forests

MDPI

Article

Specificity and Sensitivity of a Rapid LAMP Assay for Early Detection of Emerald Ash Borer (*Agrilus planipennis*) in Europe

Donnie L. Peterson [1,*], Kathleen Kyle [2], Aurélien Sallé [3], Francesco Pecori [4], Duccio Migliorini [4], Alberto Santini [4], Nicola Luchi [4] and Michelle Cleary [1]

[1] Southern Swedish Forest Research Centre, Swedish University of Agricultural Sciences, 230 53 Alnarp, Sweden
[2] Department of Ecology, Evolution, and Natural Resources, Rutgers University, New Brunswick, NJ 08901, USA
[3] Laboratoire de Biologie des Ligneux et des Grandes Cultures, INRAE, Université d'Orléans, F-45067 Orléans, France
[4] Institute for Sustainable Plant Protection-National Research Council (IPSP-CNR), 50019 Sesto Fiorentino, Italy
* Correspondence: donnie.peterson@slu.se

Abstract: Buprestids are an emerging threat to broadleaf forests across the world. Species such as emerald ash borer (EAB, *Agrilus planipennis*) seriously threaten ash (*Fraxinus* spp.) in North America and Europe. As it continues spreading west from European Russia, native European ash populations will suffer dramatic losses. Due to their cryptic lifestyle of the egg and larval stages on developing bark and vascular tissue, buprestids and other wood borers can be difficult to detect. Early detection tools are vital to implement fast eradication measures, and prevent the establishment of invasive species populations. Detection methods using polymerase chain reaction (PCR) assays to target specific taxa can be extremely timely to obtain results especially since samples need to be transported to the laboratory first. However, loop-mediated isothermal amplification (LAMP) eDNA assays are highly specific and sensitive providing results within 30 min after sample extraction. In this study, we investigated the specificity and sensitivity of an EAB LAMP assay as an early detection tool in Europe. The assay was specific to EAB when tested against 12 European *Agrilus* spp., five buprestids, two Scolytinae, and five cerambycids (n = 24). The LAMP assay sensitivity amplified DNA from a concentration as low as 0.02 pg/μL. These results demonstrate that the LAMP assay is a highly specific, sensitive tool that can be used to detect and monitor EAB in European forests and urban settings.

Keywords: early detection; emerald ash borer; buprestid; eDNA; LAMP

Citation: Peterson, D.L.; Kyle, K.; Sallé, A.; Pecori, F.; Migliorini, D.; Santini, A.; Luchi, N.; Cleary, M. Specificity and Sensitivity of a Rapid LAMP Assay for Early Detection of Emerald Ash Borer (*Agrilus planipennis*) in Europe. *Forests* **2023**, *14*, 436. https://doi.org/10.3390/f14020436

Academic Editor: Maarten De Groot

Received: 4 January 2023
Revised: 14 February 2023
Accepted: 15 February 2023
Published: 20 February 2023

1. Introduction

Non-native insects and microorganisms that are introduced to new environments can cost billions of US dollars annually in lost production and value, and threaten native biodiversity and the provision of ecosystem services upon which humans rely [1–3]. Global trade is the main source of these accidental introductions which have increased exponentially during the last century [4–6] and unfortunately, there appears to be no sign of saturation in the number of alien species introductions that will be introduced around the world in the future [7]. Failure to intercept and quickly eradicate a new invasion allows the organism to establish a viable breeding population when environmental barriers are overcome and subsequently spread making eradication efforts impossible [8]. The ecological and economic consequences of biological invasions can be reduced or avoided by investing in biosecurity tools that prevent the establishment of potentially harmful pests by detecting them early which will allow for rapid response to curb their establishment and potential impact [8–10].

Buprestids are an emerging group of pests affecting a broad range of tree species worldwide. Historically, buprestids in their native ranges are secondary pests that attack

and kill native host trees stressed by other biotic or abitoics factors [11,12]. Over the last several decades, these beetles have been increasingly observed and detected due to major damage inflicted on evolutionarily naïve hosts. Bronze birch borer (*Agrilus anxius* Gory), for example, started killing healthy Eurasian birch (*Betula* spp.) trees when these plants were introduced to North America [13]. Similarly, gold-spotted oak borer (*A. coxalis* Waterhouse) was discovered in the 2000s attacking and killing healthy oak (*Quercus* spp.) trees in southern California [14]. The main example of a buprestid pest is emerald ash borer (EAB), *A. planipennis* (Fairmaire). This insect was discovered in 2002 after an accidental introduction to Michigan, USA and Ontario, Canada, where it was found killing evolutionarily naïve ash (*Fraxinus* spp.) by girdling the plant via larval feeding on the phloem [15]. Since then, EAB has become one of the most costly biological invasions of forests in North America [2,16]. In East Asia, EAB is a secondary pest and only attacks and kills stressed Manchurian ash (*F. mandshurica* Rupr.) and Chinese ash (*F. chinensis* Roxb.). But in North America, EAB attacks and readily kills more than 99% of North American ash trees [17]. A separate introduction of EAB to European Russia occurred around 2003 [18], which has since spread in a westward direction, recently arriving in Ukraine [19]. Based on evidence from North America, EAB is expected to continue spreading unhindered as a result of natural dispersion and the unintentional movement of wood products [20], putting the fate of European ash (*F. excelsior* L.) across the rest of Europe at risk if no measures are taken to contain its spread. The potential impact of EAB on European ash, a valuable and ecologically important species, is critically important as it is known as a keystone species in temperate broadleaf forest ecosystems. European ash is already under threat due to the fungal pathogen *Hymenoscyphus fraxineus* (Baral, Queloz & Hosoya) which was introduced to Europe decades earlier and has substantially reduced the ash population across Europe, impacting critical biodiversity that depends on ash for its habitat [21]. In addition, there is a strong likelihood that the expanding EAB population will heavily impact other European *Fraxinus* species including *F. angustifolia* (Vahl) and *F. ornus* (L.) as well as other Oleaceae species such as olive (*Olea europaea* L.) in the southern parts of Europe and possibly other related ornamental landscape trees [22,23].

Biosurveillance tools that are rapid, accurate, and portable allowing for on-site detection are a critical way forward to prevent the introduction and establishment of harmful pests and pathogens [8,24]. Detection of Buprestids is difficult due to their cryptic lifestyle, with larvae feeding on the vascular tissue of their host trees under the bark. Species such as EAB often can be present for 3–8 years in a novel environment before they are discovered [20,25]. A major limitation of using traditional surveys is to detect and identify taxa that are rare, in low abundance, or both [8,24,26]. The morphological identification of some buprestid adults and larvae, such as *Agrilus* species, can be extremely challenging for non-experts, which is made worse by the fact that the group is highly speciose [27]. Traditionally, plant diagnostics are also labor-intensive and time-consuming since it requires a high level of sampling and multiple processing steps that must be handled by experienced staff in laboratories. This makes it both expensive and difficult to detect potentially invasive forest insects at an early enough stage that would allow mitigation measures to be implemented quickly and effectively [28]. However, surveys using environmental DNA (eDNA) can be a reliable tool for improving the detection probability of target species [29,30]. Multiple studies in recent years have demonstrated the power of eDNA over traditional survey methods for monitoring terrestrial pest populations [31–33]. While significant advances have been made with portable molecular techniques to detect organisms directly in the field and offer the possibility to scale up their usage in practice, progress has been largely curtailed by the need to optimize and validate on-site methods, ensure specificity and accuracy of detection, and increasing high throughput capacity for screening large numbers of pests. Portable DNA-based equipment that operates with great simplicity, sensitivity, specificity, and high speed, that can detect pests in the early stages of attack when symptoms are not yet entirely visible, or detect traces of their presence in other forms, would significantly enhance both diagnostic and surveillance applications to help control outbreak

populations. Environmental DNA, which can detect trace evidence of DNA shed by pests on different plant substrates [32,34], shows high promise to advance current biosecurity efforts and the capacity to detect cryptic species such as Buprestids, including EAB. This technology has the potential to be adapted to rapid and throughput in-field detection using a portable system called loop-mediated isothermal amplification (LAMP) [35]. A LAMP assay was recently developed for EAB in North America [36], but in order for it to be used for early detection in European conditions, the protocol must be able to discriminate EAB from European insect fauna, and its sensitivity needs to be determined to understand the limit of detection (LOD), i.e., the lowest DNA concentration that can be determined by the molecular assay. The objective of this study was to determine the ability of the EAB LAMP assay to be specific in relation to European wood borers, other *Agrilus* spp., Cerambycids, and Scolytinae; and to determine a lower threshold of DNA detection for field application.

2. Methods

2.1. Collection and Extraction of Specimens

In this study, all insect specimen used for specificity testing were adults and trapped in broadleaf forests in Europe and North America (Table 1). Insects were collected in multifunnel traps in a mix of 50/50 monopropylene glycol and water; a common method used for the collection of buprestids and EAB [37,38] and in connection with the Euphresco project (2020-A-337-'Developing and assessing surveillance methodologies for *Agrilus* beetles') in Europe.

Table 1. Insect species used for specificity testing for EAB LAMP primers for use in European conditions including insect collection location, collection date, and the DNA extraction kit and PCR primer set to amplify DNA. * represents Figure S1 showing PCR amplification in gels.

Insect Species	Label Name	Collection Date (mm-yr)	Insect Locality	Longitude Latitude	Extraction Kit	PCR Primers (A–F *)	EAB LAMP Detection
Buprestidae							
1. *Agrilus angustulus*	AA	21 May	Vierzon, France	2.0327° 47.2611°	Invitrogen	LCO1490-HCO2198 (B)	-
2. *Agrilus anxius*	BBB	14,15 June	CT, USA; ON, Canada		Qiagen	LCO1490-HCO2199 (A, D, E)	-
3. *Agrilus ater*	AAT	2020	Vierzon, France	2.0327° 47.2611°	Qiagen	28 s up–28 s low (F)	-
4. *Agrilus convexicollis*	AC	21 June	Orléans campus, France	1.9383° 47.8456°	Qiagen	16a–16b (F)	-
5. *Agrilus curtulus*	Acu	20 August	France		Qiagen	28 s up–28 s low (F)	-
6. *Agrilus graminis*	AG	20 August	Orléans campus, France	1.9383° 47.8456°	Qiagen	LCO1490-HCO2199 (D)	-
7. *Agrilus hastulifer*	AH	21 June	Orléans campus, France	1.9383° 47.8456°	Qiagen	28 s up–28 s low (F)	-
8. *Agrilus laticornis*	AL	21 June	Orléans campus, France	1.9383° 47.8456°	Qiagen	28 s up–28 s low (F)	-
9. *Agrilus obscuricollis*	AO	21 June	Orléans campus, France	1.9383° 47.8456°	Qiagen	28 s up–28 s low (F)	-
10. *Agrilus olivicolor*	AD	21 June	Orléans campus, France	1.9383° 47.8456°	Qiagen	LCO1490-HCO2198 (B)	-
11. *Agrilus planipennis*	EAB		NY, RI, VT, USA		Qiagen	LCO1490-HCO2198 (A) EAB_COIF EAB_COIR (C)	+

Table 1. *Cont.*

Insect Species	Label Name	Collection Date (mm-yr)	Insect Locality	Longitude Latitude	Extraction Kit	PCR Primers (A–F *)	EAB LAMP Detection
12. *Agrilus roscidus*	AR	2020	France		Qiagen	LCO1490-HCO2199 (E)	-
13. *Agrilus sulcicollis*	AS	21 May	Vierzon, France	2.0327° 47.2611°	Invitrogen	LCO1490-HCO2198 (A)	-
14. *Agrilus viridis*	AV	21 June	Orléans campus	1.9383° 47.8456°	Qiagen	28 s up–28 s low (F)	-
15. *Anthaxia nitidula*	AN	21 May	Marcenat	3.3619° 46.2461°	Qiagen	LCO1490-HCO2199 (E)	-
16. *Chrysobothris affinis*	CA	20 June	Vierzon, France	2.1544° 47.2899°	Qiagen	LCO1490-HCO2198 (E)28 s up–28 s low (F)	-
17. *Coraebus undatus*	CU	21 June	Orléans campus, France	1.9383° 47.8456°	Qiagen	LCO1490-HCO2198 (B)28 s up–28 s low (F)	-
18. *Lamprodila mirifica*	LM	Summer 2021	Friuli Venezia Giulia Italy	13.120926° 45.791784°	Qiagen	28 s up-28 s low (F)	-
Curculionidae: Scolytinae							
19. *Anisandrus dispar*	A dis	Summer 2021	Friuli Venezia Giulia, Italy	13.120926° 45.791784°	Qiagen	LCO1490-HCO2198 (C)	-
20. *Xyleborinus saxesenii*	XS	Summer 2021	Friuli Venezia Giulia Italy	13.120926° 45.791784°	Qiagen	LCO1490-HCO2199 (C)	-
Cerambycidae							
21. *Aegomorphus clavipes*	Aeg	Summer 2021	Friuli Venezia Giulia, Italy	13.120926° 45.791784°	Qiagen	28 s up–28 s low (F)	-
22. *Exocentrus punctipennis*	EP	Summer 2021	Friuli Venezia Giulia, Italy	13.120926° 45.791784°	Qiagen	LCO1490-HCO2199 (C)	-
23. *Leiopus nebulosus*	LN	Summer 2021	Friuli Venezia Giulia Italy	13.120926° 45.791784°	Qiagen	LCO1490-HCO2199 (C)	-
24. *Saperda punctata*	SP	Summer 2021	Friuli Venezia Giulia Italy	13.120926° 45.791784°	Qiagen	LCO1490-HCO2199 (C)	-
25. *Trichoferus pallidus*	TN	21 June	Orléans campus, France	1.9383° 47.8456°	Qiagen	LCO1490-HCO2199 (E)28 s up–28 s low (F)	-

Genomic DNA was extracted from EAB and *A. anxius* specimens from North America at Rutgers University (New Brunswick, NJ, USA) in December 2021 using the DNeasy Blood and Tissue Kit (Qiagen) and stored in a −20 °C freezer. Extracted DNA was shipped on dry ice in January 2022 to the Institute for Sustainable Plant Protection—National Research Council (IPSP-CNR, Florence, Italy) and then transferred to a −20 °C freezer until subsequent processing. All other specimens from France and Italy were shipped to IPSP-CNR and stored in 70%–95% ethanol. For all specimens (Table 1), each sample was triple rinsed with DI water to wash any potential exterior DNA contamination. Using flame-sterilized forceps, we removed legs with attached muscle tissues, and total genomic DNA was extracted. The number of specimen legs that were used ranged from three for Cerambycid adults to a maximum of six for smaller *Agrilus* individuals. A full body extraction was performed with the two smaller ambrosia beetles (*Xyleborinus saxesenii* and *Anisandrus dispar*) to obtain enough genetic material for subsequent PCR amplification. For EAB and *A. anxius* (three-five individuals/species), at least three legs were manually removed and crushed with a sterilized metal forceps inside the 1.5 mL microcentrifuge tube. For all other specimens (one per insect species), legs or full body tissue was pulverized by adding two metal sterile beads to 1.5 mL tubes and running the tubes in a TissueLyser (Qiagen) for 1–2 min at 300 oscillations/min. DNA extraction was performed from homogenized samples by using the standard protocol for insect extractions from two different commercial kits: Invitrogen JetQuick Blood and Cell Culture and DNeasy Blood and Tissue Kit (Qiagen) (Table 1). DNA quantity and quality were checked using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

2.2. PCR Amplification and Sequencing of Samples

DNA extracted from insects were amplified with PCR in a final volume of 20 µL containing, 12.5 µL Reaction buffer, GoTaq (Promega), 0.4 µL Primer Forward (10 µM final concentration, Eurofins Genomics, Ebersberg, Germany, see Table 1), 0.4 µL Primer Reverse (10 µM final concentration, Eurofins Genomics, see Table 1), 2 µL of extracted DNA, and 4.7 µL of nuclease-free distilled water. EAB DNA was amplified using the primer set EAB_COIF and EAB_COIR designed to capture potential polymorphism of EAB within a conserved region for this species [36] (Table 2). The EAB PCR reactions were run with an initial denaturation step (94 °C for 1 min) followed by five cycles of denaturation (94 °C for 40 s), annealing (45 °C for 40 s), and extension (72 °C for 60 s), and then 35 cycles of denaturation (94 °C for 45 s), annealing (60 °C for 40 s), and extension (72 °C for 60 s) and a final extension (72 °C for 10 min). All other species used the primers LCO1490-HCO2198 [39], rrnl mtDNA (16A)-rrnl mtDNA (16B), and 28S_D1D2.3.a_up-28S_D1D2.3.a_low (Table 2) with an initial denaturation (94 °C 2 min), denaturation (94 °C 30 s), annealing (45 °C 1 min), extension (72 °C 2 min), 34 cycles, and a final extension step (72 °C 5 min). Amplification products were separated by electrophoresis on gels containing 1% (*w*/*v*) of agarose LE (Genespin). Each sample (2.5 µL amplified DNA and 2.5 µL SYBR™ Safe DNA Gel Stain-Invitrogen) was run at 90 V for 30 min. The approximate length (bp) of the amplification products was determined using the 1 kbp DNA ladder Ready to Load (Genespin). For a several species that did not initially amplify, we ran the DNA at a lower concentration (1/10 dilution) to show successful amplification (Figure S1).

Table 2. General and specific PCR and LAMP primers used to amplify and confirm insect DNA.

PCR Primer	Sequence (5′–3′)	Gene Type	Reference
EAB_COIF	AGG AAT AGT AGG AAC AGC CCT TAG A	COI	[36]
EAB_COIR	TAT TTC ATC TAA GGT AGG CAT CTG G	COI	
LCO1490	GGT CAA CAA ATC ATA AAG ATA TTG G	COI	[39]
HCO2198	TAA ACT TCA GGG TGA CCA AAA AAT CA	COI	
28S_D1D2.3.A_UP	GGA ATC CGC TAA GGA GTG TGT AA	28S	[40]
28S_D1D2.3.A_LOW	AGG GCC TCG CTG GAG TAT TT	28S	
RRNL MTDNA (16A)	CGC CTG TTT AAC AAA AAC AT	mtDNA	[41]
RRNL MTDNA (16B)	CCG GTC TGA ACT CAG ATC ATG T	mtDNA	
LAMP PRIMERS			
EAB1_F3	CTC CCT CCC TCT TTA ACA TTA C	COI	[36]
EAB1_B3	GAT CAG ACT AGT AGA GGT GT	COI	
EAB1_FIP	ATA TTA GCC GCT AAT GGT GGG AAT AGT CGA AAG AGG AGC AG	COI	
EAB1_BIP	GGC TCT GTT GAC TTA GCA AAG GTT ATT CCT ATT GCT CGC	COI	
EAB1_LF	ATA TAC TGT CCA ACC AGT CC	COI	
EAB1_LB	CTG GAA TCT CCT CAA TTC TAG G	COI	

2.3. Specificity and Sensitivity Testing of EAB Assay

The LAMP assay (see Table 2) was tested using the extracted DNA from closely related species (Table 1). The reactions were carried out in a final volume of 25 µL containing 15 µL of isothermal master-mix (OptiGene, Horsham, UK); 0.5 µL of F3 and B3 (Table 2; final conc. 0.2 µM each) (Eurofins Genomics, Vienna, Austria); 1.0 µL of Loop F and Loop B (Table 2; final conc. 0.4 µM each) (Eurofins Genomics, Vienna, Austria); 2.0 µL FIP and BIP (Table 2; final conc. 0.8 µM each) (Eurofins Genomics, Vienna, Austria); and 3.0 µL of extracted DNA. LAMP amplification reactions were performed at 65 °C for 30 min using the Genie® II (OptiGene Limited, Horsham, UK) according to methods in [42]. Each species was run in duplicate. In each run, one positive (EAB) control and one negative (No Template Control, NTC) were also included to verify the success of the reaction and ensure there was no DNA contamination.

A standard curve was generated from five-fold serial dilutions (ranging from 2 ng/µL to 0.02 pg/µL) to determine the lower limit of detection. Each dilution was run in duplicate under the same conditions previously described.

3. Results and Discussion

To adequately detect target species, molecular assays need to be specific to the species of concern, highly sensitive to low or degraded DNA quantities, and free from environmental inhibitors [43]. Specificity is key to successfully designing eDNA assays. Since *Agrilus* is the largest described animal genus [27], it is particularly important to demonstrate that specific primers can be designed to readily and accurately detect the target taxa. In the study by Kyei Poku et al. [36], the similarity of the targeted gene region for EAB was compared to other congeners and other taxa from an online database to make the assay species-specific; however, specificity of a molecular assay, such as LAMP, needs to be validated with extracted DNA of other taxa. Kyei Poku et al. [36] conducted specificity testing with extracted DNA of mostly North American co-occurring fauna including four *Agrilus* and two Scolytinaes, and none amplified with the EAB LAMP primers. In order for EAB LAMP to be specific in European conditions and to better understand the risks of obtaining false positives as a result of having a different suite of co-occurring species within European forests, we tested the extracted DNA of additional *Agrilus* spp. native to Europe to determine specificity.

The EAB LAMP assay was tested on DNA extracted from 25 species (24 non-target; DNA concentration range of 5 to 160 ng/µL): 13 *Agrilus* (12 native to Europe) and 12 non-*Agrilus* (Table 1). We confirmed by PCR amplification that the DNA of EAB and the non-EAB specimens were suitable for specificity and sensitivity testing using the LAMP assay (Table 1). All EAB DNA samples were successfully amplified using the PCR primers EAB_COIF and EAB_COIR [36]. Three sets of primers were used to amplify the DNA of other specimens because DNA can be difficult to amplify for specific *Agrilus* spp. (personal communication Amanda Roe, Great Lakes Forestry Centre, Sault Ste. Marie, ON, Canada). The majority of the insect species were amplified with LCO1490-HCO2199 (Table 1), a common, universal primer set, and ten samples were successfully amplified with other common insect primers.

Of the non-target species tested, none were amplified by LAMP primers (Table 1). Our findings provide additional data supporting that the LAMP assay for EAB is highly specific and can be used to distinguish EAB DNA from other Buprestids originating from both North America and Europe. Overall, 30 species have been examined for specificity testing including those from Kyei-Poku et al. [36]. While these results are promising for two geographic regions of the world, other locations warrant further investigation because closer relatives of EAB, such as those in eastern Asia, can be more genetically similar [27].

Similar to Kyei-Poku et al. [36], we demonstrated that the DNA of *A. sulcicollis* (Lacordaire) and *A. anxius* did not amplify by the LAMP assay. In addition, we also found that non-buprestids that co-occur in European broadleaf forests did not amplify using the designed EAB LAMP primers, and DNA extracts of two additional Scolytinaes and five Cerambycid were negative for amplification. Overall, our data and that by Kyei-Poku et al. [36] demonstrate that the LAMP primers appear highly specific to the targeted fauna (EAB), even among very speciose genera *Agrilus*, and can be used as a diagnostic tool for early detection and monitoring on both continents.

Critical to the use of eDNA assays is the ability to detect low concentrations of target DNA. If the threshold for adequate detection is too high, then the ability to detect a species at the early stages of invasion may be jeopardized. Insect DNA can be detected at quite low levels; for example, spotted lanternfly (*Lycorma delicatula* White) DNA reached a limit of detection of 0.14 pg/µL with qPCR [33]. For LAMP studies, levels have reached as low as 0.61–16 pg/µL using the DNA of long-horned beetles [44,45]. Our study revealed similar levels of sensitivity with a serial dilution experiment detecting as low as 0.02 pg/µL with the EAB LAMP assay when starting from a higher level of 2.0 ng/µL (Figure 1).

This concentration is lower but comparable to those results found when running qPCR assays [33]. Our findings are similar to other studies where LAMP outperforms traditional PCR methods [46,47]. The results clearly demonstrate that LAMP assays can not only be specific, portable, and provide rapid results, but also detect lower DNA amounts than some qPCR assays. The benefits and trade-offs of using eDNA LAMP assays in early detection and surveillance efforts for EAB are evident. Conventional PCR is more expensive due to the thermocycler equipment and reagent needs, the necessity of highly trained staff, and the time needed to prepare samples. This results in an overall higher cost per sample than running LAMP protocols [48]. In contrast, LAMP equipment (i.e., Genie® II) is generally cheaper to produce, smaller in size, portable due to isothermal amplification, and amplification are much faster (generally less than 30 min). Thus, this LAMP assay is providing a user-friendly tool for stakeholders to use. One potential advantage of PCR over LAMP is the influence of plant-produced chemicals that can inhibit DNA amplification. qPCR can outperform LAMP in terms of sensitivity when inhibitors are present [49] and LAMP is known to be influenced by some plant chemicals [50]. Thus, future research should consider the influence of host plant chemistry, or other inhibitors present in the environmental sample, on the sensitivity and false negatives.

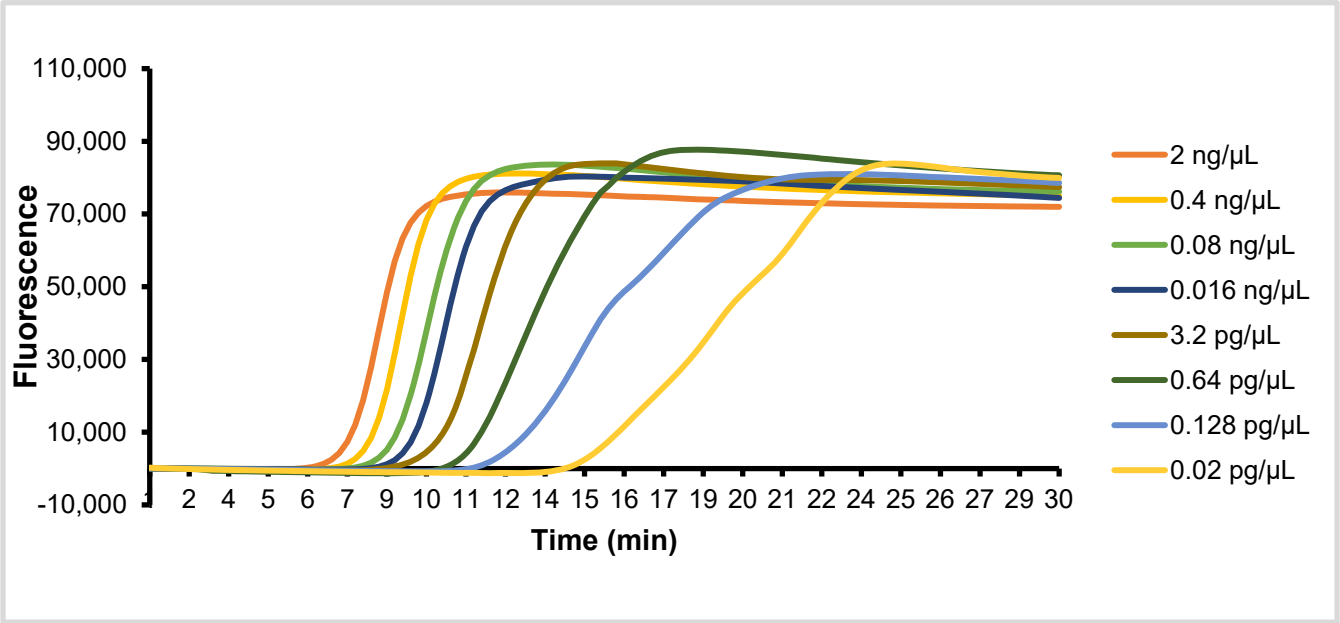

Figure 1. Fluorescence of EAB DNA in a 1:5 serial dilution to determine the lower detection threshold of the EAB LAMP assay.

Insects are a hyper-diverse group of organisms; beetles (Coleoptera) being the largest order of all described insects and the genus *Agrilus*, is the largest among animals [27]. In recent decades, *Agrilus* spp. have become major forest pests where they have been introduced outside of their native range, whereas in their native range, they are usually considered only secondary pests, killing or attacking weakened or stressed host plants. With so many species, ~3000 in the genus *Agrilus*, it is often difficult to accurately identify taxa to a species level and often taxonomic experts are needed, but even then species may go undetected for decades as was the case with EAB in North America [51]. Reliable methods such as LAMP, as demonstrated in our study, are therefore needed to rapidly detect the species of concern, and reduce the time from incursion to detection and subsequent mitigation efforts. As EAB continues to spread throughout Europe, the developed and validated LAMP assay can play an important role to monitor for satellite populations or delimit the expanding range in forests and urban settings. With the current location of EAB in eastern Europe, now on the fringe of invading the rest of Europe, LAMP could

provide a critical role for sampling to assist in containment efforts, since the Genie® II or III instruments allow users to conduct on-site testing which can lead to more rapid decisions for mitigation.

Targeted sampling methods for eDNA collection of woodboring insects such as EAB are still needed for larval stages but some methods for collecting eDNA of adults already exist for sampling foliage and bark from trees in forests (e.g., using a backpack sprayer or a paint rolling pole) [31,34]. Overall, our results demonstrated, validated, and expanded the usability of the EAB LAMP assay for European conditions, and is highly recommended that the LAMP assay be adopted by plant protection organizations throughout Europe to aid in the early detection of *A. planipennis* in surveillance efforts that ultimately aim to save European ash from its demise.

4. Conclusions

In conclusion, our study determined that the emerald ash borer LAMP assay appears specific to EAB with none of the non-target species DNA being amplified. The LAMP assay has the capacity to detect *A. planipennis* in European and North American conditions. Furthermore, the assay is quite sensitive, being able to detect low concentrations of DNA. Future validation of this or other LAMP assays in the field is being developed and needs to be implemented to demonstrate the capacity to obtain this technology for detection in both forested and urban settings.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f14020436/s1, Figure S1: Gels of amplified PCR DNA of insects used for specific testing of EAB LAMP primers. All primers used to confirm DNA were LCO1490-HCO2198 (A–E) in gel except the final gel (F) shows DNA amplified with 28S_D1D2.3.a_up-28S_D1D2.3.a_low (Top) and rrnl mtDNA (16A)-rrnl mtDNA (16B) (Bottom). Species with plus mark (+) were considered amplified. See Table 1 for the species codes.

Author Contributions: Conceptualization, D.L.P., A.S. (Alberto Santini), N.L. and M.C.; methodology, D.L.P., K.K., A.S. (Aurélien Sallé), F.P., D.M., A.S. (Alberto Santini), N.L. and M.C.; investigation, D.L.P. and F.P.; resources, D.L.P., K.K., A.S. (Aurélien Sallé), F.P., D.M., A.S. (Alberto Santini), N.L. and M.C.; writing—original draft preparation, D.L.P. and M.C.; writing—review and editing D.L.P., K.K., A.S. (Aurélien Sallé), F.P., D.M., A.S. (Alberto Santini), N.L. and M.C.; visualization, D.L.P. and F.P.; funding acquisition, D.L.P., A.S. (Alberto Santini), N.L. and M.C. 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.

Funding: This research was partially funded by the Marie Skłodowska-Curie Action Postdoctoral Fellowship project "EMERALD" #101027324 and Lilla Fonden travel grant from the Swedish University of Agricultural Sciences, and the FORMAS The Swedish Research Council grant #2018-00966.

Data Availability Statement: The data from this study is available upon reasonable request to the corresponding author.

Acknowledgments: We would like to thank Anthony Vastano at Rutgers University for coordination and shipment of DNA extractions from USA to Europe, Amanda Roe at the Great Lakes Forestry Centre, Canada for advice on DNA amplification and primers and BBB samples, Toby Petrice from USDA Forest Service for BBB specimen, and Davide Rassati from University of Padova and the Euphresco project 2020-A-337-'Developing and assessing surveillance methodologies for *Agrilus* beetles' for other non-target specimen.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Gandhi, K.J.K.; Herms, D.A. Direct and Indirect Effects of Alien Insect Herbivores on Ecological Processes and Interactions in Forests of Eastern North America. *Biol. Invasions* **2010**, *12*, 389–405. [CrossRef]
2. 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.; et al. Economic Impacts of Non-Native Forest Insects in the Continental United States. *PLoS ONE* **2011**, *6*, e24587. [CrossRef] [PubMed]

3. Bradshaw, C.J.A.; Leroy, B.; Bellard, C.; Roiz, D.; Albert, C.; Fournier, A.; Barbet-Massin, M.; Salles, J.-M.; Simard, F.; Courchamp, F. Massive yet Grossly Underestimated Global Costs of Invasive Insects. *Nat. Commun.* **2016**, *7*, 12986. [CrossRef] [PubMed]
4. Santini, A.; Ghelardini, L.; De Pace, C.; Desprez-Loustau, M.L.; Capretti, P.; Chandelier, A.; Cech, T.; Chira, D.; Diamandis, S.; Gaitniekis, T.; et al. Biogeographical Patterns and Determinants of Invasion by Forest Pathogens in Europe. *New Phytol.* **2013**, *197*, 238–250. [CrossRef] [PubMed]
5. Epanchin-Niell, R.; McAusland, C.; Liebhold, A.; Mwebaze, P.; Springborn, M.R. Biological Invasions and International Trade: Managing a Moving Target. *Rev. Environ. Econ. Policy* **2021**, *15*, 180–190. [CrossRef]
6. Venette, R.C.; Hutchison, W.D. Invasive Insect Species: Global Challenges, Strategies & Opportunities. *Front. Insect Sci.* **2021**, *1*, 650520.
7. Seebens, H.; Blackburn, T.M.; Dyer, E.E.; Genovesi, P.; Hulme, P.E.; Jeschke, J.M.; Pagad, S.; Pyšek, P.; Winter, M.; Arianoutsou, M.; et al. No Saturation in the Accumulation of Alien Species Worldwide. *Nat. Commun.* **2017**, *8*, 14435. [CrossRef]
8. Tobin, P.C.; Kean, J.M.; Suckling, D.M.; McCullough, D.G.; Herms, D.A.; Stringer, L.D. Determinants of Successful Arthropod Eradication Programs. *Biol. Invasions* **2014**, *16*, 401–414. [CrossRef]
9. *NISC 2016–2018 NISC Management Plan*; National Invasive Species Council: Washington, DC, USA, 2016.
10. Martinez, B.; Reaser, J.K.; Dehgan, A.; Zamft, B.; Baisch, D.; McCormick, C.; Giordano, A.J.; Aicher, R.; Selbe, S. Technology Innovation: Advancing Capacities for the Early Detection of and Rapid Response to Invasive Species. *Biol. Invasions* **2020**, *22*, 75–100. [CrossRef]
11. Sallé, A.; Nageleisen, L.-M.; Lieutier, F. Bark and Wood Boring Insects Involved in Oak Declines in Europe: Current Knowledge and Future Prospects in a Context of Climate Change. *For. Ecol. Manag.* **2014**, *328*, 79–93. [CrossRef]
12. Haack, R.A.; Petrice, T. Historical Population Increases and Related Inciting Factors of Agrilus Anxius, Agrilus Bilineatus, and Agrilus Granulatus Liragus (Coleoptera: Buprestidae) in the Lake States (Michigan, Minnesota, and Wisconsin). *Great Lakes Entomol.* **2019**, *52*, 7.
13. Muilenburg, V.L.; Herms, D.A. A Review of Bronze Birch Borer (Coleoptera: Buprestidae) Life History, Ecology, and Management. *Environ. Entomol.* **2012**, *41*, 1372–1385. [CrossRef]
14. Coleman, T.W.; Seybold, S.J. Previously Unrecorded Damage to Oak, Quercus Spp., in Southern California by the Goldspotted Oak Borer, Agrilus Coxalis Waterhouse (Coleoptera: Buprestidae). *Pan-Pac. Entomol.* **2008**, *84*, 288–300. [CrossRef]
15. Cappaert, D.; McCullough, D.G.; Poland, T.M.; Siegert, N.W. Emerald Ash Borer in North America: A Research and Regulatory Challenge. *Am. Entomol.* **2005**, *51*, 152–165. [CrossRef]
16. Kovacs, K.F.; Haight, R.G.; McCullough, D.G.; Mercader, R.J.; Siegert, N.W.; Liebhold, A.M. Cost of Potential Emerald Ash Borer Damage in U.S. Communities, 2009–2019. *Ecol. Econ.* **2010**, *69*, 569–578. [CrossRef]
17. Klooster, W.S.; Herms, D.A.; Knight, K.S.; Herms, C.P.; McCullough, D.G.; Smith, A.; Gandhi, K.J.K.; Cardina, J. Ash (*Fraxinus* Spp.) Mortality, Regeneration, and Seed Bank Dynamics in Mixed Hardwood Forests Following Invasion by Emerald Ash Borer (Agrilus Planipennis). *Biol. Invasions* **2014**, *16*, 859–873. [CrossRef]
18. Baranchikov, Y.; Mozolevskaya, E.; Yurchenko, G.; Kenis, M. Occurrence of the Emerald Ash Borer, Agrilus Planipennis in Russia and Its Potential Impact on European Forestry. *EPPO Bull.* **2008**, *38*, 233–238. [CrossRef]
19. Orlova-Bienkowskaja, M.J.; Drogvalenko, A.N.; Zabaluev, I.A.; Sazhnev, A.S.; Peregudova, E.Y.; Mazurov, S.G.; Komarov, E.V.; Struchaev, V.V.; Martynov, V.V.; Nikulina, T.V. Current Range of Agrilus Planipennis Fairmaire, an Alien Pest of Ash Trees, in European Russia and Ukraine. *Ann. For. Sci.* **2020**, *77*, 29. [CrossRef]
20. Herms, D.A.; McCullough, D.G. Emerald Ash Borer Invasion of North America: History, Biology, Ecology, Impacts, and Management. *Annu. Rev. Entomol.* **2014**, *59*, 13–30. [CrossRef]
21. Hultberg, T.; Sandström, J.; Felton, A.; Öhman, K.; Rönnberg, J.; Witzell, J.; Cleary, M. Ash Dieback Risks an Extinction Cascade. *Biol. Conserv.* **2020**, *244*, 108516. [CrossRef]
22. Cipollini, D.; Rigsby, C.M.; Peterson, D.L. Feeding and Development of Emerald Ash Borer (Coleoptera: Buprestidae) on Cultivated Olive, Olea Europaea. *J. Econ. Entomol.* **2017**, *110*, 1935–1937. [CrossRef] [PubMed]
23. Peterson, D.L.; Cipollini, D. Larval Performance of a Major Forest Pest on Novel Hosts and the Effect of Stressors. *Environ. Entomol.* **2020**, *49*, 482–488. [CrossRef] [PubMed]
24. Epanchin-Niell, R.S.; Haight, R.G.; Berec, L.; Kean, J.M.; Liebhold, A.M. Optimal Surveillance and Eradication of Invasive Species in Heterogeneous Landscapes. *Ecol. Lett.* **2012**, *15*, 803–812. [CrossRef] [PubMed]
25. Siegert, N.W.; McCullough, D.G.; Liebhold, A.M.; Telewski, F.W. Resurrected from the Ashes: A Historical Reconstruction of Emerald Ash Borer Dynamics through Dendrochronological Analysis. In *Proceedings of the Emerald Ash Borer and Asian Longhorned Beetle Research and Development Review Meeting*; Cincinnatti, OH, USA, 29 October–2 November 2006, FHTET 2007-04; Mastro, V., Lance, D., Reardon, R., Parra, G., Eds.; US Forest Service, Forest Health Technology Enterprise Team: Morgantown, WV, USA, 2007; pp. 18–19.
26. Epanchin-Niell, R.S.; Hastings, A. Controlling Established Invaders: Integrating Economics and Spread Dynamics to Determine Optimal Management. *Ecol. Lett.* **2010**, *13*, 528–541. [CrossRef]
27. Kelnarova, I.; Jendek, E.; Grebennikov, V.V.; Bocak, L. First Molecular Phylogeny of Agrilus (Coleoptera: Buprestidae), the Largest Genus on Earth, with DNA Barcode Database for Forestry Pest Diagnostics. *Bull. Entomol. Res.* **2019**, *109*, 200–211. [CrossRef]
28. Luchi, N.; Ioos, R.; Santini, A. Fast and Reliable Molecular Methods to Detect Fungal Pathogens in Woody Plants. *Appl. Microbiol. Biotechnol.* **2020**, *104*, 2453–2468. [CrossRef]

29. Darling, J.A.; Blum, M.J. DNA-Based Methods for Monitoring Invasive Species: A Review and Prospectus. *Biol. Invasions* **2007**, *9*, 751–765. [CrossRef]
30. Jerde, C.L.; Mahon, A.R.; Chadderton, W.L.; Lodge, D.M. "Sight-Unseen" Detection of Rare Aquatic Species Using Environmental DNA. *Conserv. Lett.* **2011**, *4*, 150–157. [CrossRef]
31. Valentin, R.E.; Fonseca, D.M.; Nielsen, A.L.; Leskey, T.C.; Lockwood, J.L. Early Detection of Invasive Exotic Insect Infestations Using EDNA from Crop Surfaces. *Front. Ecol. Environ.* **2018**, *16*, 265–270. [CrossRef]
32. Valentin, R.; Kyle, K.; Allen, M.; Welbourne, D.; Lockwood, J. The state, transport, and fate of aboveground terrestrial arthropod eDNA. *Environ. DNA* **2021**, *3*, 1081–1092. [CrossRef]
33. Allen, M.C.; Nielsen, A.L.; Peterson, D.L.; Lockwood, J.L. Terrestrial EDNA Survey Outperforms Conventional Approach for Detecting an Invasive Pest Insect within an Agricultural Ecosystem. *Environ. DNA* **2021**, *3*, 1102–1112. [CrossRef]
34. Peterson, D.L.; Allen, M.C.; Vastano, A.; Lockwood, J.L. Evaluation of Sample Collection and Storage Protocols for Surface EDNA Surveys of an Invasive Terrestrial Insect. *Environ. DNA* **2022**, *4*, 1201–1211. [CrossRef]
35. Notomi, T.; Okayama, H.; Masubuchi, H.; Yonekawa, T.; Watanabe, K.; Amino, N.; Hase, T. Loop-Mediated Isothermal Amplification of DNA. *Nucleic Acids Res.* **2000**, *28*, e63. [CrossRef]
36. Kyei-Poku, G.; Gauthier, D.; Quan, G. Development of a Loop-Mediated Isothermal Amplification Assay as an Early-Warning Tool for Detecting Emerald Ash Borer (Coleoptera: Buprestidae) Incursions. *J. Econ. Entomol.* **2020**, *113*, 2480–2494. [CrossRef]
37. Francese, J.A.; Rietz, M.L.; Mastro, V.C. Optimization of Multifunnel Traps for Emerald Ash Borer (Coleoptera: Buprestidae): Influence of Size, Trap Coating, and Color. *J. Econ. Entomol.* **2013**, *106*, 2415–2423. [CrossRef]
38. Rassati, D.; Marini, L.; Marchioro, M.; Rapuzzi, P.; Magnani, G.; Poloni, R.; Di Giovanni, F.; Mayo, P.; Sweeney, J. Developing Trapping Protocols for Wood-Boring Beetles Associated with Broadleaf Trees. *J. Pest Sci.* **2019**, *92*, 267–279. [CrossRef]
39. Folmer, O.; Black, M.; Hoeh, W.; Lutz, R.; Vrijenhoek, R. DNA Primers for Amplification of Mitochondrial Cytochrome c Oxidase Subunit I from Diverse Metazoan Invertebrates. *Mol. Mar. Biol. Biotechnol.* **1994**, *3*, 294–299.
40. Büsse, S.; von Grumbkow, P.; Hummel, S.; Shah, D.N.; Tachamo Shah, R.D.; Li, J.; Zhang, X.; Yoshizawa, K.; Wedmann, S.; Hörnschemeyer, T. Phylogeographic Analysis Elucidates the Influence of the Ice Ages on the Disjunct Distribution of Relict Dragonflies in Asia. *PLoS ONE* **2012**, *7*, e38132. [CrossRef]
41. Katoh, K.; Standley, D.M. MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability. *Mol. Biol. Evol.* **2013**, *30*, 772–780. [CrossRef]
42. Aglietti, C.; Luchi, N.; Pepori, A.L.; Bartolini, P.; Pecori, F.; Raio, A.; Capretti, P.; Santini, A. Real-Time Loop-Mediated Isothermal Amplification: An Early-Warning Tool for Quarantine Plant Pathogen Detection. *AMB Express* **2019**, *9*, 50. [CrossRef]
43. 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.; et al. Critical Considerations for the Application of Environmental DNA Methods to Detect Aquatic Species. *Methods Ecol. Evol.* **2016**, *7*, 1299–1307. [CrossRef]
44. Rizzo, D.; Taddei, A.; Da Lio, D.; Bruscoli, T.; Cappellini, G.; Bartolini, L.; Salemi, C.; Luchi, N.; Pennacchio, F.; Rossi, E. Molecular Identification of Anoplophora Glabripennis (Coleoptera: Cerambycidae) from Frass by Loop-Mediated Isothermal Amplification. *J. Econ. Entomol.* **2020**, *113*, 2911–2919. [CrossRef] [PubMed]
45. Rizzo, D.; Luchi, N.; Da Lio, D.; Bartolini, L.; Nugnes, F.; Cappellini, G.; Bruscoli, T.; Salemi, C.; Griffo, R.V.; Garonna, A.P.; et al. Development of a Loop-Mediated Isothermal Amplification (LAMP) Assay for the Identification of the Invasive Wood Borer Aromia Bungii (Coleoptera: Cerambycidae) from Frass. *3 Biotech* **2021**, *11*, 85. [CrossRef] [PubMed]
46. Foo, P.C.; Nurul Najian, A.B.; Muhamad, N.A.; Ahamad, M.; Mohamed, M.; Yean Yean, C.; Lim, B.H. Loop-Mediated Isothermal Amplification (LAMP) Reaction as Viable PCR Substitute for Diagnostic Applications: A Comparative Analysis Study of LAMP, Conventional PCR, Nested PCR (NPCR) and Real-Time PCR (QPCR) Based on Entamoeba Histolytica DNA Derived from Faecal Sample. *BMC Biotechnol.* **2020**, *20*, 34. [CrossRef]
47. Gunasegar, S.; Neela, V.K. Evaluation of Diagnostic Accuracy of Loop-Mediated Isothermal Amplification Method (LAMP) Compared with Polymerase Chain Reaction (PCR) for Leptospira Spp. in Clinical Samples: A Systematic Review and Meta-Analysis. *Diagn. Microbiol. Infect. Dis.* **2021**, *100*, 115369. [CrossRef]
48. World Health Organization. *The Use of Loop-Mediated Isothermal Amplification (TB-LAMP) for the Diagnosis of Pulmonary Tuberculosis: Policy Guidance*; World Health Organization: Geneva, Switzerland, 2016.
49. Nixon, G.; Garson, J.A.; Grant, P.; Nastouli, E.; Foy, C.A.; Huggett, J.F. Comparative Study of Sensitivity, Linearity, and Resistance to Inhibition of Digital and Nondigital Polymerase Chain Reaction and Loop Mediated Isothermal Amplification Assays for Quantification of Human Cytomegalovirus. *Anal. Chem.* **2014**, *86*, 4387–4394. [CrossRef]
50. Francois, P.; Tangomo, M.; Hibbs, J.; Bonetti, E.-J.; Boehme, C.C.; Notomi, T.; Perkins, M.D.; Schrenzel, J. Robustness of a Loop-Mediated Isothermal Amplification Reaction for Diagnostic Applications. *FEMS Immunol. Med. Microbiol.* **2011**, *62*, 41–48. [CrossRef]
51. Siegert, N.W.; McCullough, D.G.; Liebhold, A.M.; Telewski, F.W. Dendrochronological Reconstruction of the Epicentre and Early Spread of Emerald Ash Borer in North America. *Divers. Distrib.* **2014**, *20*, 847–858. [CrossRef]