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METHOD

Development of novel LAMP and qPCR assays for rapid and specific identification of Bronze birch borer (*Agrilus anxius*)

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Abstract

Buprestids are an emerging threat to broadleaf forests across the world. Bronze birch borer (*Agrilus anxius*, BBB) poses a serious threat to European birch species if the insect were to be introduced. Due to their cryptic lifestyle feeding on the vascular tissue of their host plants, buprestids and other wood borers can be difficult to observe or detect. Early detection tools are vital to swiftly implement eradication measures and prevent the establishment of introduced species. In this study, we developed novel qPCR and LAMP assays for BBB and investigated the specificity and sensitivity for their use as early detection tools in European forests. Plant chemicals may limit these assays, so we conducted sensitivity testing with extracted foliage and plant vascular tissues to determine potential inhibition effects on DNA amplification. Both assays were specific to the target species when tested against the DNA of 17 other European *Agrilus*/buprestid species, two Scolytinae, and five Cerambycids ($N = 24$). Both assays varied in sensitivity with the qPCR assay amplifying at a concentration as low as 20 fg/μL, whereas the LAMP assay amplified as low as 3.2 pg/μL. Plant chemicals in DNA extracts from leaves did not impact the sensitivity of either assay, reaching similar detection levels. In contrast, vascular tissue reduced the sensitivity of the LAMP assay, amplifying as low as 0.04 ng/μL compared with 0.008 ng/μL in the control. These results demonstrate that both assays are highly specific and sensitive tools that can be used to detect frass and identify larvae as well as monitor the spread of *A. anxius*. qPCR resulted in more sensitive than LAMP overall. Thus, if results are needed quickly to make fast management decisions or as an initial screening of samples, the LAMP method is optimal. However, if fine detection is critical, then qPCR is preferential.

KEYWORDS
bronze birch borer, early detection, emerald ash borer, forest entomology

1 | INTRODUCTION

Invasive pests cost billions of US dollars yearly in forest production, killing ecologically and economically important trees, and leading to reduced ecosystem services (Bradshaw et al., 2016). As these insects as pests and pathogens circumvent geographical barriers, they can establish viable populations (Paap et al., 2022; Tobin et al., 2014), which can grow rapidly to economically damaging levels that quickly decrease the chance of eradication (Tobin et al., 2014). Early detection tools play a critical role in reducing the impact of invasions by preventing pest introductions (Luchi et al., 2020) or, if the pest is already present, by rapidly delineating their range and monitoring their dispersal for more targeted management efforts (Martinez et al., 2020; NISC, 2016; Tobin et al., 2014). Epanchin-Niell et al. (2012) and Tobin et al. (2014) have demonstrated that biosurveillance tools can lower the environmental and economic costs of invasive species by increasing the probability of detecting these pests at an early enough stage that mitigation measures could be feasible. Yet, a major ongoing issue with detecting pests and pathogens is the limited ability of traditional surveys (e.g., traps, visual checks) and especially sufficient resources (personnel) to determine the presence of target taxa when those organisms are uncommon, rare, or in low abundance (Epanchin-Niell & Hastings, 2010; Epanchin-Niell et al., 2012; Tobin et al., 2014). However, surveys using environmental DNA (eDNA) detection tools offer a solution to aid in finding target populations at low or rare abundance. These methods have advanced aquatic sampling research for decades by reliably increasing detection probabilities through their ability to pick up DNA signatures left behind by target organisms without ever having to see them (Darling & Blum, 2007; Jerde et al., 2011) and have also gained traction in invasive species detection (Allen et al., 2021; Valentin et al., 2018; Valentin, Fonseca, et al., 2020; 2021) and biodiversity monitoring of terrestrial landscapes more recently (Allen et al., 2023).

In terrestrial systems, DNA inhibitors, that is, compounds that can prevent or limit the amplification of DNA, are present throughout the environment. These compounds may include pectins, polyphenols, polysaccharides, xylan, or humus, all of which can inhibit PCR reactions (Opel et al., 2010; Schrader et al., 2012; Stoeckle et al., 2017), leading to false negative results (Edagawa et al., 2009). The volume and number of inhibitor compounds can vary among tree species and tissue types, thus it is worth evaluating the potential inhibition of DNA and setting the limits of detection in the laboratory and under natural conditions (McKee et al., 2015; Opel et al., 2010).

Buprestids are wood borers that as larvae feed on vascular tissues in the inner bark and as adults become sexually mature from feeding on foliage. They are historically known as secondary pests, attacking and killing stressed hosts (e.g., Haack & Petrice 2019; Sallé et al., 2014), but most recently been recognized as a highly destructive group especially in forests where they have been introduced. In the past several decades, these beetles have been increasingly observed causing major damage to evolutionarily naïve hosts. *Agrilus planipennis* (emerald ash borer, EAB) is the primary example of a buprestid being extremely destructive and costly (Aukema et al., 2011). This species causes high mortality rates that nearly 99% on some evolutionarily naïve species of North American ash (*Fraxinus* spp.) that dominate broadleaf forests in Canada and the USA (Klooster et al., 2014). Not only has *A. planipennis* cost billions of US dollars, but it has had a detrimental ecological effect on ash forests from local extirpation of specialist herbivorous insects (e.g., Gandhi & Herms, 2010a, 2010b; Wagner & Todd, 2016) to wider ecosystem-level changes such as gap formations and influencing biogeochemical cycling of nutrients (Gandhi & Herms, 2010a).

Agrilus anxius (bronze birch borer, BBB) is a specialist buprestid that is native to North America and a major pest of birch (*Betula* spp.) where it occurs in boreal and north temperate forests, and of ornamental birch in urban landscapes (Muilenburg & Herms, 2012). This insect generally attacks trees that have been weakened or injured by biotic agents or other abiotic factors. Symptoms of BBB are typically characterized by crown thinning, early discoloration of foliage, branch and crown dieback, and swelling of the bark on the branches and trunk of trees attacked by the insect (Figure 1a,b). Signs of BBB include serpentine galleries bore by larvae under the bark in the vascular tissue and D-shaped exit holes from adult emergence (Figure 1c–f). Often in areas such as western Canada, BBB is part of a complex known as "birch decline," where consecutive years of attack are triggered by extreme stress events such as drought and can predispose trees to attack by BBB, causing a progressive decline of the branches in the crown, and eventual tree mortality.

If introduced to Europe, BBB would threaten to cause catastrophic damage in European birch populations (Muilenburg & Herms, 2012) similar to the damage EAB has caused in ash forests in North America (Herms & McCullough, 2014; Klooster et al., 2014). This is particularly true in European birches, that is, *Betula pendula* and *B. pubescens*, as these species are highly susceptible to BBB infestation due to evolutionary naivety (Muilenburg & Herms, 2012). In a common garden study in the USA, mortality in planted European birch was as high as 100%. Birch is one of the most important and dominant species in forest stands in Latvia, accounting for as much as 28% of the standing forest (Hynynen et al., 2010). Similarly, in Sweden and Finland, birch comprises between 12% and 16% of the total volume of growing stock, respectively (Hynynen et al., 2010; Skogsdata, 2023) and it is one of the most represented broadleaf species in Scandinavian forests. At present, BBB is a quarantine pest for the European Union (2014/78/EU) and is on the European Plant Protection Organization (EPPO, 2022) A1 list of quarantine pests (EPPO A1 List). In a global study, Seebens et al. (2017) emphasized that the increase in numbers of alien species worldwide shows no sign of saturation in the near future. Thus, to increase our readiness to deal with a potential invasion by BBB to Europe, early detection and monitoring tools are needed that would allow for mitigation measures to be swiftly implemented to prevent its establishment and potential spread.

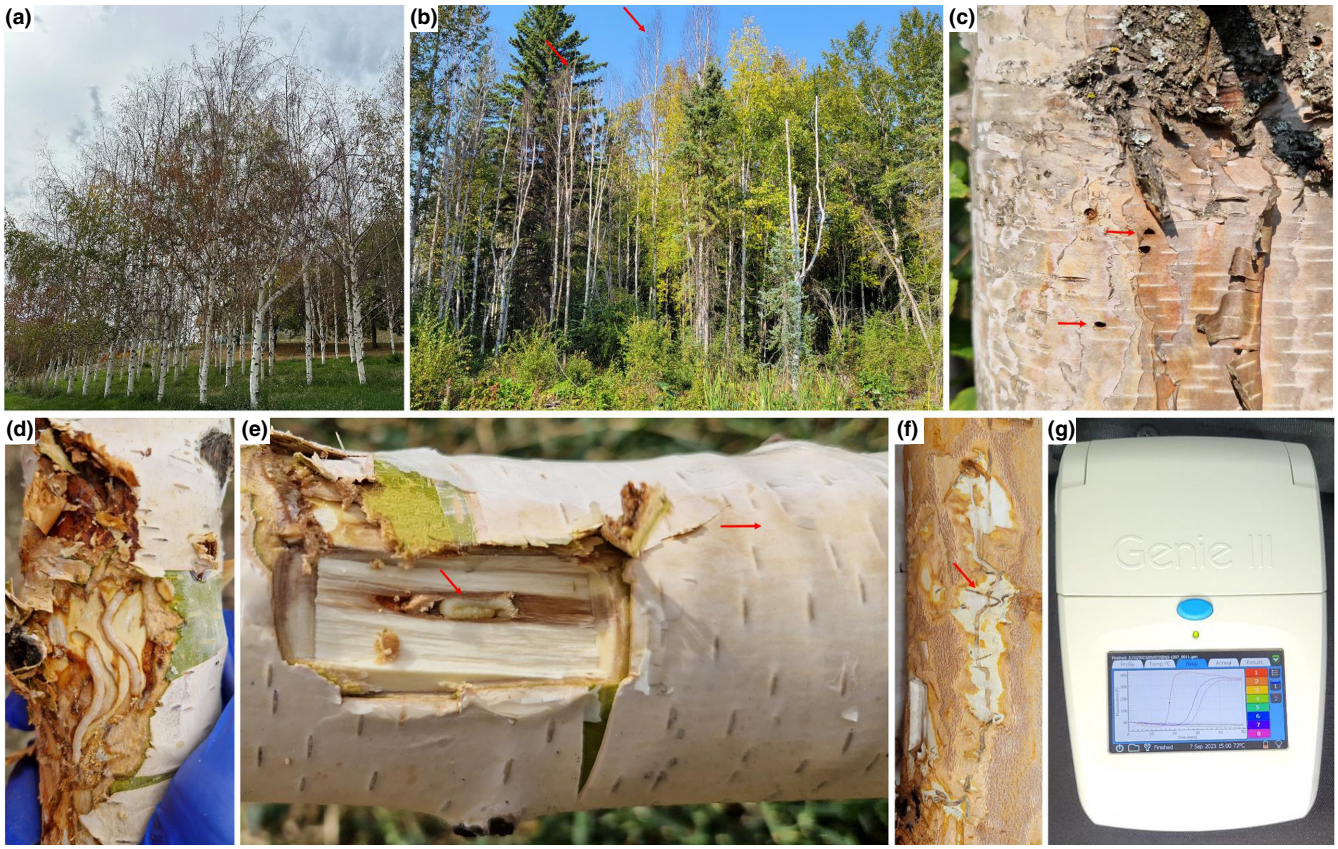

FIGURE 1 (a) BBB-infested silver birch (*Betula pendula*) trees at the Bailey Seed Orchard in British Columbia showing extensive dieback of the crown and tree mortality; (b) dead and dying paper birch (*B. papyrifera*) trees infested with BBB near Smoky Lake, Alberta; (c) characteristic D-shaped exit holds on a paper birch tree; (d) swellings on the branch of a silver birch tree and BBB larvae feeding in phloem; (e) BBB larva in overwintering J-shaped stage and branch swelling on silver birch; (f) galleries on silver birch filled with BBB frass; (g) Genie III (Optigene) instrument indicating positive run, field-validating the LAMP assay on eDNA samples.

Recently, an increasing number of molecular diagnostic methods have been developed, which have allowed for significant advances to be made to detect and identify harmful pests, thereby improving plant biosecurity. In particular, portable DNA-based equipment, which operates with great simplicity, sensitivity, specificity, and high speed, can detect pests in the early stages of attack when symptoms are not yet clearly visible or detect traces of their presence in other forms (Kyei-Poku et al., 2020; Luchi et al., 2020; Peterson et al., 2023). The application of portable detection tools in plant biosecurity is a real game-changer when it comes to preventing new invasions. One technology that has been adapted for rapid in-field detection using a portable PCR is loop-mediated isothermal amplification (LAMP) (Notomi et al., 2000), which is known for its robust and highly sensitive and specific amplification of target DNA, making it a prime candidate for low-cost, rapid diagnostics for point-of-need testing. In this work, we designed quantitative real-time polymerase chain reaction (qPCR) and LAMP assays for early detection of bronze birch borer eDNA. Buprestids, like BBB, spend up to 2 months as adults on foliage where the eDNA can accumulate from feeding while resting on foliage and similarly accumulate in vascular tissue where larvae spend the majority of their lives. For this reason, we also tested whether eDNA of BBB detected using the developed LAMP and qPCR assays is influenced by plant chemicals derived from those tissues.

2 | METHODS

2.1 | Insects collection and genomic DNA extraction

All insects used in this study were trapped in broadleaf forests in Europe and North America (Table 1). The targeted species were woodboring insects including bark beetles and cerambycids which are common forest pests. Buprestids and *Agrilus* were especially targeted since they are the closest relatives of BBB. Insects were collected in multifunnel traps which are commonly used for the collection of buprestids (Francese et al., 2013; Rassati et al., 2019; Sallé et al., 2020). A total of 29 insect specimens were collected: 6 *A. anxius*, 13 different *Agrilus* species, 3 other Buprestidae, 2 Curculionidae, and 5 Cerambycidae. Insects from Italy were kindly provided by Davide Rassati (UNIPD). Samples of *A. pensus*, a close relative of *A. anxius* were attempted to be gathered from collaborations since

TABLE 1 Insect species used for specificity testing for *Agrilus anxius* LAMP and qPCR assays.

Insect species	Sample name	Geographic origin	Locality/longitude latitude	PCR primer pairs[a]	Accession nos.	LAMP/qPCR results[b]
Buprestidae						
Agrilus angustulus	AA	France	2.0327° 47.2611°	LCO1490/HCO2198	OQ318853	−/−
Agrilus anxius	BBB1	USA	41.7662° 72.6746°	LCO1490/HCO2198	OQ318858	+/+
Agrilus anxius	BBB2	USA	41.7662° 72.6746°	LCO1490/HCO2198	OQ318859	+/+
Agrilus anxius	BBB3	USA	41.3544° 72.1000°	LCO1490/HCO2198	OQ318860	+/+
Agrilus anxius	BBB11	USA	41.8705° 72.3676°	LCO1490/HCO2198	OQ318861	+/+
Agrilus anxius	BBB12	USA	41.4466° 72.5459°	LCO1490/HCO2198	OQ318862	+/+
Agrilus anxius	BBB13	USA	41.3544° 72.1000°	LCO1490/HCO2198	OQ318863	+/+
Agrilus anxius	BBB20	Canada	46.5034° 84.3054°	LCO1490/HCO2198	NA	+/+
Agrilus anxius	BBB21	Canada	46.5034° 84.3054°	LCO1490/HCO2198	NA	+/+
Agrilus anxius	BBB22	Canada	46.5034° 84.3054°	LCO1490/HCO2198	NA	+/+
Agrilus ater	AAT	France	2.0327° 47.2611°	28S up/28S low	NA	−/−
Agrilus convexicollis	AC	France	1.9383° 47.8456°	16a/16b	NA	−/−
Agrilus curtulus	Acu	France	2.1187° 47.2653°	28S up/28S low	NA	−/−
Agrilus graminis	AG	France	1.9383° 47.8456°	LCO1490/HCO2198	OQ318855	−/−
Agrilus hastulifer	AH	France	1.9383° 47.8456°	28S up/28S low	NA	−/−
Agrilus laticornis	AL	France	1.9383° 47.8456°	28S up/28S low	NA	−/−
Agrilus obscuricollis	AO	France	1.9383° 47.8456°	28 s up-28 s low	NA	−/−
Agrilus olivicolor	AD	France	1.9383° 47.8456°	LCO1490/HCO2198	NA	−/−
Agrilus planipennis	EAB	USA		LCO1490/HCO2198 EAB_COIF/EAB_COIR	OQ318865	−/−
Agrilus roscidus	AR	France	13.120926° 45.791784°	LCO1490/HCO2198	OQ318856	−/−
Agrilus sulcicollis	AS	France	2.0327° 47.2611°	LCO1490/HCO2198	OQ318857	−/−
Agrilus viridis	AV	France	1.9383° 47.8456°	28S up/28S low	NA	−/−
Anthaxia nitidula	AN	France	3.3619° 46.2461°	LCO1490/HCO2198	NA	−/−
Chrysobothris affinis	CA	France	2.1544° 47.2899°	28S up/28S low	NA	−/−
Coraebus undatus	CU	France	1.9383° 47.8456°	LCO1490/HCO2198 28S up/28S low	OQ318864	−/−

TABLE 1 (Continued)

Insect species	Sample name	Geographic origin	Locality/longitude latitude	PCR primer pairs[a]	Accession nos.	LAMP/qPCR results[b]
Lamprodila mirifica	LM	Italy	13.120926° 45.791784°	28S up/28S low	NA	−/−
Curculionidae: Scolytinae						
Anisandrus dispar	A dis	Italy	13.120926° 45.791784°	LCO1490/HCO2198	OQ318854	−/−
Xyleborinus saxesenii	XS	Italy	13.120926° 45.791784°	LCO1490/HCO2198	OQ318869	−/−
Cerambycidae						
Aegomorphus clavipes	Aeg	Italy	13.120926° 45.791784°	28S up/28S low	NA	−/−
Exocentrus punctipennis	EP	Italy	13.120926° 45.791784°	LCO1490/HCO2198	OQ318866	−/−
Leiopus nebulosus	LN	Italy	13.120926° 45.791784°	LCO1490/HCO2198	OQ318867	−/−
Saperda punctata	SP	Italy	13.120926° 45.791784°	LCO1490/HCO2198	OQ318868	−/−
Trichoferus pallidus	TN	France	1.9383° 47.8456°	LCO1490/HCO2198 28S up/28S low	NA	−/−

Note: All DNA samples were checked for amplifiability by using different PCR primer sets.

[a]PCR was performed by using different primer pairs to amplify different target genes: LCO1490/HCO2198 (COI gene, Folmer et al., 1994) (COI gene, Folmer et al., 1994); 28S up/28S low (28S gene, Büsse et al., 2012); EAB_COIF/EAB_COIR (COI gene, Kyei-Poku et al., 2020).

[b]Each DNA sample was tested in two replicates. +: positive amplification; −: negative amplification.

DNA sequences deposited in NCBI Genbank display a 99% similarity; however, no samples could be found due to this species appearing to be uncommon or rare (pers. observation C. Rutledge).

Each individual specimen (Table 1) was washed three times in distilled sterile water and then the legs were removed using flame-sterilized forceps. The number of legs used for specimen extractions ranged from three on larger specimens like EAB and BBB, and up to all six for smaller individuals like *A. ater* and *A. laticornis*. A full body extraction was conducted with the two ambrosia beetles (*Xyleborinus saxesenii* and *Anisandrus dispar*), due to small size, to obtain enough genetic material for PCR amplification. For EAB and BBB only, legs were manually crushed with a mini-pestle inside the 1.5 mL tube. All other specimens (legs or full body) tissues were crushed by adding two sterile metal beads to 2.0 mL microcentrifuge tubes and running the tubes in a TissueLyser (Qiagen) for 1–2 min at 300 oscillations/min. DNA extractions from collected insects (Table 1) were then performed by using JetQuick Blood and Cell Culture (Invitrogen) and DNeasy Blood and Tissue kits (Qiagen) (Peterson et al., 2023).

2.2 | PCR amplification and molecular identification of insect species

The DNA extracts obtained from the insect's body were tested on all specimens (Table 1) using different general insect primer sets (LCO1490/HCO2198, 16A/16B, and 28S_up/28S_low) in order to amplify different target genes as described in Peterson et al. (2023). PCR amplification and visualization of the amplified fragments by electrophoresis were performed according to Peterson et al. (2023).

Part of the COI region was also sequenced for a subset of insect samples (see Table 1). Amplified DNA of those individual samples was purified using *mi*-PCR purification Kit (Metabion International) and sent to Macrogen Europe for sequencing. The identification of each specimen was performed by using The Basic Local Alignment Search Tool (BLAST) searches in the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to find the region of local similarity between GenBank sequences and our PCR amplicons. All sequences obtained in this study have been published in GenBank and the accession numbers are reported in Table 1.

2.3 | LAMP and real-time qPCR assays to detect *Agrilus anxius*

Two new marker sets, one for the LAMP-based assay and another for a qPCR assay, were designed to amplify *A. anxius* DNA. Six LAMP primers were designed using LAMP Designer software (OptiGene Limited) to target a fragment of the cytochrome oxidase subunit I (COI) gene (sample BBB2 sequence; Accession no. OQ318859, Table 1). Forward and Reverse qPCR primers and TaqMan® MGB probe were designed using Primer Express Software 3.0 (Applied Biosystems) in the same genetic region (BBB2 sample). Primers and TaqMan probe sequences are shown in Table 2. All primers were provided by Eurofins Genomics while, the TaqMan® MGB probe was from Life Technologies Italy. The

TABLE 2 LAMP and qPCR markers designed for *Agrilus anxius*.

Molecular assay	Oligo name	Tm (°C)	Sequence (5′–3′)	Length (bp)
LAMP	Aanx_F3	55.9	TTAATATTAGGCGCACCTGAC	21
	Aanx_B3	56.5	GCTCGCATATTAATTACAGTGG	22
	Aanx_LoopF	57.3	TCCCGCACCTCTTTCTACTA	20
	Aanx_LoopB	58.4	GGCTCCTCTGTAGATTTAGCAA	22
	Aanx_FIP	73.3	AGGCGGGTAAACAGTTCAACCCCTCCATCACTGACTTTACTTT	43
	Aanx_BIP	72.2	TTAGCCGCTAACATTGCTCACAAGAAATACCAGCTAAATGGAGG	44
qPCR	Aanx_For	59	GTGCGGGAACTGGTTGAACT	20
	Aanx_Rev	58	AGCCACTGTGAGCAATGTTAGC	22
	Aanx_Probe[a]	68	TTTACCCGCCTTTAGC	16

Note: LAMP primers were designed using the software LAMP Designer (OptiGene) on the basis of the consensus sequence of cytochrome oxidase subunit I (COI) gene. The *A. anxius* TaqMan® MGB probe and primers were designed using the Primer Express® Software 3.0 (Applied Biosystems) on the basis of the same previously described sequences (COI).

[a]TaqMan® MGB probe was labeled with 6-carboxy-fuorescein (FAM) at the 5′ end and a nonfuorescent quencher (NFQ) with minor groove binder (MGB) ligands as quencher, at the 3′ end.

specificity of the designed LAMP and qPCR markers (Table 2) was initially tested using BLAST® (Basic Local Alignment Search Tool; http://www.ncbi.nlm.nih.gov/BLAST) software in silico. Sequences with high similarity to the *A. anxius* LAMP and qPCR amplicons were retrieved from the GenBank database and aligned with the Consensus Alignment software implemented in Geneious 10.2.6, set with default parameters.

The specificity of the LAMP and qPCR assays was also tested by using DNA extracted from the selected co-occurring insects from European fauna (Table 1). For LAMP assay testing, the reaction volume consisted of 25 μL total with 0.5 μL of each F3 and B3 (at final concentration of 0.2 μM each), 1.0 μL of each Loop F and Loop B (at final concentration of 0.4 μM each), 2.0 μL of each FIP and BIP (at final concentration of 0.8 μM each), 3.0 μL of extracted DNA, and 15 μL of isothermal master-mix (OptiGene). The reactions took place in a Genie II (Optigene) portable device used for isothermal DNA amplification. Each sample was run at least in duplicate reactions. In each run, one positive (BBB) control and one negative (NTC, No Template Control) were also included. LAMP amplification reactions were run at 65°C for 30 min, followed by an annealing analysis from 98 to 80°C with ramping at 0.05°C per second to allow the generation of derivative melting curves. To assess the positivity of a sample, the following parameters were determined: amplification time (t_{amp}; expressed in min) and amplicon annealing temperature (T_a; expressed in °C).

Real-time PCR assay reactions were performed by using the Step One Plus™ Real-Time PCR System (Applied Biosystems). PCR amplification was performed in a 25 μL final volume containing: 12.5 μL TaqMan Universal Master Mix (Applied Biosystems), 300 nM forward primer, 300 nM reverse primer, 200 nM TaqMan MGB probe, and 5 μL genomic DNA (DNA from insect body was diluted to 0.4 ng/μL). Each DNA sample was tested in two replicates. Two wells, each containing 5 μL of sterile water, were used as the NTC. The PCR protocol cycling parameters were 50°C (2 min), 95°C (10 min), 50 cycles of 95°C (30 s), and 60°C (1 min). Results were analyzed with an SDS 1.9 sequence detection system (Applied Biosystems) after manual adjustment of the baseline and fluorescence threshold.

2.4 | Sensitivity LAMP and qPCR assays

The lower limit of detection (LOD) of the LAMP and qPCR assays was determined by generating a standard curve from 1:5 serial dilutions of a known concentration of *A. anxius* DNA (BBB1 sample—Table 1). For LAMP standards, the DNA concentration ranged from 2.0 ng/μL to 0.02 pg/μL, while for qPCR standards, it ranged from 0.4 ng/μL to 1 fg/μL. Each concentration was run in duplicate in each of the LAMP and qPCR protocols. DNA samples were run at cycling parameters as previously described for LAMP and qPCR.

2.5 | Sensitivity of *A. anxius* LAMP and qPCR assays in plant tissue

In late spring 2022, fully expanded fresh leaves (for adult detection) and vascular (phloem) tissue, after removing the outer bark, were collected from healthy silver birch (*B. pendula*) trees and immediately processed for extraction. Plant tissues (100 mg wet weight) were ground in liquid nitrogen and extracted following the EZNA Plant DNA kit (Omega Bio-tek) protocol. The concentration of extracted DNA samples was measured using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies). To evaluate the possible PCR interference from the presence of plant material, each extracted plant material (leaf and vascular/phloem tissue) had an aliquot of *A. anxius* DNA (BBB1 sample) in a series of fivefold dilutions (ranging from 1 to 0.0016 ng/μL). Background extractions of

healthy silver birch vascular tissue and leaf (at 10 ng/tube nucleic acid each) were run on the same qPCR plate as dilutions used for the standard curve (insect DNA diluted in sterile ddH_2O). All samples were run in triplicate, using the LAMP and qPCR protocols previously described.

2.6 | Field validation of LAMP and qPCR assays

To validate the LAMP assay on eDNA collected in the field, in September 2023, we collected larvae and frass samples from native paper birch (*Betula papyrifera*) in Alberta and British Columbia, Canada and exotic European silver birch (*B. pendula*) in British Columbia. Three sites were selected that showed clear evidence of BBB infestation; one forest site near Smoky Lake, Alberta (Figure 1b) and two seed orchards in the southern interior of British Columbia (Figure 1a). Site, species, and sample information is given in Table 4. At Smoky Lake, mature paper birch trees exhibiting branch dieback and D-shaped exit holes (Figure 1c) were felled using a chainsaw and bucked to shorter lengths. Several logs were debarked in the field to reveal BBB galleries containing frass and larvae (Figure 1d–f). Logs, seven to eight, were brought to the laboratory at the Canadian Forest Service—Northern Forestry Centre in Edmonton for further dissection and collection of samples.

At Skimikin Seed Orchard in British Columbia, a provenance trial established in 2008 containing both paper birch and silver birch (Finnish accessions) was inspected for BBB damage. The paper birch did not have fresh signs of BBB but displayed older signs of callusing wounds and aged exit holes. However, the planted silver birch showed severe symptoms of BBB in the form of branch and crown dieback and D-shaped exit holes. Symptomatic European birch trees were sampled by debarking the stem with a chisel and knife to collect frass and larvae. Another seed orchard trial at Bailey (BC), planted only with silver birch (same accessions as at Skimikin), was inspected for BBB where it was found to be severely affected and a large proportion of trees had been killed (Figure 1a). Several symptomatic branches were cut from the still-alive trees using an electric hand saw. The branches were debarked using a chisel and knife, and frass and larvae samples were collected in the field.

In some trees, larvae appeared to be in an overwintering stage and were under the vascular cambium. Most larvae were easily found by removing the vascular tissues using a hammer and a chisel or a knife around areas of swollen bark or exit holes and careful attention was paid to avoid crushing the larvae. Once galleries along sections of the logs or branches were revealed, frass was collected into 2 mL tubes by carefully scratching the frass using forceps or a scalpel along the length of the gallery. Larvae were placed into 2 mL tubes containing 70% ethanol for long-term preservation of the samples during shipping. Fresh gloves were worn throughout the sampling and tools were disinfected to avoid contamination between samples.

2.7 | DNA extraction and LAMP validation in the field

DNA extraction was conducted separately for both frass and larvae in the field using the plant material lysis kit (OptiGene Limited). Each sample was put into a fresh tube with a steel ball bearing. Approximately 40–80 mg of frass was used for DNA extractions. One whole larva per DNA extraction was used. To each tube with a ball bearing, 500 μL of lysis buffer was added and the tube was shaken vigorously for approximately 1 min. After the sample was crushed and a homogeneous solution obtained, approximately 200 μL of the sample solution was pipetted into a 1 mL dilution vial that is supplied within the DNA extraction kit.

The LAMP reaction was carried out in a Genie III instrument (Optigene, Figure 1g) following the specific LAMP protocol for BBB mentioned previously. The mastermix was prepared at the Canadian Forest Service laboratory the day before sampling in the field and stored frozen overnight. One positive (BBB) and one negative control (NTC, no template control) were included in each run. Each sample was run in duplicate. Field-extracted DNA samples were also intended to be tested with qPCR for confirmation of the LAMP field results; however, delays that occurred during shipping caused degradation of the DNA samples and they could not be used.

2.8 | Optimization of the field extraction protocol to detect frass with LAMP and qPCR

After the field validation of the LAMP assay in Canada, frass and larvae samples collected from the field and DNA extracts were shipped to the Forest Pathology Laboratory at SLU in Alnarp, Sweden for further analysis. For standardization of the DNA extraction for the field protocol, the LAMP reaction was carried out in a Genie II (Optigene) for larvae and frass using different DNA extraction dilution ratios and different amounts of starting weight for frass samples collected from each site in Canada. Three fresh tubes with ball bearings were prepared per site, each containing 20, 40, and 80 mg of frass. To each tube, 1 mL of lysis buffer was added and then tubes were shaken vigorously for approximately 1 min. Thereafter, two dilution vials were prepared per sample by adding 100 or 200 μL of the crushed sample solution. In total, six different ratios were tested per site; 18 samples in total. Each sample was run in duplicate for LAMP and in triplicate for qPCR.

3 | RESULTS

3.1 | LAMP and real-time qPCR assays to detect *A. anxius*

All three general insect primers successfully amplified the DNA of BBB and other non-BBB specimens (Table 1), allowing the DNA extracts to be used to fine-tune the new *A. anxius* LAMP and qPCR methods.

Six LAMP primers were designed to amplify BBB DNA (Table 2). The first two were external primers (Aanx_F3 and Aanx_B3), the second set was two inner primers (Aanx_FIP and Aanx_BIP), and the final two were loop primers (Aanx_LoopF and Aanx_LoopB). A set of qPCR primers and a TaqMan MGB probe were also designed to target a species-specific region within the BBB genome (Table 2).

The BLAST search in NCBI demonstrated high specificity of the LAMP and qPCR amplicon sequences with *A. anxius* (LAMP amplicon: percentage of Identity = 100%; E-value = 4e-70; qPCR amplicon: percentage of Identity = 100%; E-value = 2e-20). No significant homology was found with any other closely related species (Figures S1 and S2).

Both the LAMP and qPCR assays confirmed positive amplification for *A. anxius* DNA (Table 1), while all non-target DNA samples did not amplify (Table 1). The LAMP assay exhibited a single melting peak with diagnostic temperature (85.0 ± 0.2°C) for *A. anxius* samples, allowing specific identification of the target pest (Figure 2b).

The qPCR standard curve had a slope of −3.315, an R^2 coefficient of 0.994, and a *Y*-intercept of 34.02. Efficiencies of the qPCR runs were 1.04 ± 0.05 (SD) (Figure 2d). Reproducibility of the *A. anxius* standard curve was evaluated for each standard dilution point on the basis of *Ct* values. The %CV obtained from six different standard curves ranged from 0.8% to 4%. Measurements of linearity such as slope, *y*-intercept, and correlation coefficient (R^2) did not vary significantly among the qPCR runs (%CV < 3.5%).

The lowest limit of DNA detection in these assays was 3.2 pg/μL for LAMP (Figure 2a) and 20 fg/μL for qPCR (Figure 2c).

3.2 | Sensitivity of *A. anxius* LAMP and qPCR assays in plant tissue

Two experiments were conducted to determine the influence of leaf and vascular tissues on the detectability of target DNA and sensitivity of the BBB LAMP assay. In the first experiment, the LAMP assay was able to amplify a DNA concentration as low as 0.008 ng/μL for pure *A. anxius* DNA and for leaf tissues mixed with *A. anxius* DNA (Table 3), while the limit of detection for vascular tissue mixed with *A. anxius* DNA was 0.04 ng/μL.

In the second experiment, the qPCR assay was able to detect (from 1 to 0.0016 ng/μL) the pure *A. anxius* DNA, leaf, and vascular host tissues mixed with *A. anxius* DNA samples at each dilution step of DNA (Table 3). At each step, we detected the same concentration at the same cycle for each dilution (Table 3).

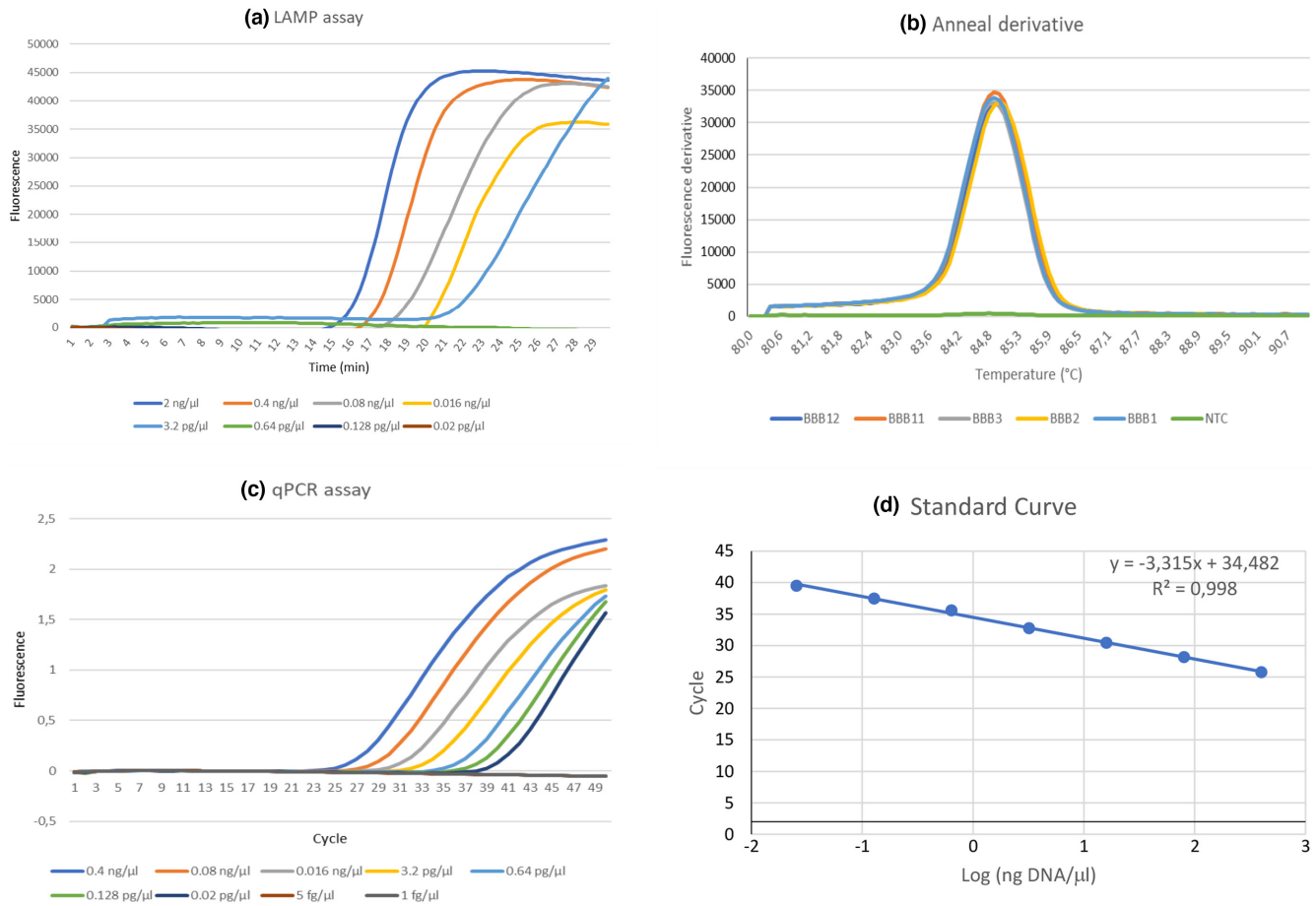

FIGURE 2 Detection of 1:5 serial dilution *Agrilus anxius* DNA by using LAMP and qPCR assays. (a) LAMP amplification plots with (b) melting curve. (c) Amplification plots of TaqMan qPCR assay (d) with standard curve.

TABLE 3 qPCR and LAMP detection of *Agrilus anxius* DNA 5-fold serial dilution mixed with (a) dd-H_2O, (b) birch leaf DNA (10 ng/μL), (c) birch vascular tissue DNA (10 ng/μL) (qPCR: Ct mean ± SE; LAMP: t_a = amplification time).

		A. anxius DNA serial dilution (ng/μL)				
Artificially mixed samples	**Molecular assay**	**1**	**0.02**	**0.04**	**0.008**	**0.0016**
A. anxius DNA + dd-H_2O	qPCR (Ct)	20.69 ± 0.06 (3/3)	23.22 ± 0.03 (3/3)	25.58 ± 0.04 (3/3)	28.81 ± 0.14 (3/3)	30.81 ± 0.08 (3/3)
	LAMP (t_a)	13:00 (3/3)	14:30 (3/3)	16:30 (3/3)	18:00 (3/3)	–(0/3)
A. anxius DNA + birch leaf DNA	qPCR (Ct)	20.74 ± 0.1 (3/3)	23.08 ± 0.06 (3/3)	25.28 ± 0.02 (3/3)	28.22 ± 0.09 (3/3)	30.30 ± 0.05 (3/3)
	LAMP (t_a)	13:30 (3/3)	15:00 (3/3)	17:15 (3/3)	–(0/3)	–(0/3)
A. anxius DNA + birch vascular tissue DNA	qPCR (Ct)	20.71 ± 0.1 (3/3)	23.04 ± 0.03 (3/3)	25.41 ± 0.01 (3/3)	28.16 ± 0.04 (3/3)	30.41 ± 0.04 (3/3)
	LAMP (t_a)	23:30 (3/3)	25:00 (3/3)	29:15 (3/3)	–(0/3)	–(0/3)

Note: Each sample was run in triplicate noted in brackets.

3.3 | Sensitivity of *A. anxius* LAMP and qPCR assays

The LAMP assay was field validated on eDNA samples (Figure 1g) collected from trees attacked by *A. anxius* in western Canada. Both frass and larvae resulted in positive detection of *A. anxius* from paper birch and silver birch at two sites (Table 4). In addition, *A. anxius* was detected from frass samples in each of the three different starting weights and dilutions that were tested with both qPCR and LAMP (Table 5).

4 | DISCUSSION

Insects are a hyperdiverse group of organisms. Beetles (Coleoptera) are the most speciose order of insects with 100,000s described species, and the Buprestidae contain *Agrilus*, the most speciose genus of beetles and animals, with 3000 known species. *Agrilus* spp. when in their native range are commonly known as secondary pests, typically attacking and killing weakened or stressed host plants (e.g., Haack & Petrice, 2019; Sallé et al., 2014). In recent decades, however, *Agrilus* spp. have crossed geographical barriers establishing in evolutionary naïve host tree populations, becoming major forest pests in the invaded region. With so many species in this genus, it is difficult to accurately identify species and often taxonomists that specialize in this species are needed. Additionally, the cryptic life cycle of these insects means that species may go undetected for decades, as was the case with emerald ash borer in North America (Siegert et al., 2014). Consistent methods are needed to quickly detect species of concern at ports of entry and for surveillance purposes to be able to rapidly implement management efforts and mitigation tactics. Molecular tools like conventional PCR and qPCR can be used to target specific regions of DNA when they are genetically conserved. A recent database has been built targeting three genetic regions to begin an easier method for the identification of *Agrilus* spp. (Kelnarova et al., 2019). To adequately detect a given species, target assay regions need to be unique to the species of concern while also being highly sensitive to low DNA quantities and not being adversely influenced by environmental inhibitors (Goldberg et al., 2016). For example, the EAB LAMP assay set up by Kyei-Poku et al. (2020) was targeted for use in North American forests. Specificity testing was done with mostly North American co-occurring *Agrilus* species. Peterson et al. (2023) explored the use of this LAMP protocol further by conducting specificity testing with European fauna since EAB is also becoming a pest in European ash forests (Orlova-Bienkowskaja et al., 2020). They found the assay to be specific when tested against 24 European insect species. In addition to specificity, Peterson et al. (2023) evaluated the lower detection limit of the EAB LAMP assay finding DNA concentrations as low as 0.02 pg/μL could be amplified, suggesting the assay is highly sensitive.

In this study, we successfully developed two molecular assays to detect *A. anxius*: an LAMP assay that can provide highly specific and rapid (max 30′) detection in the field and a laboratory qPCR

TABLE 4 LAMP detection of *Agrilus anxius* from field samples (frass and larvae) at three sites in Alberta and British Columbia.

			LAMP field result	
Site	**Coordinates**	**Sample type**	**Amplification time**	**Replications**
Smoky Lake, AB	Lat/Long	Larvae	t_a 28:15	(2/2)
	54.095152°, −112.251518°	Frass	t_a 35:30	(2/2)
Bailey Seed Orchard, BC	Lat/Long	Larvae	nt	
	50.188265°, −119.347089°	Frass	nt	
Skimikin Seed Orchard, BC	Lat/Long	Larvae	t_a 30:00	(2/2)
	50.784497°, −119.422754°	Frass	t_a 30:00	(2/2)

Note: LAMP samples were run in duplicate.

Abbreviation: nt, not tested.

TABLE 5 qPCR and LAMP detection of *Agrilus anxius* from frass samples collected in Canada with variable starting weights (20, 40, and 80 mg) and extract dilutions (100 µL and 200 µL).

	Tested variables of DNA frass extracts					
	20 mg		**40 mg**		**80 mg**	
Site	**100 µL**	**200 µL**	**100 µL**	**200 µL**	**100 µL**	**200 µL**
Smoky Lake, AB						
LAMP (t_a)	26:30 (2/2)	33:15 (1/2)	31:00 (2/2)	22:45 (1/2)	n.d. (0/2)	35:00 (1/2)
qPCR (Ct)	30.69 ± 0.19 (3/3)	29.07 ± 0.51 (3/3)	29.67 ± 0.29 (3/3)	29.62 ± 0.26 (3/3)	29.75 ± 0.29 (3/3)	29.56 ± 0.46 (3/3)
Bailey Seed Orchard, BC						
LAMP (t_a)	30:00 (2/2)	36:00 (1/2)	39:15 (2/2)	45:00 (1/2)	42:00 (1/2)	25:30 (2/2)
qPCR (Ct)	33.85 ± 0.74 (3/3)	34.44 ± 0.15 (3/3)	30.13 ± 0.19 (3/3)	29.58 ± 0.14 (3/3)	28.33 ± 0.16 (3/3)	28.15 ± 0.25 (3/3)
Skimikin Seed Orchard, BC						
LAMP (t_a)	24:45 (2/2)	31:45 (2/2)	44:00 (1/2)	32:00 (2/2)	25:15 (2/2)	24:00 (2/2)
qPCR (Ct)	31.24 ± 0.14 (3/3)	31.12 ± 0.05 (3/3)	31.01 ± 0.05 (3/3)	29.64 ± 0.27 (3/3)	30.88 ± 0.30 (3/3)	31.74 ± 0.27 (3/3)

Note: LAMP and qPCR samples were run in duplicate and triplicate, respectively. LAMP: t_a = amplification time; qPCR: Ct mean ± SE; replications shown in brackets.

assay that has a higher sensitivity and it is less impacted by the presence of plant inhibitors found in mock environmental samples but takes longer to obtain results. These assays for BBB were tested against 24 nontarget species belonging to the families Buprestidae, Curculionidae, sub-family Scolytinae, and Cerambycidae, chosen because they are common in European deciduous forests and, if BBB was introduced, would share the same habitat. No other species DNA amplified when tested. Together, both the qPCR and LAMP assays appear highly specific and provide two molecular methods for DNA detection of the target organism within environmental samples. These assays can be used to distinguish BBB from other buprestids in European ports of entry and forests.

Bronze birch borer is native to North American forests, where its closest relatives are found (Kelnarova et al., 2019). As such, future research needs to examine the specificity of the assays developed in Europe with these closely related North American species. In particular, *Agrilus pensus* is closely related to *A. anxius* and appears to have a high genetic similarity (99%) in the target region of our assays according to the few sequences publicly available on GenBank. However, this species is recorded to occur on hosts other than *Betula*, with the exception of *B. nigra* (Paiero, 2012), a North American birch species highly resistant to or is not a suitable host of *A. anxius* (Nielsen et al., 2011). Additionally, the few sequences of *A. pensus* present in the GenBank database may have been improperly identified and may actually be *A. anxius*, and ongoing research is being conducted to obtain and extract *A. pensus* specimens to adequately resolve this uncertainty (pers. comm. A. Roe).

Similar to Peterson et al. (2023), our study confirms that DNA from other cerambycids and ambrosia beetles failed to amplify with both assays, further supporting the specificity of the assay. Overall,

these data strongly support the findings of Kyei-Poku et al. (2020) and Peterson et al. (2023) that the LAMP primers appear highly specific to the target species, even when used among very speciose genera, such as *Agrilus*.

Critical to the use of eDNA assays is the ability to detect low concentrations of DNA since target DNA is often in a degraded state from breakdown in the environment or is scarce within the landscape. If the threshold of detection cannot be overcome, then our ability to effectively manage an organism at the early stage of invasion is threatened. Many studies have shown that insect DNA can be detected at quite low levels: for example, spotted lanternfly (*Lycorma delicatula*) DNA was detected at concentrations as low as 0.14 pg/μL with an optimized qPCR protocol (Allen et al., 2021). In addition, some developed LAMP assays were found to detect DNA concentrations as low as 0.61–16 pg/μL (Rizzo et al., 2020, 2021). Our results revealed that the BBB LAMP assay is 100× less sensitive (3.2 pg/μL) than the EAB LAMP assay (0.02 pg/μL; Peterson et al., 2023). The designed qPCR assay was much more sensitive for the detection of BBB (1000X higher) than the LAMP assay (0.005 pg/μL).

The results varied when we attempted to amplify BBB DNA with the LAMP assay in the presence of host tissue. The LAMP assay was successful at four of five dilution steps for control and leaf tissue. However, vascular tissue seemed to inhibit the detection ability of the LAMP assay, limiting to it to 0.04 ng/μL; additionally, the DNA took longer to amplify than it did in control and leaf tissue test groups. Our findings here demonstrate, along with other studies (Peterson et al., 2023; Rizzo et al., 2020), that LAMP assays can be specific and sensitive with the capability for portable and rapid solutions in the field, especially with DNA extracted from larvae. However, the BBB LAMP assay is less sensitive when run with vascular tissues. Thus, we expect an increase in false negatives when target DNA is low which may be due to PCR inhibitors, i.e., plant chemical compounds that can prevent or limit amplification (John, 1992; Singh & Singh, 1996; Singh et al., 2002) yielding to false negative results (Edagawa et al., 2009). The qPCR assay demonstrated that it may be more robust since the same BBB DNA is easily amplified compared to LAMP samples, seemingly unaffected by the vascular tissue compounds, suggesting it as a better choice when searching for forest pests that occupy or inhabit the vascular tissue.

With these designed assays, stakeholders including national plant protection organizations (NPPOs) have two valuable molecular tools at their disposal to aid in the detection of *A. anxius*. The LAMP assay can be run in a portable machine in the field or in a laboratory providing a tool for stakeholders like NPPOs to quickly detect the presence of the pest in imported plant material where an unknown larvae or frass sample is encountered. The major advantage of the LAMP protocol is the quick turnaround time for sample processing (<1 h) that can be used for implementing swift actions to prevent the introduction and establishment of pests. While PCR equipment is bulky and generally needs a dedicated laboratory space, the qPCR assay we designed can also be used by stakeholders that already have the facilities or equipment, giving a second option of an assay to detect BBB. Additionally, due to the increased sensitivity to detect BBB at lower concentrations of target DNA or in samples where the DNA may be more degraded, the qPCR assay is better for eDNA, where small amounts or more degraded DNA is being used for detection.

Sampling eDNA from infested trees to detect BBB or other buprestids/wood borers may prove to be challenging. As opposed to sampling eDNA in aquatic systems, sampling taxa in terrestrial systems using eDNA methodology is a developing field. Recent studies by Valentin et al. (Valentin et al., 2020, 2021) and Peterson et al. (2022) have explored the feasibility of collecting eDNA in agricultural and forestry systems with sap-sucking, phloem-feeding insects that produce higher quantities of frass (i.e., honeydew). The viability of detecting these taxa in environmental samples is higher because of the higher abundance of eDNA. However, detecting woodboring insects like EAB and BBB poses greater difficulty. When buprestids emerge from their host plants, these insects generally remain on the landscape for a relatively short period. For example, EAB adults have an average longevity of approximately 20 days (Wang et al., 2010), but they can survive for over 30 days (Peterson et al., 2020). As adults, they feed on foliage, which is a tissue suitable for eDNA collection; hence the motivation for testing potential inhibitors on foliage. Sampling the foliage of trees on seedlings or saplings in consignments or other trade plant products is relatively simple compared to sampling a mature overstory tree in the field. Therefore, future research is necessary to address this issue.

Buprestids/wood borers spend most of their life concealed as larvae under the bark of host trees, feeding internally from approximately late spring or early summer until early autumn (September to October in Northern climates). At this stage, larvae produce an abundance of frass, eDNA. Kyei-Poku et al. (2020) found that EAB DNA was detectable in frass using the LAMP assay. Similarly, we found that extracts of frass and larvae collected in the field could successfully detect BBB DNA using LAMP in two of the BBB-infested sites sampled in western Canada. Furthermore, we found that even small amounts, as little as 20 mg, of frass in the field extraction kits could successfully detect BBB with LAMP and qPCR. Frass is relatively easy to collect from logs or other woody materials and should make for easy testing for BBB DNA and other target organisms in suspect material.

Another aspect of the sampling that poses some uncertainty is whether one can detect pest eDNA at distal or proximal lengths from the source location of the pest. In a preliminary experiment complementary to this study, we injected the phloem of birch branches with BBB DNA and tested if BBB could be detected at distal locations (up to 16 cm from the point of injection) and found that BBB could be detected from eDNA in branches and their leaves with qPCR, but not LAMP (unpublished data DLP). These data would suggest that the detection of BBB with qPCR is a viable method using phloem tissue in infected birch trees. However, further studies are needed to validate and replicate this preliminary experiment.

Overall, this study delivers two molecular assays to detect a potentially lethal pest of European birch, providing valuable tools for NPPOs. The assays have been field validated and demonstrated that

both larvae and eDNA from frass are easily detectable, even at low quantities. We think that wide adoption of specific and rapid molecular assays, like those designed here, can increase the capacity to detect and help prevent the introduction of BBB and other non-native and potentially invasive pest species, ultimately safeguarding forest resources for the future.

AUTHOR CONTRIBUTIONS

D.L.P., A.S. (Alberto Santini), N.L., and M.C. conceived the study; all authors contributed to the methodology; D.L.P., N. L., and F.P. conducted laboratory investigations; D.L.P., S. O. K., T. R., and M. C. conducted field investigations; D.L.P., K.K., A.S. (Aurélien Sallé), C. R., F.P., D.M., A.S. (Alberto Santini), N. L., and M.C. contributed resources for the study; D.L.P. and M.C. wrote the initial draft of the manuscript; F.P. and S.O.K. created visuals; D.L.P., A.S. (Alberto Santini), N.L., T.R., and M.C. provided funding for the study; all authors have reviewed, read, and accept the published version of the manuscript. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data from this study are available upon reasonable request to the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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