Project title:	**Rapid DNA-based identification of brown marmorated stink bug, *Halyomorpha halys***
Project number:	CP 197
Project leader:	Glen Powell, NIAB EMR
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Key staff:	NIAB EMR Glen Powell, Celine Silva QMUL Elizabeth Clare, Joseph Trafford, Rosie Drinkwater
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Industry Representative:	N/A
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The results and conclusions in this report are based on an investigation conducted over a 4-four-month period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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GROWER SUMMARY

Headline

A highly sensitive and specific DNA-based test has been developed and validated for detection of the invasive pest insect, brown marmorated stink bug (*Halyomorpha halys*), enabling us to rapidly diagnose any material (e.g. dried and fragmentary eggs) which may be found in agricultural settings.

Background

The UK faces the emergence of a new, invasive crop pest recently confirmed to be present in South-East England, in the wild. The brown marmorated stink bug (BMSB) (Pentatomidae: *Halyomorpha halys*) is a serious and generalist pest native to Asia but spreading globally with serious impacts on agricultural productivity. Modeling based on regional climate data and the insect life cycle suggests that South-East England is the most suitable region of the UK for establishment. Adult BMSB were found in Hampshire in 2018/2019, but other life stages (indicating breeding populations) have not yet been reported. The insect poses a likely imminent threat to UK horticulture as it can feed on and damage a wide range of plant species, including soft fruit, ornamentals, field vegetables and tree fruit. The adults and immature stages of this new invasive pest are very similar in appearance to those of native UK shield bug species making it difficult to differentiate between them. Indeed, when egg masses are found in crops it is currently impossible to identify them to species.

This project was designed with four objectives:

1) Develop a high-throughput, rapid DNA-based method for forensic detection of BMSB.

2) Evaluate the reliability of this test for species-level identification of adults, nymphs and egg masses.

3) Investigate the feasibility of combining the new test with monitoring (e.g. using pheromone-baited traps) as part of an early BMSB surveillance programme.

4) Evaluate this test for secondary detection of parasitoids which may contribute to natural biocontrol.

A key deliverable of the project was to make the test available to UK growers for the 2020 season in the event that BMSB is suspected or detected and mass screening required.

Summary

In this project, we assembled an extensive database of DNA sequences from BMSB and other shield bug species that are commonly confused with this pest. This reference database will be made available as a resource internationally to aid other researchers in DNA-based identification of BMSB. The database has enabled the design and testing of new PCR primers as part of this project. Two new sets of primers (named 8XT and 13XT) have a high affinity for BMSB DNA. The 8XT primers show particularly high sensitivity for amplification of BMSB DNA and work even with trace material at extremely low DNA concentrations (with effective detection following 10,000 times dilution of the original DNA) and degraded samples (e.g. empty egg cases following hatching which were dried and not otherwise preserved). In combination with high-throughput sequencing this provides a robust and rapid (diagnosis within a few days) tool for BMSB identification of degraded fragmentation trace material.

A secondary goal of the project was to test existing 'general' primers for insects, with the potential to amplify DNA from BMSB but also from other insect species in mixed samples. Such general primers will aid the development of a less sensitive assay for BMSB detection, but one with the additional advantage of simultaneously detecting other insect species that are economically important (e.g. parasitoids and other natural enemies of BMSB, or other shield bug pest species that pose a risk to crops). The project has therefore also identified and tested two additional sets of PCR primers (Beth and Zeale) with potential for application to more complex insect species mixes. One of these primers (Beth) shows wide success with other insect species and some potential for detection of egg parasitoids of BMSB and other shield bug pests (subfamily Scelioninae), although some redesign of one of the primer pairs would be necessary to allow effective and reliable species differentiation of parasitoids.

In summary, this project provides a robust DNA-based test for BMSB and the basis for commercialisation of a diagnostic tool for BMSB and other insect species relevant to this invasive pest. Samples can already be processed at QMUL on a contract basis (http://research.sbcs.qmul.ac.uk/e.clare/Elizabeth_Beth_Clare_BMSB.html). The company that carried out all sequencing analysis as part of the project (NatureMetrics, UK) are currently in discussion with us regarding full commercialisation of the technology to provide a rapid diagnostic test for UK growers.

Financial benefits

Brown marmorated stink bug has expanded its global range in recent years and become a major agricultural pest of a wide range of crops. As a generalist insect, able to feed on more than 100 different host-plant species, outbreaks often result in substantial economic damage to multiple crops. Adult and immature (nymph) stages of the pest inflict damage when they

insert their stylet mouthparts into plant tissue for feeding and injection of toxic saliva. The insects particularly target flower buds and fruiting bodies on a variety of crops, resulting in the marketable produce becoming scarred, discoloured and deformed. The pest causes substantial losses in arable field crops (e.g. sweetcorn) in addition to vegetables (e.g. tomatoes, peppers, beans), tree fruit (e.g. apples and pears) and soft fruit (e.g. raspberries). UK apples and pears are at a particularly high risk of damage. In 2010, BMSB damage to apple crops alone in the Mid-Atlantic region of the USA was estimated to result in losses of 37 million US Dollars while stone fruit growers in the same region and period lost more than 90% of their crop. In one region of Italy (Emilia-Romagna), combined losses to pear, peach and nectarine crops have been estimated to exceed 350 million Euros in one year (2018 figures).

Effective control of BMSB requires action to be taken at an early stage following colonisation of new areas. Attract-and-kill approaches, combining applications of aggregation pheromone and a contact insecticide to selected trees, are showing promise for control in USA orchards. However, any effective mitigation strategies are reliant on rapid and accurate identification of the pest. The detection methodology developed during this project can facilitate significant advances in the speed, accuracy and sensitivity of DNA-based identification of this invasive pest species, with particularly useful application to material which is not morphologically identifiable (e.g. eggs).

Action points

- Crops at potential risk of BMSB damage following its establishment in the UK include top fruit (particularly apples), soft fruit and field vegetables (including sweetcorn and legumes). Growers should be vigilant for signs of shield bug life stages (adults, nymphs and eggs) in and around crops. To aid identification and check whether samples are BMSB, a diagnostic test is now available via the Clare Lab at QMUL on a contract basis (http://research.sbcs.qmul.ac.uk/e.clare/Elizabeth_Beth_Clare_BMSB.html). Further discussions with commercial partners are ongoing to establish the analysis as a permanent commercially-available diagnostic test for growers.
- The development of this test required construction of an extensive database of DNA sequences (BMSB and closely-related / potentially-confused species). This will be made available to other researchers via the BOLD website (http://www.boldsystems.org/) as a web-accessible public project. It will be included as a downloadable supplement to any academic publications arising from the project.

- New PCR primers have been designed. One primer set has extremely high affinity for BMSB DNA and was therefore suitable for the development of a specific detection test targeting the invasive pest. A set of generalist primers could be more suitable for simultaneous detection of parasitoid DNA, although some modification of the primer design would be required to achieve this.
- Further research is required to determine false positive rates. This is currently being undertaken with a second round of DNA sequencing which includes a number of samples of insect DNA but excludes BMSB DNA. These will be analysed blind and we will look for BMSB identifications where none should be possible (no BMSB DNA added, therefore false positives). Results are anticipated in October 2020.

SCIENCE SECTION

Introduction

An invasive pest spreading globally. The UK faces the emergence of a new invasive crop pest recently confirmed to be present in South-East England in the wild. The brown marmorated stink bug (BMSB, *Halyomorpha halys*) is an invasive shield bug from Asia that has spread around the world, causing millions of dollars in crop damage in North America and Europe (Haye et al., 2015; Leskey & Nielsen, 2018). Once introduced, BMSB is able to spread rapidly and may have agricultural impacts very quickly following establishment. For example, BMSB was first detected in Georgia in 2015 (Gapon, 2016) and caused US$24 million losses in hazelnut production in the first full year it was present. Similarly, it has spread through 44 US states and was classified as an "outbreak" in 2010 when it caused US $37million per year in damage to the apple crop alone in Mid-Atlantic states (Rice et al., 2014; Morrison et al., 2019). Stone fruit growers in the same region and period lost more than 90% of their crop (Leskey and Hamilton, 2010).

The insect now poses an imminent threat to UK horticulture as it is able to feed on and damage a wide variety of plant species, including soft fruit, ornamentals, field crops and tree fruit. In addition to this significant threat to crops, BMSB targets houses and other buildings for over-wintering. Large aggregations of adults in autumn lead to urban nuisance problems and a propensity for global "hitch-hiking" with exported cargo and passenger luggage.

In the UK, top fruit crops are at particular risk from this new pest, but cane fruit and glasshouse / field crops including sweetcorn, tomato, pepper and brassicas are also likely to be damaged. The pest has been intercepted with imported goods on several occasions in the UK over the last 10 years, but has been reported in the field only recently, with two adults found at separate locations in Hampshire during the last 18 months. Bioclimatic modeling suggests that South-East England is the most suitable region of the UK for establishment (Kriticos et al., 2017), although breeding populations (evidenced by nymphs and egg masses) have not yet been discovered and reported.

The need for early detection. BMSB is a highly-mobile insect, and its global range expansion has allowed it to escape the influence of native predators. The pest is also relatively tolerant to insecticides and unfortunately the active ingredients with highest efficacy are broad-spectrum products (e.g. pyrethroids and neonicotinoids) (Rice et al., 2014). Successful control of BMSB may require multiple applications of such insecticides, leading to major disruption of integrated pest management programmes (Short et al., 2017). BMSB is therefore extremely difficult to control. Strategies such as the use of semiochemicals and an

“attract and kill” approach have shown some promise (Weber et al., 2017; Morrison et al. 2019) but mitigation strategies are most effective early in an outbreak. Thus the key is early detection, when a local field invasion is in the low to moderate stage of attack (Morrison et al., 2019).

The need for accurate identification. NIAB EMR have taken a leadership role in the surveillance for invasive pests of UK horticulture. Pheromone-baited traps have been deployed for BMSB monitoring during the 2018 and 2019 growing seasons, as part of AHDB Project TF 223, but this project has now ended (February 2020). As part of our surveillance activities, we also launched an appeal to growers, agronomists and members of the public to look out for any shield bugs resembling BMSB (Figure 1) and send specimens and images for identification and early detection (e.g. Powell, 2018).

Unfortunately, BMSB is similar in appearance to other shield bug species found in the UK and this has caused extensive confusion, false alarms and misidentifications. Two native UK species (*Dolycoris baccarum* and *Pentatoma rufipes*) are widespread, found frequently in cropping systems, and are being confused with BMSB on a regular basis (Powell & Fountain, 2019). In addition, the invasive species *Rhaphigaster nebulosa* superficially resembles BMSB, has become established in South-East England recently, and is the source of further false alarms. While it is difficult for non-experts to identify BMSB adult and nymphs with any confidence, distinguishing BMSB egg masses from those of other species of shield bug presents an even greater challenge to effective biosecurity and crop protection.

DNA-based detection. Previous work has made some progress towards development of a DNA barcoding approach, using PCR primers to amplify DNA from a variety of Canadian species within the shield bug (Pentatomidae) family. Gariepy et al. (2014; 2019) used traditional Sanger sequencing to correctly identify >90% of recovered material (egg masses, nymphs, adults). However, this diagnostic test has not been validated for the UK where the community of pentatomid species are different and the method has not been adapted for high-throughput analysis, which will reduce diagnostic costs and increase the rate and scale of sample processing. One of the aims of this project is to refine and adapt this method for use with a high-throughput sequencing platform, substantially reducing the diagnostic costs and making the detection test compatible with future large-scale pest monitoring programmes.

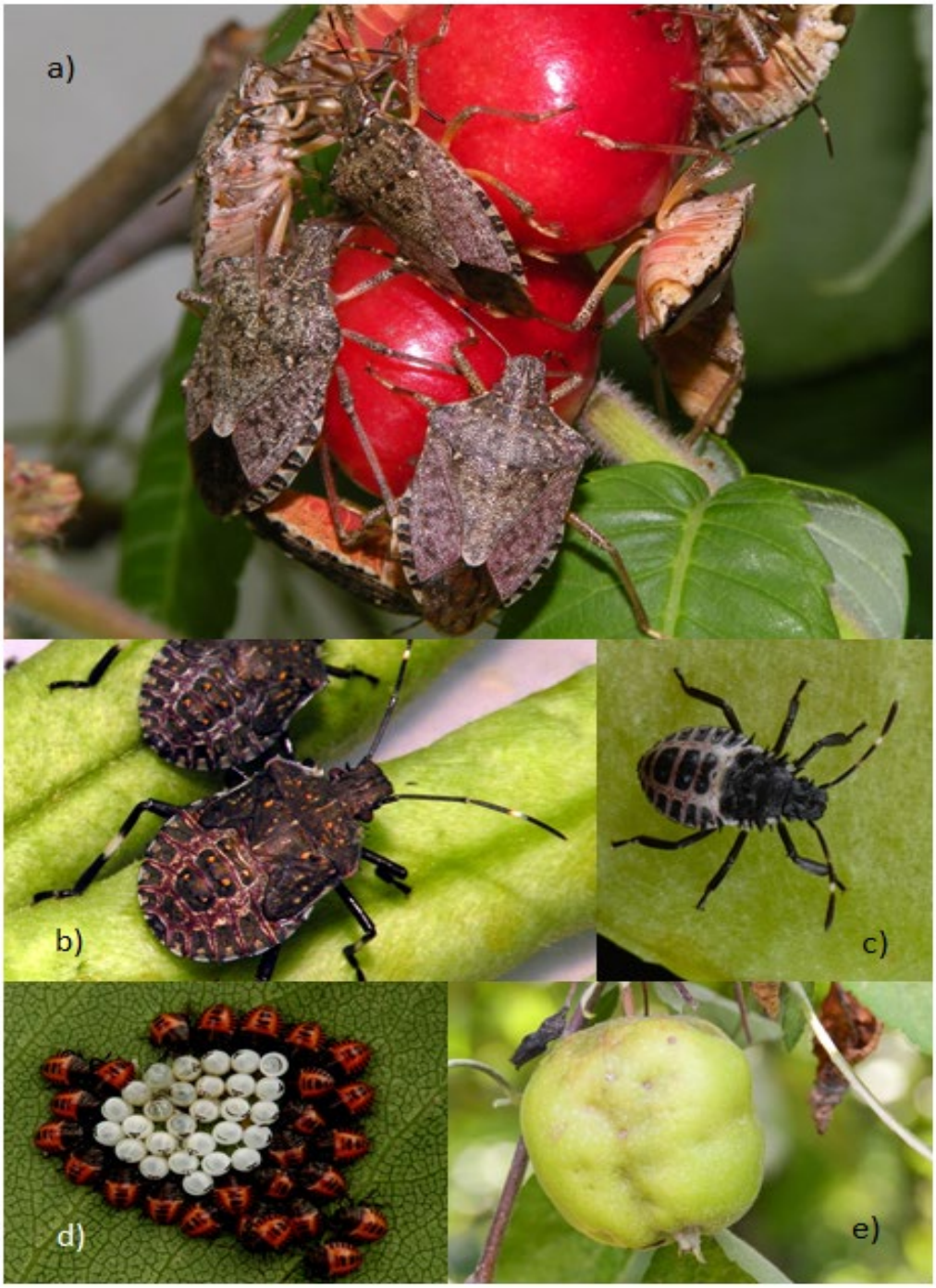

Figure 1. BMSB life stages and damage (not to scale). a) Adults; b) last immature stage (fifth instar nymphs); c) second instar nymph; d) first-instar nymphs clustered around empty egg cases; e) feeding damage to apple. All images kindly provided by Tim Haye (CABI) and used with permission.

In this project we aim to develop and validate a high-throughput DNA-based test to identify BMSB, allowing reliable and rapid discrimination from other shield bugs within the UK. Because our approach is designed to employ the latest in high-throughput sequencing and to therefore access trace and environmental DNA (eDNA) along with the target species, a possible secondary benefit will be the detection of potential parasites preying on BMSB and leaving trace material, for example egg parasitoids which leave DNA traces in egg masses. The Gariepy approach also correctly diagnoses these and we also seek to validate the anticipated high-throughput diagnostic for the detection of these potential agents of bio-control as a secondary target of DNA sequencing.

This project therefore aims to develop and validate a rapid diagnostic DNA test for use in the UK horticultural sector and capitalise on opportunities to monitor the first phase of the BMSB invasion as it unfolds and help inform mitigation actions at an early stage. We further aim to evaluate the potential to detect native agents of bio-control using the same diagnostic as a by-product of BMSB identification. Once completed, our intention is to establish a new pipeline for high-throughput analysis of BMSB (e.g. with the commercial eDNA analysis company NatureMetrics), ready for deployment during the 2020 field season for both commercial and academic applications in screening for BMSB in the wild.

Materials and Methods

WP1. Develop a high-throughput, rapid DNA-based method for forensic detection of BMSB

Reference database generation

A reference collection of sequences was assembled for BMSB and 48 other species (other Heteroptera likely to be confused with BMSB and misidentified; Table 1). This database was targeted to include 2,514 reference sequences and specimens which were obtained from collaborator Tara Gariepy (2091 sequences), the BOLD database (Ratnasingham & Hebert, 2007; www.boldsystems.org; 404 sequences), and specimens supplied by NIAB EMR recent or historic collections (19 sequences).

Table 1. Numbers of specimens and sequences targeted for the creation of a reference collection. “NIAB New Specimens” were field collected in the UK (April 2018 – November 2019); “NIAB Old Specimens” represent single-leg samples taken from a historic (1930s-1940s) collection of pinned UK-collected insects. Sequences were obtained from public BOLD records or from records assembled by Agriculture and Agri-Food Canada (collaborator Tara Gariepy).

Common name if applicable	Genus	Species	NIAB New Specimens	NIAB Old Specimens	Sequences from Agriculture and Agri-Food Canada	Sequences from BOLD
Hawthorn shield bug	*Acanthosoma*	*haemorrhoidale*	2			8
Bishop's Mitre shield bug	*Aelia*	*acuminata*		1		14
	Anasa	*sp.*			6	
	Banasa	*calva*			11	
	Banasa	*dimidiata*			93	
Juniper shield bug	*Banasa*	*euchlora*			1	
Four-humped stink bug	*Brochymena*	*quadripustulata*			33	
Green stink bug	*Chinavia*	*hilaris*			329	
	Chlorochroa	*persimilis*			7	
Dock bug	*Coreus*	*marginatus*	3			24
	Cosmopepla	*sp.*			2	
Juniper shield bug	*Cyphostethus*	*tristriatus*				2
Sloe bug/Hairy Shield bug	*Dolycoris*	*baccarum*	5		3	25
Birch shield bug	*Elasmostethus*	*interstinctus*		1		29
Parent bug	*Elasmucha*	*grisea*	2			10
Scarlet shield bug	*Eurydema*	*dominulus*				81
Crucifer shield bug	*Eurydema*	*oleracea*				28
	Eurydema	*sp.*			22	
Scarce Tortoise shield bug	*Eurygaster*	*maura*				9
Tortoise shield bug	*Eurygaster*	*testudinaria*				11
Brown stink bug	*Euschistus*	*servus*			113	

Common name if applicable	Genus	Species	NIAB New Specimens	NIAB Old Specimens	Sequences from Agriculture and Agri-Food Canada	Sequences from BOLD
	Euschistus	*sp.*			35	
Dusky stink bug	*Euschistus*	*tristigmus*			230	
One-spotted stink bug	*Euschistus*	*variolarius*			52	
Woundwort shield bug	*Eysarcoris*	*venustissimus*				2
Brown marmorated stink bug	*Halyomorpha*	*halys*	1		989	
Western conifer seed bug	*Leptoglossus*	*occidentalis*	1			81
Small Grass shield bug	*Neottiglossa*	*pusilla*				3
Southern green shield bug	*Nezara*	*viridula*			8	
Common green shield bug	*Palomena*	*prasina*	3	1		19
Arboreal stink bug	*Parabrochymena*	*arborea*			30	
Forest bug/red-legged shield bug	*Pentatoma*	*rufipes*	1			12
	Pentatomidae	*sp.*			4	
Spiked Shield bug	*Picromerus*	*bidens*		1		
Gorse shield bug	*Piezodorus*	*lituratus*	1			15
	Plautia	*stali*			17	
Predatory stink bug	*Podisus*	*brevispinus*			3	
Spined soldier bug	*Podisus*	*maculiventris*			60	
Predatory stink bug	*Podisus*	*placidus*			2	
	Podisus	*sp.*			3	
Turtle or Knobbed shield bug	*Podops*	*inuncta*				1
Heather shield bug	*Rhacognathus*	*punctatus*				1
Mottled shield bug	*Rhaphigaster*	*nebulosa*	2		3	12
Sand-runner shield bug	*Sciocoris*	*cursitans*				5
Red-shouldered stink bug	*Thyanta*	*accerra*			7	
Red-shouldered stink bug	*Thyanta*	*pallidovirens*			28	
Pied shield bug	*Tritomegas*	*bicolor*				3
Bronze shield bug	*Troilus*	*luridus*		1		2
Blue shield bug	*Zicrona*	*caerulea*				7

Reference specimen collections

UK field-collected specimens of 14 species were assembled at NIAB EMR (Table 1). Specimens collected in 2018 /19 were stored at -20ºC between collection and DNA extraction. Additional specimens were sourced from a pinned insect collection at NIAB EMR (one leg taken per specimen, insects originally collected by A.M. Massee between 1935 and 1943). The BMSB sample included in the NIAB collection represents a single leg taken from the first adult reported in the UK (collected in Hampshire, November 2018 and kindly supplied by Mr Melvin Knapp of Killgerm Ltd.).

Test material collection

In addition to the creation of a reference database (see above) we obtained a large number of specimens which were used to test laboratory protocols (DNA extraction, evaluation of primers, sequencing). Where possible these steps were tested on as wide a variety of samples as possible. The material assembled for this purpose was as follows:

From our collaborator at CABI Switzerland (Tim Haye) we acquired dried samples of adults, nymphs and eggs of BMSB with a small number of additional eggs and adults of *Nezara viridula*, and *Palomena prasina*. These samples were dry and not preserved under ideal conditions. Adults, “moults” (cast insect cuticles or “skins”), and nymphs had been dead for approximately two months prior to DNA extraction. Egg masses had been collected in May of 2019 so were approximately 8 months old. Samples had been stored at 10°C and then at room temperature. They were used to test the sensitivity of the analysis against “difficult” samples.

From our collaborator at Agriculture and Agri-food Canada (Tara Gariepy) we acquired dried samples of unhatched egg masses (freeze killed) and hatched egg masses (empty egg cases) from BMSB (5 & 5 unhatched and hatched samples respectively), *Chinavia hilares* (3 & 3 samples), *Podisus maculiventris* (4 & 5), *Cosmopepla spp.* (3 & 2), *Euschistus variolarius* (3 & 5), *Euschistus tristigmus* (3 & 5). These were freshly killed when collected but subsequently stored dry.

Fifty samples of over-wintering adults (~600 individuals) were collected from Italy with help from collaborator Lara Maistrello in September, October and November 2019. These were collected across a wide geographical area, from the province of Bali (Apulia region) in the south, up to the Pordenone province north of Venice. An additional adult was collected in Germany (Cologne). Nymphs are not available in autumn and winter, but our collaborators made available seven stored samples of nymphs (66 individuals). An additional 89 samples

of nymphs and egg masses (collected and frozen) were also made available by our colleagues in Italy. These were predominantly *H. halys*, although a few other pentatomid species (*Nezara viridula* and *Dolycoris baccarum*) were included. Samples of adults and nymphs were stored in 70% ethanol following live collection, whereas egg masses were stored dry and frozen (at -20°C) before DNA extraction.

In silico *analysis of variable region*

Existing sequences (obtained from collaborator Tara Gariepy and BOLD databases) were aligned in Geneious (www.geneious.com; Kearse et al., 2012). Variable regions were identified as potential amplicons (see primer design). We designed two sets of primers which target the most variable region and specifically amplify BMSB DNA without great affinity for other insects. We then evaluated existing primers from the literature (Elbrecht et al., 2019) and selected published primers BF3, MzplankF2, BN, BR1i, BR2, En which closely matched the identified variable region. Additionally, two general insect primers, the Zeale primers (Zeale et al., 2011) and a newly designed primer ("Beth" primer from the QMUL Clare laboratory) were selected. These primer pairs were ordered for laboratory-based testing (sequences shown in Table 2). The corresponding amplified regions were extracted from the reference collection to assess the discrimination of the region for BMSB *vs*. other insect species in the databases.

DNA extraction protocol

DNA was extracted following a Chelex-based protocol modified from Gariepy et al. (2019).
Preparation of Chelex mixture:
1) 50% bleach was used to sterilise a dry reagent spatula, and a small magnetic stir bar. These were then rinsed in DNA-free water and wiped clean with Kimwipes.
2) A 5% by weight slurry of Chelex 100 Resin (Biorad part 143-3832, 100-200 mesh Chelex, sodium form) and UV sterilised HPLC water was prepared.
3) A sterile stirbar was placed in a beaker on a magnetic stirrer. Chelex settles quickly so the slurry was kept well mixed, before aliquoting 300-500 µl into sterile 1.5ml eppendorf tubes and capping immediately.

DNA extraction:
1) Sample material was placed in sterile 1.5 ml eppendorf tubes using sterilised tweezers (To sterilise, tweezers were placed in 50% bleach for 30 seconds then rinsed with DNA-free water. Wiped with Kimwipes)
2) Each sample was macerated in 2 µl of proteinase K (20 mg/ml) and 100µl of 5% Chelex 100 Molecular Grade Resin (Bio-Rad) using a sterilised micropestle

3) One negative extraction control (no sample) was included to ensure absence of contaminants

4) Samples were incubated at 55ºC overnight

5) Samples were then incubated at 99ºC for 10 mins

6) Samples were centrifuged at 5800 g for 5 mins to pellet Chelex solution

7) 50 µl of supernatant was transferred to new sterile eppendorf tubes (this includes DNA)

8) Samples were stored at -20ºC until further analysis.

PCR primer design

Novel primers (Table 2) were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) with the restriction that the maximum product size was 350bp. Specificity checking was performed against refseq representative genomes (Hemiptera) and against our own sequence database alignments. Our match criteria included at least 3 mismatches within the last 5bps at 3' end to produce specificity for BMSB. For sequencing all primers were modified to include Nextera adapters.

Table 2. Primer evaluation

Name	Direction	Sequence (5'-3')	Reference
8XT_F	Forward	Temporary publication ban*	pending
8XT_R	Reverse	Temporary publication ban*	pending
13XT_F	Forward	Temporary publication ban*	pending
13XT_R	Reverse	Temporary publication ban*	pending
Beth_F	Forward	Temporary publication ban*	pending
Beth_R	Reverse	Temporary publication ban	pending
ZEA_F	Forward	AGATATTGGAACWTTATATTTTATTTTTGG	Zeale et al., 2010
ZEA_R	Reverse	WACTAATCAATTWCCAAATCCTCC	Zeale et al., 2010
BF3	Forward	CCHGAYATRGCHTTYCCHCG	Elbrecht et al., 2019
BR2	Reverse	TCDGGRTGNCCRAARAAYCA	Elbrecht et al., 2019
BN	Forward	CCNGAYATRGCNTTYCCNCG	Elbrecht et al., 2019
En	Reverse	GTRATNGCNCCNGCNARNAC	Elbrecht et al., 2019
MzplankF2	Forward	RGYNGGNACRGGNTGRACNGT	Elbrecht et al., 2019
BR1i	Reverse	ARYATIGTRATIGCICCIGC	Elbrecht et al., 2019

*Note: these primers are currently protected by a temporary publication ban.

PCR primer testing

Primers were tested against reference material provided by collaborators using the following amplification mix (Table 3) and protocol:

Table 3. PCR amplification mix for all primers.

Reagent	**Volume x1 rcn**
2 x Qiagen multiplex mix	7.5 µL
Primer F (10 µM)	0.5 µL
Primer R (10 µM)	0.5 µL
H_2O	4.5 µL
Total mix	13 µL
DNA added per rcn	2 µL
Total volume	15 µL

The following thermocycling procedures were used:

8XT and 13XT primers with sequencing adapters incorporated

15 mins @ 95°C
35 x 40s @ 94°C
60s @ 61°C
40s @ 72°C
10 mins @ 72°C
Hold at 10°C

Beth primers with sequencing adapters incorporated

15 mins @ 95°C
35 x 40s @ 94°C
60s @ 46°C
30s @ 72°C
10 mins @ 72°C

Zeale primers with sequencing adapters incorporated
15 mins @ 95°C
40 x 40s @ 94°C
60s @ 50°C
30s @ 72°C
10 mins @ 72°C

For primers from Elbrecht et al (2019) a variety of PCR conditions were used including regular and gradient PCRs. These were not consistently successful and are thus not reported and further analysis with these primers was abandoned.

Testing design and sequence analysis

To validate PCR testing, a full high-throughput sequencing test was designed which included mixes of BMSB DNA from adults, nymphs and eggs in undiluted and diluted form (both as single specimens and deliberately contaminated with DNA from other insect species; see plate layout, Table 4) for a total of 96 different DNA combinations. Dilution was set at 100x with the exception of the 13XT primers where dilution at this level failed consistently so was held to 10x. Deliberate insect contamination used a mix of DNA extracted from 63 individuals of hemipteran species (*Nezara viridula, Chinavia hilaris, Podisus maculiventris, Cosmopepla sp., Euschistus variolarius, Euschistus tristigmus, Leptoglossus occidentalis, Palomena prasina, Coreus marginatus, Rhaphigaster nebulosa, Pentatoma rufipes, Elasmucha grisea, Dolycoris baccarum, Piezodorus lituratus,* and *Acanthosoma haemorrhoidale*), and non-hemipteran insects (*Gryllus bimaculatus, Schistocerca gregaria, Periplaneta americana, Callosobruchus maculatus,* and *Forficula auricularia*) from orders Orthoptera, Blattodea, Coleoptera and Dermaptera. To generate this "contaminant' 4 µL of DNA extract from each individual was mixed as one general insect sample" and spiked into samples of BMSB to increase the difficulty of detection by simulating field based contamination (e.g. from a pheromone trap).

This 96-well design was then PCR amplified for each of the four selected primer sets (see above), yielding a total of 384 PCR products which were visualised on a 1.5% agarose gel and sent for sequencing at NatureMetrics (www.naturemetrics.co.uk/) on an Illumina MiSeq platform.

Table 4. Plate layout for sequencing at NatureMetrics included a combination of adult, nymph and/or egg DNA from BMSB with or without the addition of the DNA mix generated as described above.

	H	G	F	E	D	C	B	A
1	Adult 1	Adult 2	Adult 3	Adult 4	Adult 5	Adult 6	Adult 7	Adult 8
2	Nymph 1	Nymph 2	Nymph 3	Nymph 4	Nymph 5	Nymph 6	Nymph 7	Nymph 8
3	Egg 1	Egg 2	Egg 3	Egg 4	Egg 5	Egg 6	Egg 7	Egg 8
4	Adult 1 + Insect	Adult 2 + Insect	Adult 3 + Insect	Adult 4 + Insect	Adult 5 + Insect	Adult 6 + Insect	Adult 7 + Insect	Adult 8 + Insect
5	Nymph 1 + Insect	Nymph 2 + Insect	Nymph 3 + Insect	Nymph 4 + Insect	Nymph 5 + Insect	Nymph 6 + Insect	Nymph 7 + Insect	Nymph 8 + Insect
6	Egg 1 + Insect	Egg 2 + Insect	Egg 3 + Insect	Egg 4 + Insect	Egg 5 + Insect	Egg 6 + Insect	Egg 7 + Insect	Egg 8 + Insect
7	Adult + Nymph + Egg + Insect	Adult + Nymph + Egg + Insect	Adult + Nymph + Egg + Insect	Adult + Nymph + Egg + Insect	Adult + Nymph + Egg + Insect	Adult + Nymph + Egg + Insect	Adult + Nymph + Egg + Insect	Adult + Nymph + Egg + Insect
8	Adult + Nymph + Egg + Insect	Adult + Nymph + Egg + Insect	Adult + Nymph + Egg + Insect	Adult + Nymph + Egg + Insect	Adult + Nymph + Egg + Insect	Adult + Nymph + Egg + Insect	Adult + Nymph + Egg + Insect	Adult + Nymph + Egg + Insect
9	Adult + Nymph + Egg + Insect (Diluted)	Adult + Nymph + Egg + Insect (Diluted)	Adult + Nymph + Egg + Insect (Diluted)	Adult + Nymph + Egg + Insect (Diluted)	Adult + Nymph + Egg + Insect (Diluted)	Adult + Nymph + Egg + Insect (Diluted)	Adult + Nymph + Egg + Insect (Diluted)	Adult + Nymph + Egg + Insect (Diluted)
10	Difficult eggs / low quality sample	Difficult eggs / low quality sample	Difficult eggs / low quality sample	Difficult eggs / low quality sample	Difficult eggs / low quality sample	Difficult eggs / low quality sample	Difficult eggs / low quality sample	Difficult eggs / low quality sample
11	Difficult eggs / low quality sample	Difficult eggs / low quality sample	Difficult eggs / low quality sample	Difficult eggs / low quality sample	Difficult eggs / low quality sample	Difficult eggs / low quality sample	Difficult eggs / low quality sample	Difficult eggs / low quality sample
12	HH+ve + Insect	HH+ve + Insect	HH+ve + Insect	HH+ve + Insect	HH+ve + Insect	HH+ve + Insect	HH+ve + Insect	HH+ve + Insect

The DNA template plate was then used for 4 PCR reactions, one for each set of tested primers (8XT, 13XT, Zeale, Beth). Adult/nymph/egg 1-8 refer to different individual specimens of each BMSB source. +Insect refers to the addition of our deliberate insect contamination (see above), diluted refers to the DNA having been diluted before PCR, “difficult” refers to BMSB samples which were not preserved under ideal conditions (dried unhatched eggs, moults, dried empty (hatched) eggs and stored at room temperature), HH+ve refers to samples of BMSB we have amplified repeatedly and thus act as positive controls we expect to amplify and sequence efficiently (in contrast to “difficult” samples).

WP2. Evaluate the reliability of the test for species-level identification of adults, nymphs and egg masses

Because only two individuals have thus far been identified in the UK, and only one of those was available for analysis, field collecting in Italy was necessary to obtain overwintering BMSB adults for validation of the new test. The haplotype diversity across Northern Italy is particularly high (Cesari et al., 2018) and collecting samples here, and accessing reference material provided by our collaborators Drs Lara Maistrello and Roberto Guidetti (UNIMORE, Emilia Romagna region) enabled us to access genetically diverse samples of BMSB for evaluation of the test. In addition, the test design allowed comparisons between detection of different life stages (eggs, nymphs and adults) as described above (Table 4).

Dilution testing

To assess the sensitivity of the four selected primer sets (8XT, 13XT, Zeale and Beth), we constructed a dilution series from BMSB adults, nymphs and eggs which included DNA extracts of concentration 2.16 ng / µl (undiluted) and then diluted with molecular grade water to 10x, 100x, 1,000x and 10,000x. PCRs were then carried out as above and visualised on an agarose gel.

WP3. Investigate the feasibility of combining the new test with monitoring (e.g. using pheromone-baited traps) as part of an early BMSB surveillance programme

Double-sided clear sticky traps can be combined with high-dose, long-lasting (12-week) pheromone lures as part of BMSB monitoring programmes (Weber et al., 2017; Powell & Fountain, 2019). Any new DNA-based diagnostic test will be particularly useful if it can be combined with pheromone-based monitoring, enabling rapid and accurate identification of BMSB when the pest colonises new areas and cropping systems. Field-trapped BMSB are likely to be held in place for at least several days before traps can be checked, and some degree of environmental degradation of the insect DNA is therefore expected to occur before samples can be collected for diagnostic testing. In addition, pheromone lures are often combined with sticky traps or a drowning solution to immobilise the target pest for monitoring purposes, but this results in significant by-catch (trapping of other, non-target species) and samples of the pest are therefore inevitably mixed with a variety of other arthropods. In order to investigate the feasibility of DNA detection from contaminated and degraded samples we have used multiple methods to assess the sensitivity of the assay.

1) Dilution series were constructed to estimate the sensitivity of the test to degraded material (see WP2 above).

2) PCR tests were used to specifically compare well-preserved material to poorly preserved material.
3) Sequencing specifically included diluted and poorly preserved (difficult) material (Table 4) which included dried eggs.

These three approaches were incorporated to challenge the methodology of the designed DNA test with material which simulates environmental contamination and degradation.

WP4. Evaluate the test for secondary detection of parasitoids which may contribute to natural bio-control

To assess whether the newly-developed tests can also detect potential native parasitism (particularly egg parasitoids) we performed an *in silico* analysis of the most successful general primers (Beth). We extracted reference examples of the Platygastridae, in particular *Telenomus podisi* (subfamily Scelioninae) which are known egg parasitoids of stink bugs. We aligned these with the excised Beth region but alignment was problematic at the 5' end of the region. We then truncated the region to the well-aligned 3' region and constructed a neighbour joining tree to test for species differentiation.

Results

WP1. Develop a high-throughput, rapid DNA-based method for forensic detection of BMSB

DNA extraction and PCR amplification

Chelex DNA extractions were highly successful on all material including old and fragmentary insects, moults and dried hatched (empty) eggs. Newly-designed 8XT and 13XT primers proved highly-specific for the amplification of BMSB DNA from all life stages. Primer set 13XT did not amplify any non-BMSB DNA that could be visualised on a gel. Primer set 8XT amplified *Nezara viridula* and *Gryllus bimaculatus* in a few tests but this was sporadic. Primer sets from Elbrecht et al. (2019) failed to amplify any DNA extracts and were excluded from further analysis. General insect primer sets Zeale and Beth amplified BMSB and other non-target insects as expected.

Species discrimination testing

To test the discrimination ability of the four selected regions for further analysis (8XT, 13XT, Zeale and Beth) excised regions from the reference database were imported into MEGA (www.megasoftware.net; Kumar et al., 2018). A neighbour-joining tree was constructed using the Kimura-2parameter model which was then visualised in FigTree (www.tree.bio.ed.ac.uk\software\figtree). In each case the amplified region shows high discrimination ability for BMSB as well as good discrimination for all species in the reference database, suggesting that both the specific (8XT and 13XT) and generic (Zeale and Beth) amplified regions should provide good identification ability for BMSB (see Figures 2-5).

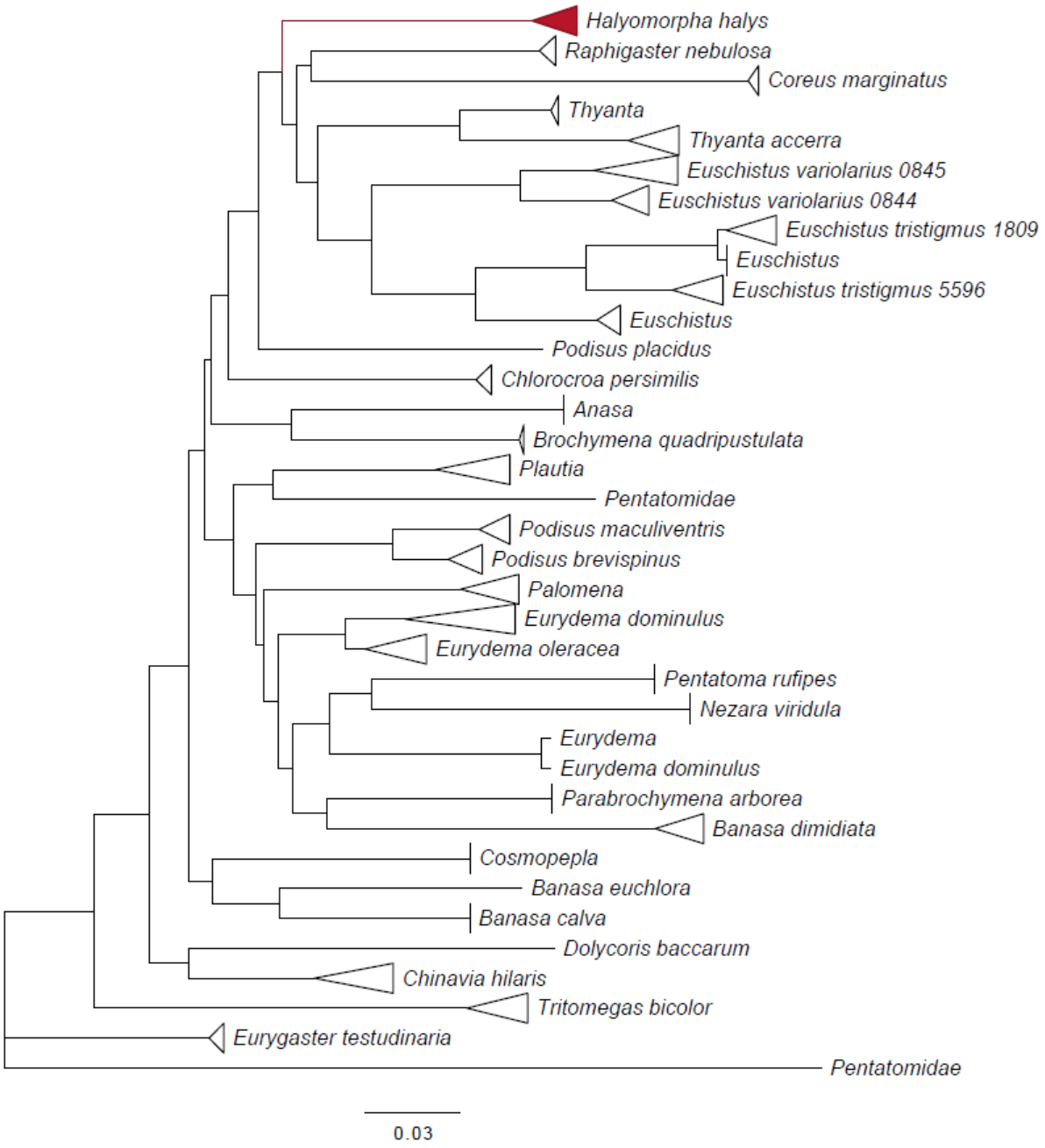

Figure 2. A neighbour-joining tree of the 8XT region showing discrimination ability for BMSB (red triangle) and other insects in the reference collection. Triangle width depicts intraspecific sequence variability.

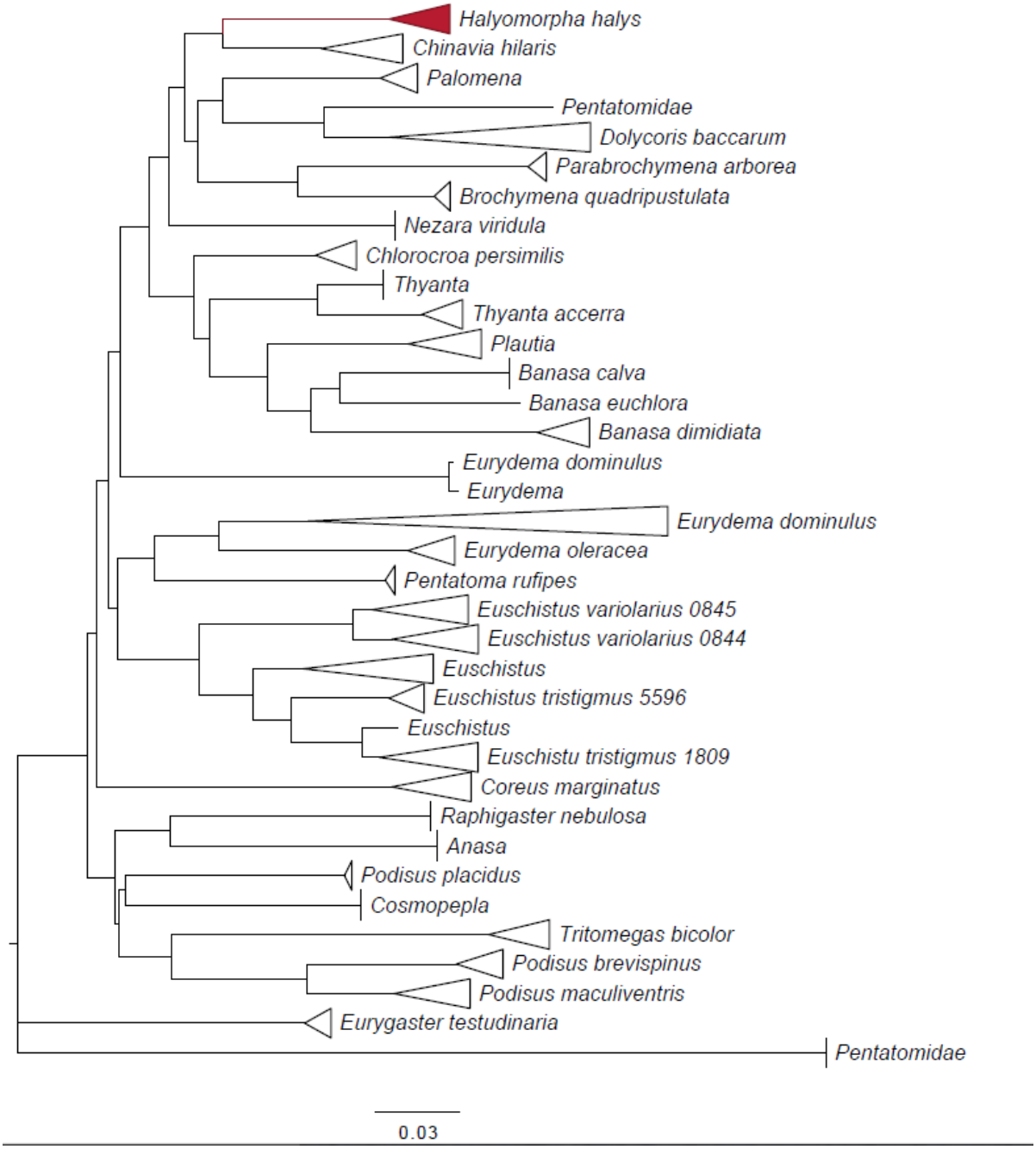

Figure 3. A neighbour-joining tree of the 13XT region showing discrimination ability for BMSB (red triangle) and other insects in the reference collection. Triangle width depicts intraspecific sequence variability.

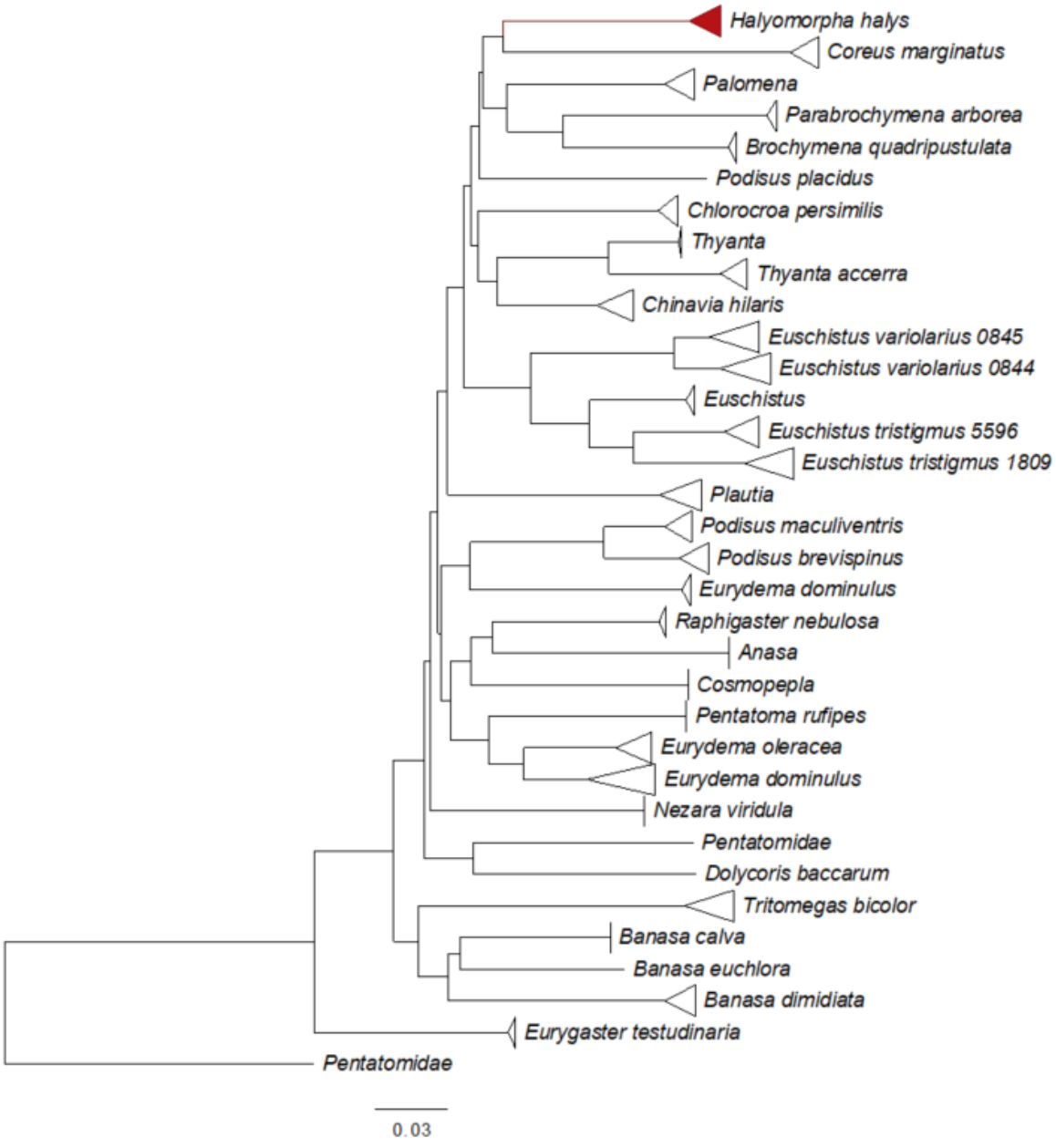

Figure 4. A neighbour-joining tree of the Beth region showing discrimination ability for BMSB (red triangle) and other insects in the reference collection. Triangle width depicts intraspecific sequence variability.

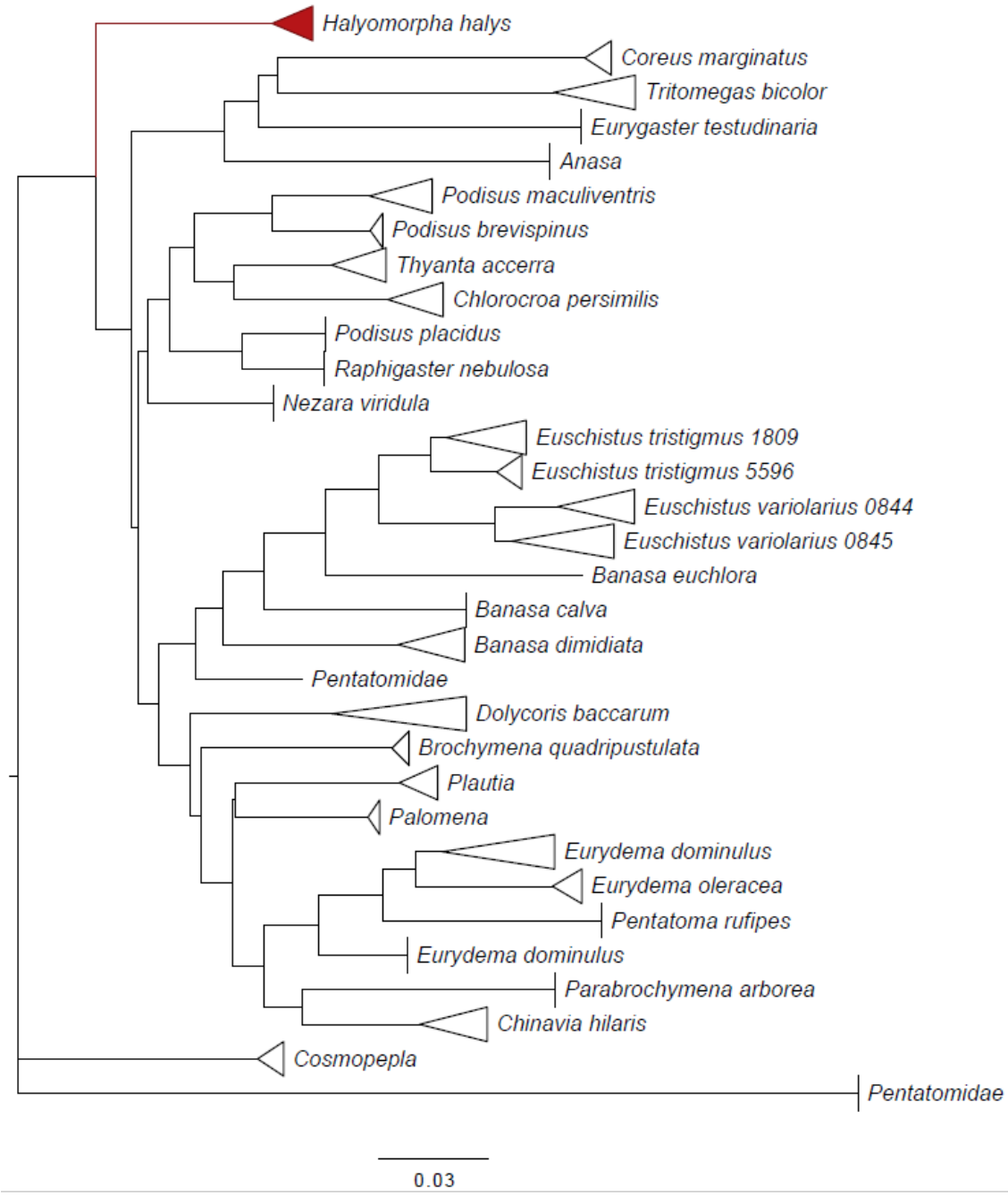

Figure 5. A neighbour-joining tree of the Zeale region showing discrimination ability for BMSB (red triangle) and other insects in the reference collection. Triangle width depicts intraspecific sequence variability.

Sequencing outcomes

All sequencing files received from NatureMetrics passed standard quality control steps. For analysis, all files were uploaded to the mBRAVE (www.mbrave.net) system. Analysis included merging R1 and R2 files, removal of primers, quality assessment and filtering and identification against selected reference datasets. The following parameters were set for initial processing: front and end trimming (specific to primers); trim length = 500 bp; primer masking = Off; Min QV = 10; Min length = 100 bp; Max bases with low QV = 75%; Max bases with ultra-low QV = 75%; Paired end merging = Merge; Min Overlap = 20 bp; Max substitution = 5 bp. Note: quality control (QV) was deliberately set to maximise read retention.

The 96 samples generated 6,001,306 sequences for primer set 8XT, 5,940,430 sequences for primer set 13XT, 12,355,140 sequences for primer set Beth and 5,457,592 sequences for primer set Zeale. Sequence quality was extremely high (QV scores ~40 for all samples).

For identification, the bespoke database of reference material created for this project was added to mBRAVE and selected as the primary identification library. This was augmented with system libraries for insects, non-insect arthropods, human contamination checking and non-arthropod invertebrates in that order. To maximize the detection of BMSB along with any contamination, identification parameters were set to report any identification within 10% of a reference sequence.

Based on previous work using control mock communities, we applied a filter to the identification results, removing any identification with fewer than 200 reads assigned to a reference which has been experimentally determined to minimize false positive assignment rates. For the 8XT sequence set, BMSB was correctly identified in 92 of 96 samples for a 4.2% false negative identification rate. For the 13XT sequence set, BMSB was correctly identified in 86 of 96 samples for a 10.4% false negative identifications. For the Beth sequence set, BMSB was correctly identified in 78 of 96 samples, for an 18.8% false negative identification rate. For the Zeale sequence set, BMSB was correctly identified in 46 of 96 samples, for a 52.1% false negative rate (see Table 7). All retained identifications for screening exceeded 98% similarity to reference sequence from the bespoke database. If the screening filter (requiring a minimum of 200 sequences to be assigned to a reference to accept the identification) is lowered, the false negative rates fall considerably. For example, in the 8XT primers the false negative rate would become zero if the filter requirement was set at 100 reads assigned rather than 200.

For the general primer sets, false negatives tended to represent swamping of the BMSB DNA by other insects. In our test, DNA from 20 other species were spiked into BMSB samples in 56 of the 96 wells to represent potential contamination events during sample collection (e.g.

using pheromone traps where other insect material would be present). Systematic false negatives (e.g. occurring in two rows in the Beth primer test set) were found when the BMSB DNA was very low (derived from eggs, or diluted) and contaminant insects DNA was high (Table 5). In these cases the sequencing results were dominated by *Palomena prasina, Pentatoma rufipes, Rhaphigaster nebulosa,* and *Schistocerca gregaria*. Systematic false negatives were not seen in the corresponding BMSB specific primers (8XT and 13XT) and it should be noted that these represent the worst possible case scenarios for detection; weak BMSB DNA, strong insect contamination and primers designed to be general in nature. False positive rates are currently being determined through a second round of sequencing (results anticipated during May 2020). If false positive rates are low the 200 read filter can be reduced and detection rates would correspondingly increase.

Table 5. False negative detections (red) of BMSB when the read assignment filter was set at 200. Any such identification where fewer than 200 reads was assigned to a reference was excluded from detections. For example in the 8XT table, the 4 false negative results (red) actually detected BMSB but with fewer than 200 reads assigned. See Table 4 for further details of samples.

8XT								**Contents Summary**
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Adults
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Nymphs
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Eggs
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Adults+Insect
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Nymphs+Insect
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Eggs+Insect
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Adults+Nymphs+Eggs+Ins
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Adults+Nymphs+Eggs+Ins
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Adults+Nymphs+Eggs+Ins
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Difficult eggs/low quality
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Difficult eggs/low quality
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	HH+ve +Insect
13XT								
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Adults
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Nymphs
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Eggs
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Adults+Insect
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Nymphs+Insect
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Eggs+Insect
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Adults+Nymphs+Eggs+Ins
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Adults+Nymphs+Eggs+Ins
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Adults+Nymphs+Eggs+Ins
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Difficult eggs/low quality
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Difficult eggs/low quality
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	HH+ve +Insect
BETH								
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Adults
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Nymphs
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Eggs
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Adults+Insect
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Nymphs+Insect
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Eggs+Insect
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Adults+Nymphs+Eggs+Ins
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Adults+Nymphs+Eggs+Ins
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Adults+Nymphs+Eggs+Ins
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Difficult eggs/low quality
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Difficult eggs/low quality
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	HH+ve +Insect

Zeal								
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Adults
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Nymphs
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Eggs
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Adults+Insect
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Nymphs+Insect
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Eggs+Insect
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Adults+Nymphs+Eggs+Ins
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Adults+Nymphs+Eggs+Ins
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Adults+Nymphs+Eggs+Ins
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Difficult eggs/low quality
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	Difficult eggs/low quality
8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	8XT_	HH+ve +Insect

WP2. Evaluate the reliability of the test for species-level identification of adults, nymphs and egg masses

Dilution series

Dilution testing suggests the 8XT primer pair amplified all samples, even in extreme dilution cases. Primer set 13XT failed to recover strong amplification of highly-diluted samples. Generic primer Beth similarly failed to amplify from highly-diluted samples. Generic primer Zeale amplified only undiluted DNA.

In general, adult DNA produced stronger amplicons than egg-derived DNA (Figure 6). The eggs and nymphs were not well preserved and while the DNA concentration was the same the DNA may have been more degraded.

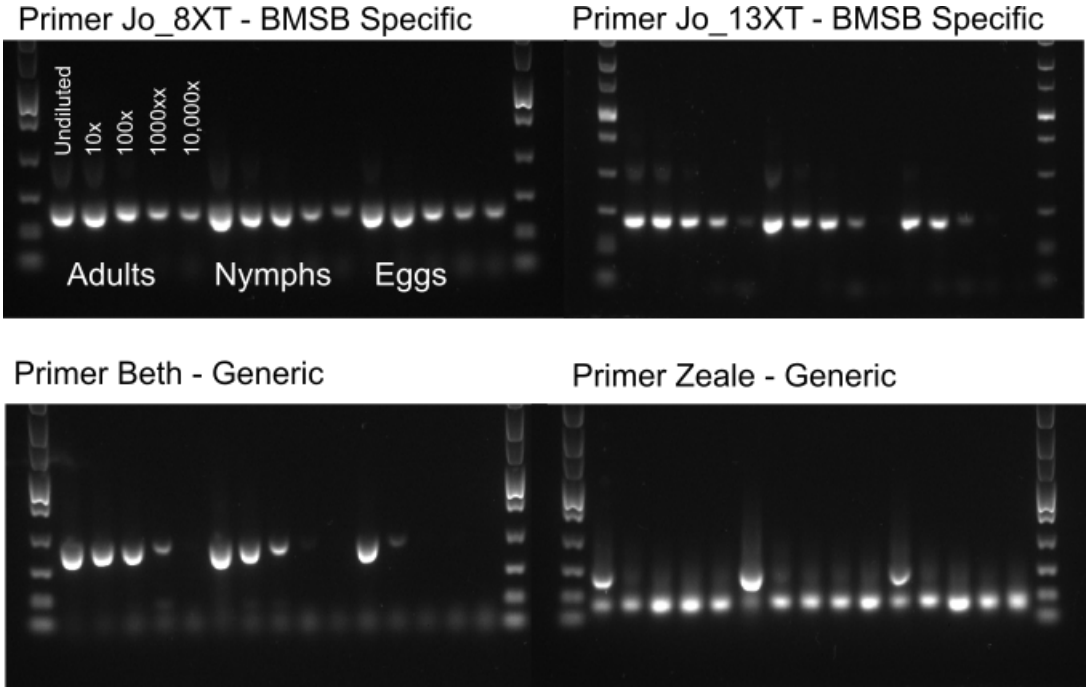

Figure 6. Dilution series of adults, nymphs and eggs for each of the selected specific (8XT, 13XT) and generic (Beth, Zeale) primer sets. Labels for BMSB life stage (adult, egg or nymph) and dilution level are only given on the top-left panel but apply in the same order to all samples and primers.

The reliability of the detection test was further evaluated using the DNAqua-Net (https://dnaqua.net/) environmental (eDNA) validation scale (https://edna-validation.com/). DNAqua-Net is a cost action as part of the EU framework supporting trans-national cooperation under the European Commission. DNAqua-Net's goal is to identify gold-standard genomic tools and novel eco-genomic indices for routine applications in biodiversity assessment and monitoring of European water bodies. As part of this framework, they have

developed a five-stage validation tool for eDNA assays (Figure 7). Although the validation criteria were developed specifically for application to eDNA in aquatic ecosystems, the validation scale is transferrable to samples collected from terrestrial habitats. To evaluate the reliability of our test, we have therefore adapted the DNAqua-Net validation scale and used it to assess the current state of development of our diagnostic test.

1	In silico analysis	PCR protocol applied but not optimised					
2	In silico analysis	PCR Reaction optimised	Some in vitro testing of related species suggests test is specific	Detection obtained from water samples in laboratory environment or mesocosm			
3	In silico analysis	PCR Reaction optimised	In vitro specificity confirmed – all closely-related co-occurring species tested	Extensive field testing at sites of known presence and absence of target species			
4	In silico analysis	PCR Reaction optimised	In vitro specificity confirmed – all closely-related co-occurring species tested	Extensive field testing at sites of known presence and absence of target species	Limits of detection established		
5	In silico analysis	PCR Reaction optimised	In vitro specificity confirmed – all closely-related co-occurring species tested	Extensive field testing at sites of known presence and absence of target species	Limits of detection established	Detection probability estimates from statistical modelling	Good understanding of ecological, temporal and spatial factors affecting detectability

Figure 7. DNAqua-Net evaluation scale (From https://edna-validation.com/) showing validation steps.

The DNAqua-Net scale comprises five steps (Figure 7). The current status of the BMSB test for each of these steps is as follows:

Step 1: Completed. *In silico* analysis and PCR protocol tested. See Figures 2-5.

Step 2: Completed. PCR optimisation and *in vitro* testing of potentially confused species.

(Detection from water samples not applicable).

Step 3: Completed or in progress to completion. Potentially-confused species have been tested and field testing of samples acquired with assistance from collaborators has been completed. Estimates of false negative rates have been established using different thresholds for identification. False positive rates are being determined via a second round of sequencing.

Step 4: Completed. Limits of detection have been established (see Figure 6).

Step 5: Completed or in progress. Detection probability estimates (from qPCR assays) are ongoing, and we cannot currently carry out ecological studies in the UK due to seasonal factors and lack of currently-confirmed establishment of BMSB.

Based on current validation, our test meets almost all criteria of a Level 4 or 5 assay as established by DNAqua-Net, with the current limitations of needing to determine false positive rates, use qPCR to refine estimates of the lower limits of detection and our inability to currently assay UK populations for seasonal and spatial factors (pests are not active and the extent of the invasion has not been determined).

WP3. Investigate the feasibility of combining the new test with monitoring (e.g. using pheromone-baited traps) as part of an early BMSB surveillance programme

To investigate the applicability of our methodology to samples from trapping campaigns we used three methods of analyses to assess contamination and degradation effects.

1. Dilution series were constructed to estimate the sensitivity of the test to trace material. This was done both by including highly-diluted (up to 10,000x) material and by including poorly preserved nymph and egg DNA based sources. We found that our newly-designed 8XT primers were able to assay for BMSB in even the most highly-diluted and degraded DNA sources (Figure 6).
2. PCR tests were used to specifically compare well preserved material to poorly preserved material. We particularly included BMSB samples which were not preserved under ideal conditions including dried eggs, moults and dried emerged eggs all of which were stored at temperatures higher than those needed for good preservation of DNA. Our primers were successful, even with the most challenging DNA sources (see Figure 8).
3. Sequencing specifically included diluted and poorly preserved (“difficult") material (Table 4) which included moults, dried eggs, and dried empty eggs stored at room temperature for extended periods of time (up to 8 months). The most specific 8XT and 13XT primers were successful for all sample types. The more generic Beth primers were successful on all sample types unless they were from poorly preserved samples that were also contaminated and diluted.

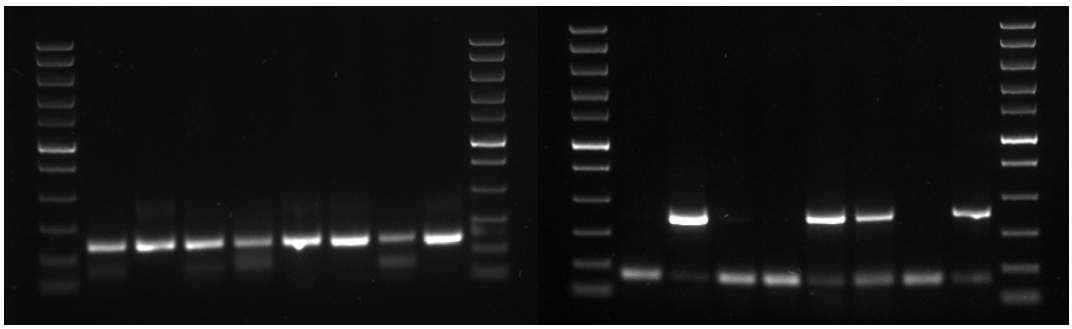

Figure 8. PCR tests of challenging material amplified with the 8XT (left) and Beth primer sets. The DNA was derived from eggs preserved in ethanol x2, old eggs dried x2, 1st instar nymphs x2, moults x2 (Left to Right). The specific 8XT primers show good amplification of all samples while the more general Beth primers failed to amplify dried eggs and some samples of ethanol-preserved eggs and moults.

These tests suggest that the primers and approach can be applied to field collected samples from contaminated and degraded samples and will be particularly effective if used in combination with both a specific and general primer (e.g. 8XT and Beth).

WP4. Evaluate the test for secondary detection of parasitoids which may contribute to natural bio-control

The aligned region and tree suggests this smaller 3' end can differentiate all species (see Figure 9) though the alignment problems of the 5' end mean a new forward primer will need to be designed to simplify this region and the resulting primer would need to be multiplexed.

Further testing of this truncated region and a new forward primer will be conducted at QMUL during the 2020 growing season.

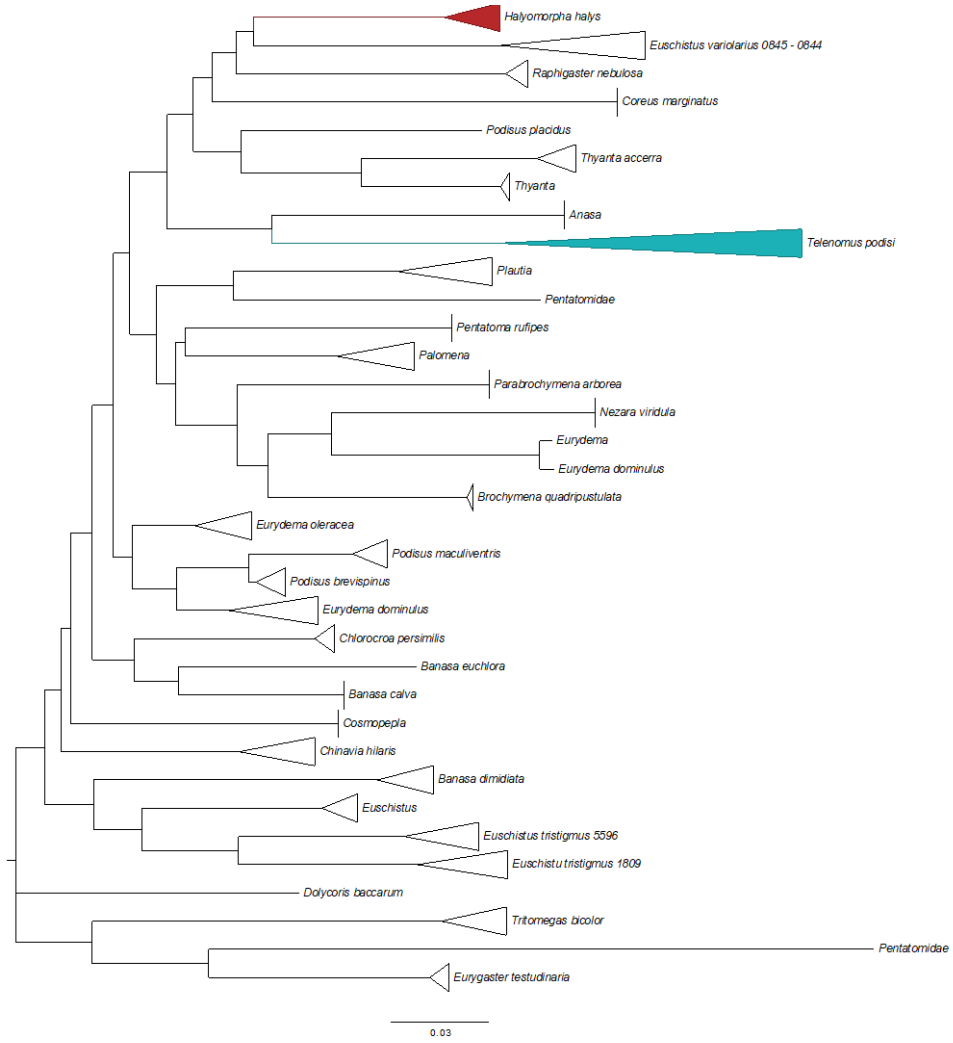

Figure 9. A neighbour-joining tree of the 5' truncated region of the most successful Beth primers showing discrimination ability for BMSB (red triangle) and the Scelionidae represented by *Telenomus podisi* (green triangle) alongside other insects in the reference collection. Triangle width depicts sequence variability.

Discussion

The aims of this project were to 1) accumulate an extensive reference database of DNA sequences from BMSB and species commonly confused with BMSB in agricultural field conditions, 2) design a high-throughput sequencing approach which can be used to identify BMSB under a wide variety of conditions (degraded, old, fragmentary material) and from a variety of sources including challenging material (e.g. dry, empty egg cases), 3) evaluate this test in the context of accepted high level standards (e.g. those developed by the EU cost-action DNAqua-Net for validation), 4) determine whether the test might detect other agricultural pests and parasitoids and 5) make the test available to UK growers for the 2020 season in the event that BMSB is suspected or detected and mass screening required.

Our analysis presented in the preceding pages has achieved all of these objectives and, while validation will continue over time, we here present a viable DNA-based diagnostic for UK and international growers ready for deployment in academic and commercial laboratories.

Test development and validation

A well-validated DNA-based test for any pest should include:

- Testing by different facilities
- Defined false negative and positive rates
- Some assessment of sensitivity
- Clearly outlining where confidence may be low
- Testing across different life stages and sample types
- Testing across a variety of environmental degradation conditions
- An extensive database of reference material including potentially confused species.

To accomplish this we sought to follow criteria set by DNAqua-Net to evaluate the test developed in this project. The test met all criteria tested with just a few of the highest level criteria currently pending (detection probability estimates and an understanding of the ecological, temporal and spatial factors determining detectability). The test can now be used with a high degree of confidence.

Outputs

There are several significant outputs from this work:

- The reference database is extensive and includes nearly 1000 representatives of BMSB and more than 1000 additional sequences from species that are commonly confused with the invasive pest target, providing extremely reliable estimates of intra- and inter-specific variation. It is currently housed in the web-accessible BOLD and mBRAVE platforms for both traditional Sanger and high-throughput sequencing use and will be freely available upon publication of our methods. Representatives in the database have a UK focus but are likely to be internationally useful in terms of geographic collection and taxonomic breadth. It is therefore likely that this reference database will be internationally applicable in aiding identification of BMSB. Because our database and analysis is housed in mBRAVE, it is augmented by additional publicly available databases including nearly a million reference sequences from more than 250,000 species of invertebrate which can be used to screen for other species not included in the newly-created reference database. These additional resources represent added value and are expanded by the global research community continually.

- New PCR primers designed during this project also represent significant outputs. The primary goal of the project was to develop an assay specific to BMSB with the ability to discriminate the pest even when present in degraded form and when contaminated with other sources of environmental DNA. We used our extensive reference database to design two new primers with high affinity for BMSB. The resulting primers 8XT and 13XT preferentially amplify BMSB DNA with 8XT showing extremely high sensitivity with diluted and degraded samples. We will make both sets of primers publicly available for general use in the detection of BMSB.

- The secondary goal was to test existing general primers for insects, as this may also be useful to co-amplify parasitoids, other insects cohabiting with BMSB, and for ecological scenarios where competition between species may be important. The tested publicly available primers from Elbrecht et al. (2019) did not prove useful. The two primer sets commonly used in the Clare Lab (Beth and Zeale) both discriminate BMSB and successfully identified it in all life stages, though as expected the false negative rate increased when environmental contamination becomes more complex (our addition of many insect species' DNA to the sample) and BMSB starting material

more degraded. Possible solutions to this would be to increase sequencing depth (decrease the number of samples being multiplexed) or combine specific and general primers in every analysis. The latter is a far more useful approach which should maximize BMSB detection potentials. Indeed, for valuable and critical situations where detection is of paramount importance, we advise that all primers be used in parallel (multiplex) and analysis include considerable technical replication to maximise detection success.

- An additional outcome will be our recommended protocols for bioinformatics which can be used for the most common scenarios to maximize detection. We have particularly employed the mBRAVE pipeline because it requires no command line or programming and can be implemented without access to a high-performance computing system, making it much more user-friendly and our analysis here has demonstrated that it performs well for this application.

Future development / ongoing research

Three of the criteria of the DNAqua-Net DNA validation scale have not yet been met. Tests under a variety of ecological, spatial and temporal factors of DNA degradation cannot currently be evaluated in the UK because we are at a very early stage of BMSB invasion and no adults were active during the effective funded life of this project (mid-November 2019 – February 2020). We attempted to overcome these constraints by including material collected (with assistance from collaborators) from Italy (September – November 2019) and by ensuring that samples were collected and stored under different circumstances and preserved using different methods (e.g. dried material from Switzerland and emerged and dried material from Canada). However, these additional criteria cannot be fully evaluated currently. Two other criteria (establishment of false positive rates and accurate quantification of sensitivity) are ongoing with estimates expected in May 2020. To evaluate the false positive rate of this test, a second round of DNA sequencing is pending which targets only the 8XT and Beth primer sets (the most successful specific and generic primers respectively). These primers are being tested on a larger number of potential insect mixes, including known negatives (no BMSB DNA included) and are being conducted using technical replication. The inclusion of negatives and technical replication will allow us to establish the false negative rate and further refine the false positive rates. The final criterion, determining the lower limit of sensitivity of this test, is being conducted using qPCR, specifically targeting the most successful 8XT primer. This should allow us to determine a lower threshold for potential BMSB detection.

A recent publication presents a global threat analysis for nearly 1,300 invasive agricultural pest species (Paini et al., 2016). Tracking and intercepting this ever-growing threat represents a global challenge to supply lines and trans-national trade. Traditional approaches cannot meet the challenge of rapid identification, however tests modelled on the one we have designed here represent a viable and scalable approach to the problem. Our project provides proof of concept and a model for development and validation of diagnostic tests for invasive pests and pathogens posing new risks to agriculture and horticulture, and we anticipate a rapid development of such tests over the coming years.

Our developed test is now available via the Clare Lab at QMUL (http://research.sbcs.qmul.ac.uk/e.clare/Elizabeth_Beth_Clare_BMSB.html) and can be implemented on a scale of individual specimens to large collections on a variety of contracted arrangements depending on the needs of a grower or other stakeholder. However, the end goal is for the test to become fully commercially available. NatureMetrics carried out all sequencing analysis as part of this project and currently operates as an SME providing eDNA and DNA based diagnostic services on a very fast commercial basis. We anticipated that BMSB diagnosis can be developed commercially as a two-stage test. PCR testing could initially be carried out to give an indication of BMSB presence or absence (feasibly within 3-4 working days). If PCR testing is positive it can be used to provide an indication of risk and as a basis to recommend sequencing, which can be accomplished in 3-7 days depending on sample type.

We are now in discussion with NatureMetrics regarding commercialisation of this technology to provide a rapid diagnostic test for UK growers and international stakeholders. They have agreed, in principle, to become a provider of this diagnostic and all protocols for DNA extraction and PCR will be provided to them with explicit training and support including laboratory protocols and the use of the mBRAVE pipeline, our bioinformatics protocols and access to our reference library. We are preparing a publication based on this analysis to be submitted to *Methods in Ecology and Evolution* which will make the protocols publicly available.

Conclusions

- Specific PCR primers were designed which preferentially amplify BMSB DNA even when the original material has degraded or been contaminated.
- Generic primers show good success at amplifying DNA from both BMSB and other insect species for research applications targeting broader diversity analyses.
- Newly-designed specific primer set 8XT showed very high sensitivity to trace BMSB DNA material. Detection was possible even after 10,000x dilution of extracted DNA samples, showing the potential of this primer for detection of trace eDNA in field samples.
- The protocol cannot immediately be used to detect egg parasitism, but a modified forward primer for the general BETH amplicon is the most likely target for multiplexing the test to detect members of the platygastrid egg parasitoids.
- The methods tested here are now available for use. Samples can be processed in the Clare lab at QMUL to detect BMSB on a contract basis with a high degree of sensitivity as described in the preceding paragraphs. Information can be found here http://research.sbcs.qmul.ac.uk/e.clare/Elizabeth_Beth_Clare_BMSB.html
- The company NatureMetrics have carried out the quality control and sequencing protocols to help validate this material with high quality and success and have expressed an interest in developing the methods presented here as part of a high-throughput sequencing-based diagnostic test for BMSB in a commercial context.

Knowledge and Technology Transfer

Glen Powell presented a talk at the Berry Gardens Research and Agronomy Conference, Ashford, 14 Nov 2019. Watch out! Brown marmorated stink bug is here!

Elizabeth Clare presented a talk at the HAPI Dissemination Event, Leeds, 10 Dec 2019.

Elizabeth Clare presented a talk at the UKDNA Working Group, London, 28 Jan 2020. Agricultural pest detection by DNA – a model system for best practices.

Glen Powell presented a talk at the AHDB Tree Fruit Day, NIAB EMR, 27 Feb 2020. Update on two shield bug pests: a native and an invader.

Glen Powell will give a presentation at a planned AHDB Protected Edibles Webinar Event (date to be confirmed). A rapid identification method for brown marmorated stink bug.

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