interpreted cautiously and in conjunction with other ecological and sampling considerations.

According to the heat maps, the high level of concordance in Maine indicated that both methods can reliably identify periods of increased target presence; however, the occurrence of molecular-only and visual-only detections highlights differences in detection sensitivity and the potential influence of imperfect detection. There were some instances of visual-only detections that can be explained by the potential presence of congeners. These specimens could appear like RPS under the microscope but could have been identified as otherwise by the assay. Another explanation could be increased inhibitors present in certain collection timepoints. It is also possible that the use of different eDNA extraction protocols caused some discrepancies between the detection outcomes in the two trap types. Overall, molecular and visual approaches provide complementary information and that combining both methods may improve detection reliability across varying levels of target abundance. However, molecular-only detections in New Hampshire suggest that molecular methods may detect target DNA even when individuals are not observed visually. This can indicate the benefits of molecular detection in low-abundance sites. This is consistent with past research on paired eDNA and visual surveys in terrestrial settings (Allen *et al*., 2021).

Molecular Detection Under Low Population Abundance

The 2025 datasets provided valuable insight into the performance of molecular detection at low population densities. In Maine, molecular detection largely tracked visual positivity through time, but I observed instances where molecular detections occurred more frequently than visual detections, particularly in canopy pitfall traps. This is consistent with findings from the HWA study showing the molecular method detected the pest more successfully than visual inspection (Kirtane *et al.,* 2022). Visually representing data at each tree in the Maine 2025 site showed that some traps performed

better at capturing RPS and producing a positive molecular signal. However, as per the statistical analysis, these differences were not very pronounced and not significant, unlike temporal effects. The greater success of detecting RPS using the assay at certain timepoints is expected as it corresponds with greater trap capture, which relates to the emergence of mobile stages.

In Maine, the GLMM showed that positive detection were approximately 18 times greater in canopy pitfall traps than in 3D-printed traps. Again, this could be due to the difference in eDNA extraction kits, and also due to increased RPS capture in canopy pitfall traps. In New Hampshire, visual captures were non-existent in 2025, and molecular detections were infrequent. Although this limited my ability to apply GLMM analysis to the dataset, descriptive comparisons showed that molecular detections occurred more frequently in canopy pitfall traps than in 3D-printed traps. This result ties back to the idea of surface area and its importance in capturing more specimens (Fidgen *et al.,* 2020; Sanders *et al.,* 2023).

Limitations and Future Directions

While this study provides a foundation for developing eDNA monitoring approaches specific to RPS, several limitations highlight important directions for future research. The geographic scope and two-year duration of the project constrained broader inference, particularly with respect to long-term population trends and wider assay applicability. As a result, conclusions regarding temporal consistency and regional generalizability remain limited. Long-term monitoring would be valuable for improving understanding of RPS phenology (Primack *et al*., 2023), as the patterns observed in one year were not consistently replicated in the next. Multi-year monitoring efforts, particularly when combined with climate data, could help clarify the timing of

emergence and activity across different RPS life stages, which would help optimize the time of deployment for better pest surveillance (Pérez-Girón *et al*., 2026).

Methodological considerations related to trap processing also warrant attention. In 2025, RPS counts from canopy pitfall traps were based on only one half of the filter paper, which differed from the processing approach used for other trap types. To make the methods more uniform, I doubled the number of trap capture by canopy pitfall trap, making the assumption that both halves of the filter paper had equal numbers of RPS.

Intraspecific genetic variation represented another important limitation. Following initial assay development, primer and probe designs were based on conserved RPS COI sequences from Maine and New Hampshire. However, near the end of the project, a SNP variant was found in the forward primer region in some RPS specimens collected from New York. This SNP variant at the terminal end of the forward primer reduced the assay's applicability across regions since it decreased efficiency. Further research at the Canadian Forest Service will continue to explore this issue to ensure the assay is applicable to RPS from other geographic regions. However, this does not impact the validity of my results. It impacts its applicability in regions where this variant would be found in higher proportions. It also highlights the importance of checking both the intraspecific and interspecific genetic variation of species of interest and their congeners to make sure that the variation does not impact assay specificity and sensitivity. Assays designed in one region often can fail or lack applicability in another region, and follow-up testing at other locations is necessary (Franklin *et al*., 2018).

Furthermore, variability observed among technical replicates indicated that the presence of PCR inhibitors remains a potential concern. Although more comprehensive inhibitor testing and removal methods exist (Hu *et al*., 2014; Schrader *et al*., 2012), these

approaches are time-consuming and costly and would require further optimization and additional resources to implement effectively.

Finally, I acknowledge broader limitations inherent to eDNA-based approaches. Environmental inhibitors, uneven eDNA distribution (particularly during early detection stages), and the potential for human error, such as accidental contamination, can all influence detection probability and accuracy (Hutchins *et al.,* 2021; Lin *et al.,* 2025). Despite these limitations, the risks associated with eDNA detection remain acceptable when compared with traditional visual identification methods, which are constrained by morphological similarity to other taxa and the need for taxonomic expertise.

Future research should prioritize expanding geographic and genetic validation of the assay to ensure robust detection across the full invaded and at-risk range of RPS. This could be done by testing the assay with congeners found in other regions and assessing the barcode for RPS from various geographical locations. Further standardization of trap processing and optimization of eDNA extraction protocols, particularly to reduce inhibition and replicate variability, would enhance detection reliability at low population densities. To achieve this, different DNA-extraction kits could be tested to determine which one reduces inhibitors the most (Hu *et al*., 2014).

Detection and monitoring are only one aspect of the solution to RPS infestation. Now that a species-specific assay is available, it's ability for early detection needs to be tested. One method to do this would be deploying the traps in different locations with variable infestation levels to see the impact on detection. If the assay can detect RPS consistently before visually identifiable, it can be used for early detection. The most important outcome of this research is stakeholders developing and applying management strategies to protect red pine stands from RPS.

Conclusion

For this thesis, I developed, optimized, and validated a species-specific qPCR assay capable of detecting RPS from eDNA collected using conventional insect traps. I met all my original objectives by evaluating the performance of three trap types for collecting both RPS specimens and eDNA, optimizing and validating a sensitive and specific molecular assay, and field-testing the assay on samples across multiple regions and infestation levels.

I found that trap design played a critical role in both visual capture and molecular detection success. Canopy pitfall traps consistently captured more RPS specimens than green sticky traps and 3D-printed traps, and they also generated stronger and more frequent molecular signals. There were less conclusive signals in eDNA collected using the 3D-printed trap. However, their benefit for use as early detection tools has yet to be determined.

I found that sampling date influenced RPS capture rates and detection probability, exceeding the effects of spatial variation among trees. Seasonal phenology differed between years, underscoring the importance of multi-year monitoring and flexible deployment schedules. My results showed that first-instar crawlers contributed most strongly to both visual captures and eDNA detection, highlighting the need to align trap deployment with peak crawler activity. These findings reinforce the limitation of relying on single-year or single-site data to inform long-term surveillance strategies, particularly in the context of climate-driven shifts in insect phenology.

The eDNA assay that I developed in this study demonstrated specificity, sensitivity and detected RPS DNA from environmental samples collected from field conditions. Although assay efficiency fell slightly below conventional quantitative PCR

standards, the assay performed well for the intended purpose of environmental detection at low and variable target concentrations. I found that molecular detection probability increased with visual abundance, confirming a positive correspondence between organismal presence and molecular signal. This means the molecular approach can be useful alone but may be used alongside visual assessment if possible. Using both methods together does not significantly increase labour costs as the traps need to be processed regardless, and the molecular assay helps improve detection accuracy as specialized taxonomic expertise in RPS is not necessary and benefits regulatory agencies that require further confirmation of the pest on the host tree at the site itself for confirmation and regulation.

Despite some limitations related to geographic scope, assay applicability across broader populations, and potential environmental inhibitors, this study provides a strong foundation for future RPS monitoring efforts. The combined use of optimized trap design, repeated temporal sampling, and molecular detection offers a practical and sensitive approach for detection and surveillance. Overall, my findings support the integration of eDNA-based methods into forest pest monitoring programs and contribute to the growing body of evidence that molecular tools can enhance the detection and management of invasive forest insects.

Bibliography

Abbott, C., Coulson, M., Gagné, N., Lacoursière-Roussel, A., Parent, G., Bajno, R., Dietrich, C., & May-McNally, S. (2021). Guidance on the use of Targeted Environmental DNA (EDNA) analysis for the management of aquatic invasive species and species at risk. In *FAO* (No. CA2107180). *Fisheries and Oceans Canada / Pêches et Océans*. https://www.fao.org/fishery/en/openasfa/8b681c42-f1d7-4e28-89a4-c85163b1e77c

Adcock, Z. C., Adcock, M. E., & Forstner, M. R. J. (2023). Development and validation of an environmental DNA assay to detect federally threatened groundwater salamanders in central Texas. *PloS One*, *18*(7), e0288282. https://doi.org/10.1371/journal.pone.0288282

Ahmed, M. Z., Ray, C. H., Moore, M. R., & Miller, D. R. (2020). *The Matsucoccus Cockerell,* 1909 of Florida (Hemiptera: Coccomorpha: Matsucoccidae): Potential pests of Florida pines. DigitalCommons@University of Nebraska - Lincoln. https://digitalcommons.unl.edu/insectamundi/1306/

Allen, M. C., Nielsen, A. L., Peterson, D. L., & Lockwood, J. L. (2021). Terrestrial eDNA survey outperforms conventional approach for detecting an invasive pest insect within an agricultural ecosystem. *Environmental DNA*, *3*(6), 1102-1112. https://doi.org/10.1002/edn3.231

Allison, J. D., Bhandari, B. D., McKenney, J. L., & Millar, J. G. (2014). Design factors that influence the performance of flight intercept traps for the capture of longhorned beetles (Coleoptera: Cerambycidae) from the Subfamilies Lamiinae and Cerambycinae. *PLoS ONE*, *9*(3), e93203. https://doi.org/10.1371/journal.pone.0093203

Anand, M., Leithead, M., Silva, L., Wagner, C., Ashiq, M., Cecile, J., Drobyshev, I., Bergeron, Y., Das, A., & Bulger, C. (2013). The scientific value of the largest remaining old-growth red pine forests in North America. *Biodiversity and Conservation*, *22*(8), 1847-1861. https://doi.org/10.1007/s10531-013-0497-1

Anderson, J. F., Ford, R.P., Kegg, J.D., & Risley, J.H. (1976). The red pine scale in North America: A report to the 1975 Eastern Plant Board. *U.S. Department of Agriculture.* https://doi.org/10.5962/bhl.title.51088

Armbruster, D. A., & Pry, T. (2008). Limit of blank, limit of detection and limit of quantitation. *The Clinical Biochemist Reviews*, *29*(Suppl 1), S49. https://pmc.ncbi.nlm.nih.gov/articles/PMC2556583/

Auger-Méthé, M., Newman, K., Cole, D., Empacher, F., Gryba, R., King, A. A., Leos-Barajas, V., Mills Flemming, J., Nielsen, A., Petris, G., & Thomas, L. (2021). A guide to state-space modeling of ecological time series. *Ecological Monographs*, *91*(4). https://doi.org/10.1002/ecm.1470

Bean, J. L., & Godwin, P. A. (1955). Description and bionomics of a new red pine scale, *Matsucoccus resinosae*. *Forest Science*, *1*(2), 164-176. https://doi.org/10.1093/forestscience/1.2.164

Bean, J. L., & Godwin, P. A. (1971). Red pine scale. Forest pest leaflet 10. *USDA Forest Service*. https://nfsl.contentdm.oclc.org/digital/collection/p17053coll1/id/137147

Bininda-Emonds, O. R. P. (2021). 18S rRNA variability maps reveal three highly divergent, conserved motifs within Rotifera. *BMC Ecology and Evolution*, *21*(1), 118. https://doi.org/10.1186/s12862-021-01845-2

Bragard, C., Baptista, P., Chatzivassiliou, E., Di Serio, F., Gonthier, P., Jaques Miret, J. A., Justesen, A. F., Magnusson, C. S., Milonas, P., Navas-Cortes, J. A., Parnell,

S., Potting, R., Reignault, P. L., Stefani, E., Thulke, H., Van der Werf, W., Vicent Civera, A., Yuen, J., Zappalà, L., & Gregoire, J. (2022). Pest categorisation of *Matsucoccus massonianae*. *EFSA Journal*, *20*(11). https://doi.org/10.2903/j.efsa.2022.7643

Bragard, C., Baptista, P., Chatzivassiliou, E., Di Serio, F., Gonthier, P., Miret, J. a. J., Justesen, A. F., Magnusson, C. S., Milonas, P., Navas-Cortes, J. A., Parnell, S., Potting, R., Reignault, P. L., Stefani, E., Thulke, H., Van Der Werf, W., Civera, A. V., Yuen, J., Zappalà, L., . . . MacLeod, A. (2024). Pest categorisation of *Matsucoccus matsumurae. EFSA Journal, 22*(7), e8970. https://doi.org/10.2903/j.efsa.2024.8970

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., & Wittwer, C. T. (2009). The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry*, *55*(4), 611-622. https://doi.org/10.1373/clinchem.2008.112797

Bustin, S. A., Van Den Hoff, M. J. B., Pfaffl, M. W., Kubista, M., & Ruijter, J. M. (2026). Quantification revisited: What qPCR efficiency models reveal about data analysis integrity. *International Journal of Molecular Sciences*, *27*(5), 2337. https://doi.org/10.3390/ijms27052337

Cain, C. (2014). Red pine scale: Insect the size of fleck of pepper killing trees in Northampton's watershed. *Gazettenet.com.* https://www.northamptonma.gov/DocumentCenter/View/14367

Canadian Food Inspection Agency. (2024). D-02-12: Phytosanitary import requirements for non-processed wood and other wooden products, bamboo and bamboo

products, originating from all areas other than the continental United States - inspection.canada.ca. *Canada.ca.* https://inspection.canada.ca/en/plant-health/invasive-pests-and-plants/directives/forest-products/02-12

Çevik, T., Çevik, N. (2025). Environmental DNA (eDNA): A review of ecosystem biodiversity detection and applications. *Biodiversity and Conservation, 34*, 2999-3035. https://doi.org/10.1007/s10531-025-03112-y

Childs, R. (2016, December 7). Hemlock woolly adelgid frequently asked questions. *Center for Agriculture, Food, and the Environment*. https://ag.umass.edu/landscape/fact-sheets/hemlock-woolly-adelgid-frequently-asked-questions

Choi, J., Cha, D., Kim, D.S., & Lee, S. (2019). Review of Japanese pine bast scale, *Matsucoccus matsumurae* (Kuwana) (Coccomorpha: Matsucoccidae), occurring on japanese black pine (*Pinus thunbergii* Parl.) and Japanese red pine (*P. densiflora* Siebold & Zucc.) from Korea. *Forests*, *10*(8), 639. https://doi.org/10.3390/f10080639

Cox, D. R. (1983). Some remarks on overdispersion. *Biometrika*, *70*(1), 269-274. https://doi.org/10.1093/biomet/70.1.269

Crouch, C. D., Hofstetter, R. W., Grady, A. M., Edwards, N. N. S., & Waring, K. M. (2024). Oystershell scale (Hemiptera: Diaspididae) population growth, spread, and phenology on aspen in Arizona, USA. *Environmental Entomology*, *53*(2), 293-304. https://doi.org/10.1093/ee/nvae006

Dagal, I., Harrison, A., Ibrahim, A.W., & Mbasso, W. (2025). Comprehensive evaluation of data preprocessing and visualization techniques for enhanced

classification and sampling. *Cluster Computing*, *28*(7). https://doi.org/10.1007/s10586-025-05512-9

Deagle, B. E., Jarman, S. N., Coissac, E., Pompanon, F., & Taberlet, P. (2014). DNA metabarcoding and the cytochrome c oxidase subunit I marker: not a perfect match. *Biology Letters*, *10*(9), 20140562. https://doi.org/10.1098/rsbl.2014.0562

Dietrich, D., Uhl, B., Sailer, V., Holmes, E. E., Jung, M., Meller, S., & Kristiansen, G. (2013). Improved PCR performance using template DNA from formalin-fixed and paraffin-embedded tissues by overcoming PCR inhibition. *PLoS ONE*, *8*(10), e77771. https://doi.org/10.1371/journal.pone.0077771

Doane, C. C. (1959) The red pine scale. *Circular 207, The Connecticut - Agricultural Experiment Station, New Haven*. https://portal.ct.gov/-/media/CAES/DOCUMENTS/Publications/Circulars/C207pdf.pdf

Doane, C. C. (1966). Evidence for a sex attractant in females of the red pine scale. *Journal of Economic Entomology*, *59*(6), 1539-1540. https://doi.org/10.1093/jee/59.6.1539

Duda, E. J. (1961). Some aspects of the biology and ecology of the red pine scale, *Matsucoccus matsumurae* B. & G. (Homoptera: Margarodidae). *UMass*. https://scholarworks.umass.edu/cgi/viewcontent.cgi?article=6602&context=dissertations_1

Dunham, K., Geller, K., Sanders, M., & Partridge, C. (2025). Assessing the use of 3D-printed traps to evaluate hemlock woolly adelgid (Hemiptera: Adelgidae) infestation levels. *Journal of Economic Entomology*, *118*(6), 2971-2982. https://doi.org/10.1093/jee/toaf174

Fidgen, J. G., Whitmore, M. C., Studens, K. D., Macquarrie, C. J. K., & Turgeon, J. J. (2020). Sticky traps as an early detection tool for crawlers of *Adelges tsugae* (Hemiptera: Adelgidae). *Journal of Economic Entomology*, *113*(1), 496-503. https://doi.org/10.1093/jee/toz257

Fidgen, J. G., Forbes, G., Roscoe, L. E., Stastny, M., Van Der Meer, B. M., Ogden, J., & Williams, M. (2025). A funnel trap for the detection of hemlock woolly adelgid (Hemiptera: Adelgidae) and a method of extracting crawlers from trap samples. *Journal of Economic Entomology*, *118*(5), 2645-2655. https://doi.org/10.1093/jee/toaf140

Frank, S. D. (2020). Review of the direct and indirect effects of warming and drought on scale insect pests of forest systems. *Forestry: An International Journal of Forest Research*, *94*(2), 167-180. https://doi.org/10.1093/forestry/cpaa033

Franklin, T. W., Dysthe, J. C., Golden, M., McKelvey, K. S., Hossack, B. R., Carim, K. J., Tait, C., Young, M. K., & Schwartz, M. K. (2018). Inferring presence of the western toad (*Anaxyrus boreas*) species complex using environmental DNA. *Global Ecology and Conservation*, *15*, e00438. https://doi.org/10.1016/j.gecco.2018.e00438

Forootan, A., Sjöback, R., Björkman, J., Sjögreen, B., Linz, L., & Kubista, M. (2017). Methods to determine limit of detection and limit of quantification in quantitative real-time PCR (qPCR). *Biomolecular Detection and Quantification*, *12*, 1-6. https://doi.org/10.1016/j.bdq.2017.04.001

Gilmore, D. W., & Palik, B. J. (2006). A revised managers handbook for red pine in the North Central Region. *Gen. Tech. Rep. NC-264. St. Paul, MN: U.S. Department*

of Agriculture, Forest Service, North Central Research Station. 55 P., *264*. https://doi.org/10.2737/NC-GTR-264

Gilmour, A. R., Anderson, R. D., & Rae, A. L. (1985). The analysis of binomial data by a generalized linear mixed model. *Biometrika*, *72*(3), 593-599. https://doi.org/10.1093/biomet/72.3.593

Griess, H., Veit, H., & Petercor, R. (2013). Risk Assessment for biotic pests under prospective climate conditions. *Management Strategies to Adapt Alpine Space Forests to Climate Change Risks*. https://doi.org/10.5772/56410

Guinn, W. 2014. "Managing forest health on NH state lands." *Presented at the Resources Resilience Renewal Restoration 94th Annual Winter Meeting, Nashua, New Hampshire.* https://extension.unh.edu/sites/default/files/migrated_unmanaged_files/Resource004119_Rep5867.pdf

Gupta, V., Antunez, L. R., Saleh-Birdjandi, S., Kumru, O. S., Pospisil, R., Lilja, A., Fuhrmann, G., Smith, L., Volkin, D. B., & Joshi, S. B. (2021). Development of a high-throughput RT-PCR based viral infectivity assay for monitoring the stability of a replicating recombinant lymphocytic choriomeningitis viral vector. *Journal of Virological Methods*, *301*, 114440. https://doi.org/10.1016/j.jviromet.2021.114440

Hajdukiewicz, H., Zielonka, A., Hajdukiewicz, M., & Walusiak, E. (2025). UAV-based fine-scale monitoring reveals species-specific riparian responses to environmental changes. *Ecological Indicators*, *182*, 114573. https://doi.org/10.1016/j.ecolind.2025.114573

Hajibabaei, M., Shokralla, S., Zhou, X., Singer, G. A. C., & Baird, D. J. (2011). Environmental barcoding: A next-generation sequencing approach for biomonitoring applications using river benthos. *PLoS ONE*, *6*(4), e17497. https://doi.org/10.1371/journal.pone.0017497

Hall, T. A. (1999). BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series, 41*, 95-98.

Hartig, F. (2016). *DHARMa: Residual Diagnostics for Hierarchical (Multi-Level / Mixed) Regression Models*. https://doi.org/10.32614/cran.package.dharma

Hoey, S. (2023). *Pinus resinosa. Trees and Shrubs Online*. https://www.treesandshrubsonline.org/articles/pinus/pinus-resinosa/

Hu, Q., Liu, Y., Yi, S., & Huang, D. (2014). A comparison of four methods for PCR inhibitor removal. *Forensic Science International Genetics*, *16*, 94-97. https://doi.org/10.1016/j.fsigen.2014.12.001

Hunter, M. E., Ferrante, J. A., Meigs-Friend, G., & Ulmer, A. (2019). Improving eDNA yield and inhibitor reduction through increased water volumes and multi-filter isolation techniques. *Scientific Reports*, *9*(1). https://doi.org/10.1038/s41598-019-40977-w

Hutchins, P. R., Simantel, L. N., & Sepulveda, A. J. (2021). Time to get real with qPCR controls: The frequency of sample contamination and the informative power of negative controls in environmental DNA studies. *Molecular Ecology Resources*, *22*(4), 1319-1329. https://doi.org/10.1111/1755-0998.13549

Integrated DNA Technologies, Inc. (n.d.). *gBlock gene fragments*. https://www.idtdna.com

Kestel, J. H., Field, D., Bateman, P. W., White, N., Allentoft, M. E., Hopkins, A., Gibberd, M. R., & Nevill, P. G. (2022). Applications of environmental DNA (eDNA) in agricultural systems: Current uses, limitations and future prospects. *Science of the Total Environment*, *847*, 157556-157556. https://doi.org/10.1016/j.scitotenv.2022.157556

Kirtane, A., Dietschler, N. J., Bittner, T. D., Lefebvre, M. B., Celis, S., O'Connor, K., Havill, N., & Whitmore, M. C. (2022). Sensitive environmental DNA (eDNA) methods to detect hemlock woolly adelgid and its biological control predators *Leucotaraxis* silver flies and a *Laricobius* beetle. *Environmental DNA*, *4*(5), 1136-1149. https://doi.org/10.1002/edn3.317

Kondo, T & Watson, G.W. (2022) Chapter 5. collection, preservation, slide-mounting, labelling and vouchering of scale insects. *In:* Kondo, T & Watson, G.W. (Eds.). *Encyclopedia of Scale Insect Pests*. CABI, Wallingford, Oxfordshire, pp.548-558.

Kreader, C. A. (1996). Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. *Applied and Environmental Microbiology*, *62*(3), 1102-1106. https://doi.org/10.1128/aem.62.3.1102-1106.1996

Kriezis, A., Vitale, M., Morselli, G., Crisanti, A., & Bernardini, F. (2024). Unravelling the role of mitochondrial DNA in hybrid incompatibility within species of the *Anopheles gambiae* complex. *Scientific Reports*, *14*(1). https://doi.org/10.1038/s41598-024-80887-0

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution, 35*(6), 1547-1549. https://doi.org/10.1093/molbev/msy096

Lee, J. H., Han, G., Fulp, W. J., & Giuliano, A. R. (2011). Analysis of overdispersed count data: application to the Human Papillomavirus Infection in Men (HIM) Study. *Epidemiology and Infection*, *140*(6), 1087-1094. https://doi.org/10.1017/s095026881100166x

Lenth, R. V. (2017). *emmeans: Estimated Marginal Means, aka Least-Squares Means*. https://doi.org/10.32614/cran.package.emmeans

Lin, W., Liu, F. C., Cho, L., & Yang, C. S. (2025). Let ants find them: Using ants as eDNA samplers for detecting the invasive spotted lanternfly. *NeoBiota*, *98*, 261-267. https://doi.org/10.3897/neobiota.98.151420

Little, Elbert L., Jr. (1971). Atlas of United States trees. Volume 1. Conifers and important hardwoods. Miscellaneous Publication 1146. Washington, DC: *U.S. Department of Agriculture, Forest Service*.

Little, T. (2015). Method validation essentials, limit of blank, limit of detection, and limit of quantitation. *BioPharm International*, *28*(4). https://www.biopharminternational.com/view/method-validation-essentials-limit-blank-limit-detection-and-limit-quantitation

Liu, W., Xie, Y., Dong, J., Xue, J., Zhang, Y., Lu, Y., & Wu, J. (2014). Pathogenicity of three entomopathogenic fungi to *Matsucoccus matsumurae*. *PLoS ONE*, *9*(7), e103350-e103350. https://doi.org/10.1371/journal.pone.0103350

Ma, H., Bell, K. N., & Loker, R. N. (2020). Development and validation of qPCR and RT-qPCR: Regulatory points to consider when conducting biodistribution and vector shedding studies of gene and cell therapy products. *Molecular Therapy - Methods & Clinical Development*, *20*. https://doi.org/10.1016/j.omtm.2020.11.007

MacDonald, A. J., & Sarre, S. D. (2016). A framework for developing and validating taxon-specific primers for specimen identification from environmental DNA. *Molecular Ecology Resources*, *17*(4), 708-720. https://doi.org/10.1111/1755-0998.12618

Maine Forest Service. (2026). Red Pine Scale (*Matsucoccus matsumarae*). *Division of Forest Health and Monitoring*, https://www.maine.gov/dacf/mfs/forest_health/insects/red_pine_scale.htm

Marino, P. (2023). A tree-destroying insect is in N.H. and Canada fears we'll accidentally send it north. *Concord Monitor.* https://www.concordmonitor.com/2023/12/26/pine-invasive-insect-52938046/

Mason, D. H., Henderson, H. A., Franklin, T. W., Young, M. K., Hernandez, J. E., Engkjer, C. L., Hatt, J., & Schwartz, M. K. (2025). Environmental DNA detects endangered Texas hornshell and its hosts. *Ecology and Evolution*, *15*(7), e71397. https://doi.org/10.1002/ece3.71397

Mauvisseau, Q., Harper, L. R., Sander, M., Hanner, R. H., Kleyer, H., & Deiner, K. (2022). The multiple states of environmental DNA and what is known about their persistence in aquatic environments. *Environmental Science & Technology*, *56*(9), 5322-5333. https://doi.org/10.1021/acs.est.1c07638

McClure, M. S. (1976). Colonization and establishment of the red pine scale, *Matsucoccus resinosae* (Homoptera; Margarodidae) in a Connecticut plantation. *Environmental Entomology*, *5*(5), 943-947. https://doi.org/10.1093/ee/5.5.943

McClure, M. S. (1977). Population dynamics of the red pine scale, *Matsucoccus resinosae* (Homoptera: Margarodidae): the influence of resinosis. *Environmental Entomology*, *6*(6), 789-795. https://doi.org/10.1093/ee/6.6.789

McClure, M. S. (1983). Population dynamics of a pernicious parasite: Density-dependent vitality of red pine scale. *Ecology*, *64*(4), 710-718. https://doi.org/10.2307/1937193

McClure, M. S. (1989). Importance of weather to the distribution and abundance of introduced adelgid and scale insects. *Agricultural and Forest Meteorology*, *47*(2-4), 291-302. https://doi.org/10.1016/0168-1923(89)90101-9

Mech, A. M., Asaro, C., Cram, M. M., Coyle, D. R., Gullan, P. J., Cook, L. G., & Kamal. (2013). *Matsucoccus macrocicatrices* (Hemiptera: Matsucoccidae): First report, distribution, and association with symptomatic eastern white pine in the southeastern United States. *Journal of Economic Entomology*, *106*(6), 2391-2398. https://doi.org/10.1603/ec13251

Montgomery, G. A., Belitz, M. W., Guralnick, R. P., & Tingley, M. W. (2021). Standards and best practices for monitoring and benchmarking insects. *Frontiers in Ecology and Evolution*, *8*. https://doi.org/10.3389/fevo.2020.579193

Moore, L. M. (2002). Red pine - plant fact sheet. *U.S. Department of Agriculture.* https://plants.usda.gov/DocumentLibrary/factsheet/pdf/fs_pire.pdf

Morrison, H. (1939). Descriptions of new species of *Matsucoccus* (Hemiptera: Coccidae). *Proceedings of the Entomological Society of Washington, 41*, 1-20.

Nagy, A., Vitásková, E., Černíková, L., Křivda, V., Jiřincová, H., Sedlák, K., Horníčková, J., & Havlíčková, M. (2017). Evaluation of TaqMan qPCR system integrating two identically labelled hydrolysis probes in single assay. *Scientific Reports*, *7*(1), 1-10. https://doi.org/10.1038/srep41392

Odero, J., Gomes, B., Fillinger, U., & Weetman, D. (2018). Detection and quantification of *Anopheles gambiae sensu lato* mosquito larvae in experimental aquatic

habitats using environmental DNA (eDNA). *Wellcome Open Research*, *3*, 26. https://doi.org/10.12688/wellcomeopenres.14193.1

Okonechnikov, K., Golosova, O., & Fursov, M. (2012). Unipro UGENE: A unified bioinformatics toolkit. *Bioinformatics, 28*(8), 1166-1167. https://doi.org/10.1093/bioinformatics/bts091

Park, S. C., West, J. R., Abrahamson, L. P., Lanier, G. N., & Silverstein, R. M. (1986). Cross-attraction between two species of *Matsucoccus*. *Journal of Chemical Ecology*, *12*(3), 609-617. https://doi.org/10.1007/bf01012096

Park, D., Suh, S., Hebert, P., Oh, H., & Hong, K. (2011). DNA barcodes for two scale insect families, mealybugs (Hemiptera: Pseudococcidae) and armored scales (Hemiptera: Diaspididae). *Bulletin of Entomological Research*, *101*(4), 429-434. https://doi.org/10.1017/s0007485310000714

Pérez-Girón, J. C., Castedo-Dorado, F., Lombardero, M. J., & Álvarez-álvarez, P. (2026). How accurately do species distribution models predict the expansion of invasive insects, and does climate data choice matter? insights from the invasion of *Dryocosmus kuriphilus*. *Journal of Applied Entomology*. https://doi.org/10.1111/jen.70103

Plumb, George H. (1950). A new and serious insect pest of red pine. *Connecticut Agricultural Experiment Station*. https://portal.ct.gov/-/media/caes/documents/publications/special_circulars/scfebruary1950pdf.pdf?rev=ffebcf5277a946bcb2623ca5e726b571&hash=CD2FFB31B2CC2DEB956D195323862232

Porco, D., Hermant, S., Purnomo, C., Horn, M., Marson, G., & Colling, G. (2022). eDNA-based detection of the invasive crayfish *Pacifastacus leniusculus* in

streams with a LAMP assay using dependent replicates to gain higher sensitivity. *Scientific Reports*, *12*(1). https://doi.org/10.1038/s41598-022-10545-w

Primack, R. B., Gallinat, A. S., Ellwood, E. R., Crimmins, T. M., Schwartz, M. D., Staudinger, M. D., & Miller-Rushing, A. J. (2023). Ten best practices for effective phenological research. *International Journal of Biometeorology*, *67*(10), 1509-1522. https://doi.org/10.1007/s00484-023-02502-7

QIAGEN. (n.d.). *DNeasy blood & tissue kit handbook*. QIAGEN. https://www.qiagen.com

Rishan, S. T., Kline, R. J., & Rahman, M. S. (2023). Applications of environmental DNA (eDNA) to detect subterranean and aquatic invasive species: A critical review on the challenges and limitations of eDNA metabarcoding. *Environmental Advances*, *12*(100370), 100370. https://doi.org/10.1016/j.envadv.2023.100370

Rogers-Broadway, K.-R., & Karteris, E. (2015). Amplification efficiency and thermal stability of qPCR instrumentation: Current landscape and future perspectives. *Experimental and Therapeutic Medicine*, *10*(4), 1261-1264. https://doi.org/10.3892/etm.2015.2712

Ruppert, K. M., Kline, R. J., & Rahman, M. S. (2019). Past, present, and future perspectives of environmental DNA (eDNA) metabarcoding: A systematic review in methods, monitoring, and applications of global eDNA. *Global Ecology and Conservation*, *17*. https://doi.org/10.1016/j.gecco.2019.e00547

Ryall, K. (2015). Detection and sampling of emerald ash borer (Coleoptera: Buprestidae) infestations. *The Canadian Entomologist*, *147*(03), 290-299. https://doi.org/10.4039/tce.2015.7

Sanders, M., Tardani, R., Locher, A., Geller, K., & Partridge, C. G. (2022). Development of novel early detection technology for hemlock woolly adelgid, *Adelges tsugae* (Hemiptera: Adelgidae). *Journal of Economic Entomology*, *116*(1), 168-180. https://doi.org/10.1093/jee/toac175

Schmidt, K. J., Soluk, D. A., Maestas, S. E. M., & Britten, H. B. (2021). Persistence and accumulation of environmental DNA from an endangered dragonfly. *Scientific Reports*, *11*(1), 18987. https://doi.org/10.1038/s41598-021-98099-1

Schrader, C., Schielke, A., Ellerbroek, L., & Johne, R. (2012). PCR inhibitors - occurrence, properties and removal. *Journal of Applied Microbiology*, *113*(5), 1014-1026. https://doi.org/10.1111/j.1365-2672.2012.05384.x

Simpson, T., Wellington, C. M., Lukehurst, S., Huerlimann, R., Veilleux, H. D., Snow, M., Dias, J., & McDonald, J. I. (2023). Development of a real-time PCR (qPCR) method for the identification of the invasive paddle crab *Charybdis japonica* (*Crustacea*, *Portunidae*). *PeerJ*, *11*, e15522-e15522. https://doi.org/10.7717/peerj.15522

Solomon, A. M., & Bartlein, P. J. (1992). Past and future climate change: response by mixed deciduous-coniferous forest ecosystems in northern Michigan. *Canadian Journal of Forest Research*, *22*(11), 1727-1738. https://doi.org/10.1139/x92-227

Taberlet, P., Bonin, A., Zinger, L., & Coissac, E. (2018). Environmental DNA. In *Oxford Scholarship Online*. Oxford University Press. https://doi.org/10.1093/oso/9780198767220.001.0001

Tang, W., He, H., & Tu, X. (2012). Applied categorical and count data analysis. *Chapman and Hall/CRC*. https://doi.org/10.1201/b12123

Taylor, D. B., Friesen, K., & Zhu, J. J. (2013). Spatial-temporal dynamics of stable fly (Diptera: muscidae) trap catches in eastern Nebraska. *Environmental Entomology*, *42*(3), 524-531. https://doi.org/10.1603/en12258

Thermo Fisher Scientific. (2024). *QuantStudio™ Design and Analysis Software* (Version 2.8) [Computer software]. Thermo Fisher Scientific. https://www.thermofisher.com

Thomsen, P. F., & Willerslev, E. (2015). Environmental DNA - An emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation*, *183*, 4-18. https://doi.org/10.1016/j.biocon.2014.11.019

Wang, Y., Dai, T., Tian, H., Wan, F., & Zhang, G. (2019). Comparative analysis of eight DNA extraction methods for molecular research in mealybugs. *PLoS ONE*, *14*(12), e0226818. https://doi.org/10.1371/journal.pone.0226818

Washaya, P., Modlinger, R., Tyšer, D., & Hlásny, T. (2024). Patterns and impacts of an unprecedented outbreak of bark beetles in central Europe: A glimpse into the future? *Forest Ecosystems*, *11*, 100243. https://doi.org/10.1016/j.fecs.2024.100243

Whitcomb, W. E. (2014). "Pest alert: red pine scale". *Maine Department of Agriculture Conservation and Forestry*, Maine Forest Service. https://www.maine.gov/dacf/mfs/forest_health/documents/condition_reports/2014/cond_2014_5.pdf

Whitney, T. D., Gandhi, K. J. K., & Lucardi, R. D. (2019). Native or non-native? Historical biogeography of an emergent forest pest, *Matsucoccus macrocicatrices*. *Journal of Biogeography*, *46*(12), 2860-2874. https://doi.org/10.1111/jbi.13702

Wu, X., Ge, T., Hu, Y., Wei, X., Chen, L., Whiteley, A., & Wu, J. (2017). Abundance and diversity of carbon monoxide dehydrogenase genes from BMS clade bacteria in different vegetated soils. *European Journal of Soil Biology*, *81*, 94-99. https://doi.org/10.1016/j.ejsobi.2017.06.012

Yemshanov, D., Haight, R. G., Chen, C., Liu, N., MacQuarrie, C. J. K., Koch, F. H., Venette, R., & Ryall, K. (2019). Managing biological invasions in urban environments with the acceptance sampling approach. *PLOS ONE*, *14*(8), e0220687. https://doi.org/10.1371/journal.pone.0220687

Zhao, F., Maren, N. A., Kosentka, P. Z., Liao, Y., Lu, H., Duduit, J. R., Huang, D., Ashrafi, H., Zhao, T., Huerta, A. I., Ranney, T. G., & Liu, W. (2021). An optimized protocol for stepwise optimization of real-time RT-PCR analysis. *Horticulture Research*, *8*(1), 179. https://doi.org/10.1038/s41438-021-00616-w

Zhu, X., Bell, K. L., Wu, H., & Gopurenko, D. (2024). Development of an environmental DNA assay for prohibited matter weed amazon frogbit (*Limnobium laevigatum*). *Environments*, *11*(4), 66-66. https://doi.org/10.3390/environments11040066

Chapter 3: Thesis Conclusion

Invasive forest pests remain a leading cause of ecological disturbance and biodiversity loss worldwide (Liebhold *et al.*, 2012; Roy *et al*., 2017**).** Among these, the red pine scale (RPS; *Matsucoccus matsumurae*) has emerged as a particularly insidious threat to red pines (*Pinus resinosa*) across parts of eastern North America (Choi *et al.*, 2019; Whitcomb, 2014).

The primary goal of my thesis was to develop and evaluate an effective surveillance tool for RPS by integrating conventional insect trapping with targeted environmental DNA (eDNA) detection. This work was motivated by the rapid spread of RPS toward the Canadian border (Marino, 2023) and the need for sensitive tools capable of detecting this cryptic insect at low population densities, prior to widespread host damage.

I successfully met all three objectives initially set for this research. First, I evaluated the performance of three conventional trap types for capturing RPS individuals and recoverable eDNA. Second, I developed and optimized an RPS-specific quantitative PCR (qPCR) assay targeting the mitochondrial COI gene with sensitivity. Third, I applied the assay to field-collected eDNA samples across multiple sites and infestation levels to assess its performance under natural conditions.

Trap design emerged as a critical determinant of both visual capture success and molecular detection probability. Across both field seasons, canopy pitfall traps consistently outperformed the 3D-printed traps and green sticky traps in capturing RPS individuals and producing eDNA signals. This pattern was especially clear under higher population densities, highlighting the importance of trap surface area for maximizing

capture numbers. Although differences among trap types were less pronounced during low-abundance conditions, canopy pitfall traps remained the most dependable option for detection as there were signals missing in some 3D-printed trap-collected eDNA.

Temporal variation strongly influenced RPS capture rates and detection outcomes, often exceeding the influence of spatial variation among trees or trap locations. Seasonal phenology differed substantially between years and sites, underscoring the risks of relying on single-year or single-site data to guide monitoring decisions. First-instar crawlers accounted for the vast majority of both visual captures and positive eDNA detections, emphasizing the importance of aligning trap deployment with peak crawler activity. These findings reinforce the need for flexible, multi-year monitoring strategies that can accommodate variation and potential climate-driven shifts in emergence timing.

The qPCR assay developed in this study demonstrated sensitivity and specificity for RPS. The assay reliably detected as few as one DNA copy per reaction and showed no cross-amplification with the primary sympatric congener in the area of study (New Brunswick, Maine, New Hampshire), the white pine bast scale (WPS; *Matsucoccus macrocicatrices*). Field validation confirmed agreement between known infestation status and molecular detections, with no false positives detected at the RPS-negative site in New Brunswick. Although amplification efficiency fell slightly below conventional quantitative PCR standards, the assay performed robustly for the intended purpose of environmental detection across low and variable DNA concentrations.

Comparison of visual counts and molecular detection outcomes revealed a positive relationship between RPS abundance and eDNA detection probability. The canopy pitfall traps had 18 times greater odds of having a positive eDNA detection than

the 3D-printed traps. Importantly, molecular detection occasionally indicated presence when visual captures were sparse or absent, particularly in canopy pitfall traps. This suggests that targeted eDNA sampling may potentially enhance sensitivity during low-density stages of infestation, when traditional visual methods are more likely to fail. At the same time, variability among qPCR technical replicates at very low DNA concentrations highlighted the inherent trade-off between sensitivity and precision near the detection limit. These results emphasize the importance of interpreting molecular data in the context of trap type, replicate consistency, and ecological conditions.

This study also identified several important limitations that inform future research and surveillance efforts. Intraspecific genetic variation, including a single nucleotide polymorphism (SNP) detected in New York RPS populations, show that assay performance can diminish outside the populations used for assay design. This finding underscores the importance of broad geographic validation during assay development and highlights the need for ongoing refinement to ensure regional applicability. In addition, variability potentially associated with PCR inhibitors and differences in eDNA extraction protocols contributed to uncertainty in a subset of samples. While these challenges are not unique to this study, they emphasize that eDNA-based approaches are most effective when complemented by appropriate quality control measures and repeated temporal sampling.

Despite these limitations, the work presented here provides a strong ecological and molecular foundation for RPS surveillance. By integrating optimized trapping strategies with targeted eDNA detection, this thesis demonstrates a practical approach for improving detection capacity in invaded and at-risk forest systems. The methods developed are compatible with existing forest pest monitoring frameworks and can be

adapted as part of broader biosecurity programs aimed at slowing the spread of invasive insects. The most important outcome of using the eDNA assay to detect and monitor RPS is the sense of urgency it can put on stakeholders to develop and implement management strategies to conserve red pine populations.

Overall, this research contributes to the growing body of evidence that molecular tools can substantially enhance invasive species monitoring when thoughtfully combined with conventional survey methods. As RPS continues to expand its range, the approaches developed in this thesis offer a field-validated option for timely detection and may help inform proactive management and response efforts before severe ecological and economic impacts occur.

Bibliography

Choi, J., Cha, D., Kim, D.S., & Lee, S. (2019). Review of Japanese pine bast scale, *Matsucoccus matsumurae* (Kuwana) (Coccomorpha: Matsucoccidae), occurring on japanese black pine (*Pinus thunbergii* Parl.) and Japanese red pine (*P. densiflora* Siebold & Zucc.) from Korea. *Forests*, *10*(8), 639. https://doi.org/10.3390/f10080639

Liebhold, A. M., Brockerhoff, E. G., Garrett, L. J., Parke, J. L., & Britton, K. O. (2012). Live plant imports: the major pathway for forest insect and pathogen invasions of the US. *Frontiers in Ecology and the Environment*, *10*(3), 135-143. https://doi.org/10.1890/110198

Marino, P. (2023). A tree-destroying insect is in N.H. and Canada fears we'll accidentally send it north. *Concord Monitor.* https://www.concordmonitor.com/2023/12/26/pine-invasive-insect-52938046/

Roy, H. E., Rabitsch, W., Scalera, R., Stewart, A., Gallardo, B., Genovesi, P., Essl, F., Adriaens, T., Bacher, S., Booy, O., Branquart, E., Brunel, S., Copp, G. H., Dean, H., D'hondt, B., Josefsson, M., Kenis, M., Kettunen, M., Linnamagi, M., & Lucy, F. (2017). Developing a framework of minimum standards for the risk assessment of alien species. *Journal of Applied Ecology*, *55*(2), 526-538. https://doi.org/10.1111/1365-2664.13025

Whitcomb, W. E. (2014). "Pest alert: red pine scale". *Maine Department of Agriculture Conservation and Forestry*, Maine Forest Service. https://www.maine.gov/dacf/mfs/forest_health/documents/condition_reports/2014/cond_2014_5.pdf

Appendix

Supporting Data for Primer-Probe Development and Trap Performance Analyses

Table A1. List of specimens analyzed along with their GenBank accession numbers, species type, date of collection and collection location, collected for development of primer probe design. Specimens 1-22 were red pine scale (RPS; *Matsucoccus matsumurae*) processed for DNA extraction by Mugdha Stone with the goal of endpoint PCR/Sanger sequencing to inform primer probe design. Specimens 23-45 were white pine bast scale (WPS; *Matsucoccus macrocicatrices*) and processed for DNA extraction by Whitney *et al*., 2019 and obtained for endpoint PCR/Sanger sequencing to inform primer probe design.

#	Specimen	Accession Number	Species	Date	Collection Location
1	RPSNH2Folmer	PZ172457	RPS	2024	Strafford, NH
2	RPSAF4DNAFolmer	PZ172458	RPS	2024	Strafford, NH
3	RPS3ProsoilFolmer	PZ172459	RPS	2024	Strafford, NH
4	RPSSe8-2Folmer	PZ172460	RPS	2024	Strafford, NH
5	RPSAFMay3MainePrimer	PZ172461	RPS	2024	Strafford, NH
6	RPSF1AugMainePrimer	PZ172462	RPS	2024	Strafford, NH
7	RPSF2AugMainePrimer	PZ172463	RPS	2024	Strafford, NH
8	RPSM3MainePrimer	PZ172464	RPS	2024	Strafford, NH
9	RPSMay29-1AFMainePrimer	PZ172465	RPS	2024	Strafford, NH
10	RPSMay29-2AFMainePrimer	PZ172466	RPS	2024	Strafford, NH
11	RPSMay29-4AFMainePrimer	PZ172467	RPS	2024	Strafford, NH
12	RPSMay29-3AFMainePrimer	PZ172468	RPS	2024	Strafford, NH
13	RPSMay29-6AFMainePrimer	PZ172469	RPS	2024	Strafford, NH
14	RPSMay29-9AMMainePrimer	PZ172470	RPS	2024	Strafford, NH
15	RPSMay29-10AMMainePrimer	PZ172471	RPS	2024	Strafford, NH
16	RPSSe8-1MainePrimer	PZ172472	RPS	2024	Strafford, NH
17	RPSSe8-3MainePrimer	PZ172473	RPS	2024	Strafford, NH
18	RPSSe8-4MainePrimer	PZ172474	RPS	2024	Strafford, NH
19	MaineRPS1	PZ653566	RPS	2024	Beddington, ME
20	MaineRPS2	PZ653567	RPS	2024	T36 MD BPP, ME
21	MaineRPS3	PZ653568	RPS	2024	T30 MD BPP, ME
22	MaineRPS5	PZ653569	RPS	2024	T30 MD BPP, ME
23	WPS1MainePrimer	PZ171127	WPS	2015	Boggs Creek, GA
24	WPS2MainePrimer	PZ171128	WPS	2015	Penobscot, ME

25	WPS3MainePrimer	PZ171129	WPS	2015	Crawford, MI
26	WPS4MainePrimer	PZ171130	WPS	2015	Crawford, MI
27	WPS6MainePrimer	PZ171131	WPS	2015	Hillsborough, NH
28	WPS7MainePrimer	PZ171132	WPS	2015	Crawford, MI
29	WPS8MainePrimer	PZ171133	WPS	2015	Habersham, GA
30	WPS9MainePrimer	PZ171134	WPS	2015	Gilmer, GA
31	WPS10MainePrimer	PZ171135	WPS	2015	Murray, GA
32	WPS11MainePrimer	PZ171136	WPS	2015	Monroe, TN
33	WPS12MainePrimer	PZ171137	WPS	2015	Monroe, TN
34	WPS13MainePrimer	PZ171138	WPS	2015	Bland, VA
35	WPS14MainePrimer	PZ178828	WPS	2015	Madison, NC
36	WPS15MainePrimer	PZ178829	WPS	2015	Madison, NC
37	WPS16MainePrimer	PZ178830	WPS	2015	Madison, NC
38	WPS17MainePrimer	PZ171139	WPS	2015	Macon, NC
39	WPS18MainePrimer	PZ178831	WPS	2015	Alleghany, VA
40	WPS19MainePrimer	PZ171140	WPS	2015	Clarion, PA
41	WPS20MainePrimer	PZ171141	WPS	2015	Pocahontas, WV
42	WPS21MainePrimer	PZ171142	WPS	2015	Bayfield, WI
43	WPS22MainePrimer	PZ171143	WPS	2015	Bayfield, WI
44	WPS23MainePrimer	PZ171144	WPS	2015	Delta, MI
45	WPS24MainePrimer	PZ171145	WPS	2015	Delta, MI

Table A2. Predicted mean RPS counts (estimated marginal means; EMMs) by trap type from generalized linear mixed-effects models. Estimates are adjusted for the random effects of sampling timepoint and location and are presented with 95% confidence intervals (CIs). Values represent expected RPS captures for each trap type in New Hampshire (2024) and Maine (2025).

Data	Trap Type	Predicted Counts	95% CI
2024 - NH	3D Printed trap	0.3	0.1 - 0.7
	Canopy Pitfall trap	41	18.9 - 89.2
	Green sticky trap	0.7	0.3 -1.7
2025 - ME	3D Printed trap	2.3	0.8 - 6.9
	Canopy Pitfall trap	5.8	1.9 - 17.1
	Green sticky trap	3.1	1.0 - 9.0

Table A3. Incidence rate ratios (IRR), 95% confidence interval (CI) values and p-values from pairwise comparisons at the sites in New Hampshire (2024) and Maine (2025).

2024 New Hampshire			
Comparison	**IRR**	**CI 95%**	**p-value**
Canopy pitfall trap vs 3D printed trap	135	82.6-222	<0.0001
Canopy pitfall trap vs green sticky trap	55.31	37.00-82.67	<0.0001
Green sticky vs 3D printed trap	2.48	1.48-4.07	0.0001
2025 Maine			
Comparison	**IRR**	**CI 95%**	**p-value**
Canopy pitfall trap vs 3D printed trap	2.48	1.40-4.41	0.0006
Canopy pitfall trap vs green sticky trap	1.897	1.106-3.256	0.015
Green sticky vs 3D printed trap	1.31	0.766-2.24	0.47

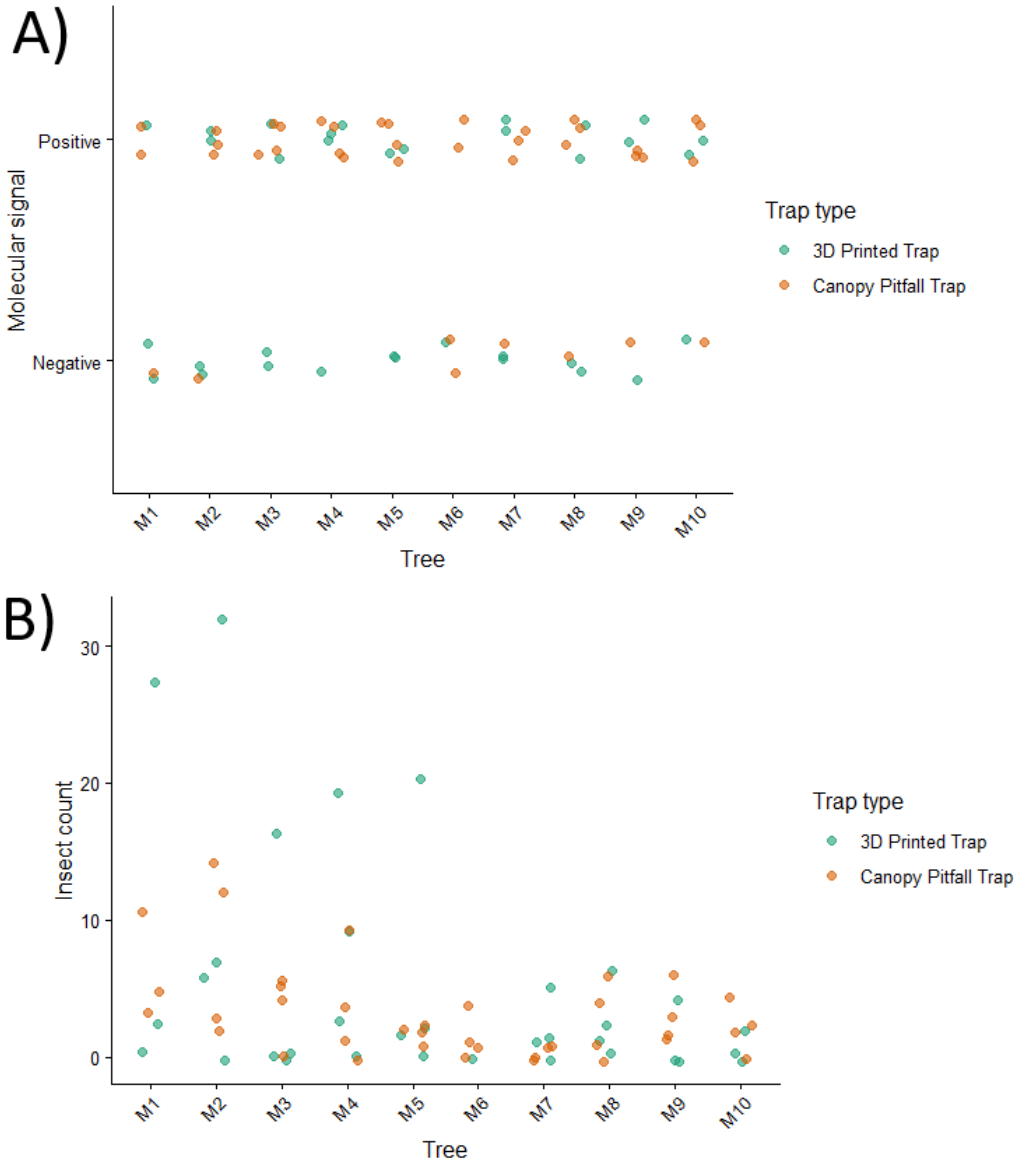


Figure A1. Total per-tree molecular signal and *Matsucoccus matsumurae* (RPS; red pine scale) capture in the Maine 2025 site. Colour-coding shows trap type. Each point represents one of four timepoints for each of the two traps - 3-D printed and canopy pitfall with some points omitted due to inconclusive molecular signals. A) Positive or negative molecular signal detected at each tree with the jitter function to reduce overplotting. B) Number of RPS captured at each tree location.

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Pureswaran DS, M Stone, C Hughes, T Swanburg, J Garnas, T Rounsville and M Williams. 2025. Threat of red pine scale, *Matsucoccus matsumurae,* to Canada. 68th National Forest Pest Management Forum. Ottawa, ON, Canada.

Williams M, M Stone, C Hughes, T Swanburg, J Garnas, T Rounsville and DS Pureswaran. 2026. Molecular surveillance of an advancing forest invasive: eDNA assay development for red pine scale (*Matsucoccus matsumurae*). International Union of Forest Research Organizations Meeting of Working Groups. Fredericton, NB, Canada.