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Molecular identification of the brown marmorated stink bug's egg parasitoids by species-specific PCR collected from Beijing, China

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Abstract

Brown marmorated stink bug (BMSB), *Halyomorpha halys* (Stål) (Hemiptera: Penta-tomidae), is a highly polyphagous and invasive insect pest with more than 300 plant species as hosts, including a wide range of economic crops. To date, several egg parasitoid species are reported to attack BMSB. In this study, two species of *Trissolcus* (Hymenoptera: Platygastridae) and one *Anastatus* (Hymenoptera: Eupelmidae) were recovered from field exposed BMSB sentinel eggs in Beijing, China. The wasps' small size of only 1.5 mm and 4 mm in length make them difficult to identify morphologically and required taxonomist for identification of specific species. In addition, these parasitoids have morphology sibling species and have been misidentified several times in China. To overcome these problems, a molecular method with species-specific primers designed for the COI gene has been developed to identify *Trissolcus*, *Anastatus* and their host samples from field collected samples. After successful morphological confirmation with experts, DNA extractions were carried out from these samples. PCR amplification using published primers for *T. japonicus*, *A. japonicus* and BMSB specimens confirmed the species. As for *T. cultratus*, species specific primers TCYF and TCYR were developed which produce 340-bp PCR products length while no positive amplifications found in other wasps and host. Sensitivity analysis of markers revealed that TCYF and TCYR primers could detect as low a DNA template concentration as 0.00025 ng/µL. This indicates that PCR with these primers specifically and sensitively differentiates *T. cultratus* specimens from other similar wasp species. All the primers tested in this study could discriminate between parasitized and non-parasitized BMSB eggs. This molecular identification method shows promise for conveniently identifying *Trissolcus* and *Anastatus* species in host-parasitoid associations and accurately evaluating parasitism rates in the field.

Keywords DNA barcoding, COI gene, *Anastatus*, *Trissolcus*, Biocontrol, *Halyomorpha halys*

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Background

Brown marmorated stink bug (BMSB), *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae), is a polyphagous species of Asian origin including China, Japan and Korea (Xu et al. 2014). BMSB has become extremely invasive in Europe and North America following accidental introduction in the 1990s (Hoebeke and Carter 2003; Wermelinger et al. 2008; Haye et al. 2014). The recent invasion was reported in South America in 2017 (Faúndez and Rider 2017). It has caused significant damage as an agricultural and horticultural pest on many economically important crops, including a variety of vegetables and fruit such as beans, capsicum, tomato, maize, apple, peach, pear, hazelnut, and kiwifruit (Rice et al. 2014; Mi et al. 2021).

Chemical pesticides are often used to control *H. halys* which can disturb natural ecosystems and are only a short-term solution that does not prevent re-infestation. Plantings of trap trees such as tree of heaven or deployment of sticky traps are alternative strategies against this pest, but these strategies are very labor intensive and costly (Park et al. 2009). In contrast, natural enemies can provide effective long-term regulation of invasive pests, especially in forests where it is difficult to apply pesticides effectively.

In its native range, *H. halys* is attacked by a complex of scelionid and eupelmid egg parasitoids, with *Trissolcus* sp. (Hymenoptera: Scelionidae) as the most prevalent species (Yang et al. 2009; Zhang et al. 2017) and *Anastatus* sp. ranked second (Zhang et al. 2017). Several species of *Trissolcus* and *Anastatus* have been considered or used for biological control of hemipteran pests around the world. In southern China, *Anastatus* sp. has been augmentatively released from egg cards to control the litchi stink bug *Tessaratoma papillosa* Drury (Hemiptera: Pentatomidae) with satisfactory control since late 1960s (Li et al. 2014). In northern China, parasitism rates of *Trissolcus* and *Anastatus* sp. on nature *H. halys* egg masses ranged from 48.7 to 77.2% in peach orchards and forests (Hou et al. 2009; Yang et al. 2009). In Europe, *Trissolcus* and *Anastatus* species are the most common native parasitoids of the invasive *H. halys* and has been investigated for potential augmentative biological control against this pest (Stahl et al. 2019a). Furthermore, *A. bifasciatus* has been released in apple and pear orchards in Switzerland and Italy, respectively against *H. halys* eggs (Stahl et al. 2019b). Similarly, *A. japonicus* has been selected as a potential candidate for inundative biological control of *H. halys* in China with parasitism rate of more than 70% in laboratory test (Mi et al. 2021, 2022).

Parasitism rate is an important parameter to evaluate biological control efficacy, and accurate assessment of parasitism of *H. halys* in the field can be a difficult task. Previously, rearing and dissection methods have been used to estimate parasitism, but both methods have some technical limitations (Greenstone 2006). First, species-level identification of the host eggs is challenging, as there are few distinguishing morphological characters in the egg stage (Bundy and McPherson 2000; Gariepy et al. 2013). In some cases, recovered egg masses are found when egg parasitoids have already emerged, which further exacerbates identification efforts. Although there are species identification methods described for parasitoids of Heteroptera, based on exit holes and morphology of the frass the parasitoid larva has left behind in the egg (Viggiani and Mineo 1973), the applicability and reliability of those methods in multispecies systems could be questioned. Even if the parasitoid was still present inside the host egg, pre-imaginal mortality, interspecific and intraguild interactions/competitions can prevent identification via rearing and even dissections are of little use once the parasitoids have disintegrated. Furthermore, the rearing method can only be used to estimate the apparent parasitism rate and does not address whether or not parasitoid emergence was from a previously parasitized host or a newly parasitized host (Gariepy et al. 2005, 2007; Jones et al. 2005) which leads to misidentification of both parasitism and particular species. For example, a species that has been used extensively in China for biocontrol of *T. papillosa*, was initially identified only as *Anastatus* species (Lu and Yang 1983; Lin and Lin 1998) and then as *A. japonicus* Ashmead, 1904 (Xin and Li 1989, 1990; Tang et al. 1993; Xian et al. 2008). Peng et al. (2020) indicates that at least in southern China this results mostly from misidentifications of *A. fulloi* Sheng and Wang, 1997. Similarly, *Trissolcus* species has also been misidentified several times in *Trissolcus-H. halys* associations (Talamas et al. 2015, 2017).

The dissection method for identification is very tedious, time and labor consuming because it is difficult to find parasitoid eggs inside host tissues or eggs (Agustí et al. 2005). To overcome some of these difficulties, a modified DNA barcode technique have also been used to identify and distinguish parasitoid species (Dowton and Austin 2009). Different mitochondrial and nuclear genes can be used alone or in combination to identify insect species. However, because mitochondrial DNA (mtDNA) is haploid and inherited maternally and does not undergo genetic recombination, it has higher mutation rates than nuclear DNA (Simon et al. 1999; Hebert et al. 2004). Among different mitochondrial genes, the cytochrome oxidase subunit I (COI) gene is considered to have high genetic diversity, and therefore is useful to detect intraspecific genetic variation (Simon et al. 1994; Hu et al. 2008). Species-specific molecular markers for parasitoids can be used to identify minute quantities

of parasitoid DNA inside the host through polymerase chain reaction (PCR), allowing for estimation of parasitism rates in host populations more rapidly than the rearing and dissection methods (Gariepy et al. 2007, 2013, 2019). Therefore, molecular markers provide a valuable tool to accurately identify parasitoid species and understand their population dynamics.

In this study, egg parasitoids attacking BMSB were sampled at three different sites in Beijing. Later on, collected parasitoid specimens were preliminary identified via morphological features. Thereafter, species-specific primers were designed for the COI gene to identify parasitoids inside host eggs of any developmental stage using PCR assays. Since hosts are normally associated with a limited number of parasitoids, primer specificity testing can be restricted to species closely related to the parasitoid the primers were already designed and species that parasitize closely related hosts (Gariepy et al. 2007). The sensitivity of a primer set measures each stages of the parasitoid because minor DNA amount can be detected. The method we present relies solely on species diagnostic PCR. Thus, we expect it to be a quick, accessible, and cost-efficient option to determine specific parasitoid used in this study. The molecular methods can also be used to estimate the parasitism rates in the field.

Materials and methods

Insects collection

Egg masses of *H. halys* were placed on 5 cm^2 piece of paper with clear liquid glue (Pritt Liquid Glue, Henkei Industry and Trade Co., Ltd, Guangdong, China) and hang on the tree at three different locations across Beijing, China. Sites included agricultural research stations with a wide variety of trees (Yangtai mountain, Beijing; N40° 04′ 15.84; E116° 04′ 53.12), fruit orchards with a mix of peach, cherry, and apple trees (Lengquan, Beijing; N40° 02′ 0 6″; E116° 12′ 41″), and natural forests and parks (Baiwang Mountain, Beijing; N40° 01′ 53″; E116° 15′ 32″) during summer in 2021. The egg masses were collected after one week of exposure and then kept in petri dishes in incubator (BluePard Series, Yiheng Technology Company, Shanghai, China) at 25 ± 1 °C and 60 ± 5% R.H. and 16:8 h L:D photoperiod until emergence. All samples collected in the field were morphologically identified up to species level, ultimately representing two families. Wasps emerging from parasitized eggs were confined individually and provided with a 20% honey solution for feeding. Furthermore, these species were reared in laboratory conditions separately for further studies (Mi et al. 2021).

Morphological identification of emerged egg parasitoids

All emerged parasitoids from BMSB eggs were identified morphologically using the keys developed by Talamas et al. (Talamas et al. 2017) and Tortorici et al. (Tortorici et al. 2019) for *Trissolcus* and Peng et al. for *Anastatus* (Peng et al. 2017, 2020), respectively. Specimens were checked under stereomicroscope (Olympus SZX10 SZX2-ILLT connected to an Olympus LG-PS2 Microscope Ring Light Illuminator and DP80 digital camera, Olympus Corp., Tokyo, Japan) and photographs were captured in super-depth-of-field three-dimensional microscopy system (VHX-2000).

Genomic DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from the adults of experimental insects from each morphologically identical species. After crushing and homogenization with a bead shaker (Hoder, China), DNA extraction was performed with 2X (2%) CTAB (Beijing Solarbio Science & Technology Co., Ltd.) following the manufacturer's protocol. The quality of extracted DNA was checked using agarose gel electrophoresis, and the quantity was measured with nanodrop spectrophotometer (DS-11, DeNovix, Wilmington, DE). Following DNA extraction, the "barcode" region of the mitochondrial COI from parasitoids and

Table 1 Primer pairs used for molecular identification of *Halyomorpha halys* and its assoicated egg parasitoids

n	Species	PCR primers	Source
1	Universal primers	LCO1490: (5′–GGTCAACAAATCATAAAGATATTGG–3′) HCO2198: (5′–TAAACTTCAGGGTGACCAAAAAATCA–3′)	(Folmer et al. 1994)
2	*Trissolcus japonicus*	TJ234F: (5′–ATCCCATCATTAATTTTATTAATCTATAGG–3′) TJ460R (5′–CATGTAAATAACGTTCAATTATTAATTGATA–3′)	(Chen et al. 2021)
3	*Trissolcus cultratus*	TCYF: (5′–ATAATTAATGCTCCTGATA–3′) TCYR: (5′–CAGCTAATACAGGGAGGGATAAA–3′)	This study
4	*Anastatus japonicus*	Ana-361F: (5′–ATCACATAGGGGTCCTTCAGTA–3′) HCO2198: (5′–TAAACTTCAGGGTGACCAAAAAATCA–3′)	(Stahl et al. 2019c)
5	*Halyomorpha halys*	Hhal1dF: (5′– GAGGATTCGGTAATTGATTA–3′) Hhal1dR2: (5′–GTGAGATATTACTTGATAAGG–3′)	(Dhami et al. 2016)

hosts was amplified using the universal forward primer LCO1490 and reverse primer HCO2198 (Table 1).

DNA amplifications were performed in 20 µL reaction volumes containing 10 µL rTaq Premix Taq™ (Takara Taq™ V 2.0 plus dye, Japan) reaction buffer, 0.5 + 0.5 µL of 10 µM of each forward and reverse primer, 1 µL of DNA template, and ddH_2O added to make a final volume of 20 µL. The PCR protocol for COI reaction was as follows: denaturation at 94 °C for 1 min, 35 cycles of 94 °C for 15 s, 55 °C for 15 s and 72 °C for 30 s and a final extension at 72 °C for 7 min. All reactions were performed on Applied Biosystems™ Veriti™ Thermal Cycler, 96-Well (Thermo Fisher Scientific, US). When verifying DNA extraction using the universal LCO1490/HCO2198 primers, an annealing temperature of 40 °C was used. Following PCR, 10 µL of the PCR product were run on a 1% agarose gel stained with PAGE GelRed® Nucleic Acid Gel Stain (Biotium, Fremont, CA, USA) together with 2µL of Trans2K® DNA Marker (Transgen, Beijing, China), and then visualized using a ChampGel 6000 Imaging System (Sage Creation Sciences, China).

Species-specificity of PCR primers

Candidate primers for specific identification of *T. cultratus* were designed based on COI sequence get after sequencing using universal primer pairs (LCO1490 and HCO2198) (Folmer et al. 1994). When designing specific primers, the selected COI template sequence must be closely related to *T. cultratus* in the NCBI Blast tool, and there are differences in the sequence comparison with other insects such as host and wasps. Species specific primers for *T. japonicus* (Chen et al. 2021), *A. japonicus* (Stahl et al. 2019c) and *H. halys* (Dhami et al. 2016) were also used in this study. COI gene sequence data from the host and parasitoid were also aligned and analyzed. Primer 3 software (Rozen and Skaletsky 2000) was used to design species-specific primers to distinguish the parasitoid from the host insect. The optimal annealing temperature for the *T. cultratus* species-specific primers was determined experimentally using a gradient PCR ranging from 40 to 60 °C and determined as 42 °C, however for other species optimal annealing temperatures were selected from references including *T. japonicus* (58 °C) (Chen et al. 2021), *A. japonicus* (58 °C) (Stahl et al. 2019c) and *H. halys* (62 °C) (Dhami et al. 2016). The PCR protocol and gel run process was same as described in Sect. "Genomic DNA extraction, PCR amplification and sequencing". All the primers used in this study are listed in Table 1.

Detection of sensitivity of primers

To assess the detection sensitivity of the *T. cultratus* species-specific primers (TCYF and TCYR), PCR reactions were performed with different concentrations of parasitoid template DNA following the same PCR conditions described in Sect. "Genomic DNA extraction, PCR amplification and sequencing". DNA stock was extracted from pooling 30 *T. cultratus* wasps which was reliably detected using a nanodrop spectrophotometer (DS-11, DeNovix, Wilmington, DE), then diluted to yield the appropriate DNA concentrations: 50, 25, 2.5, 0.025, 0.0025, 0.00025 and 0.000025 ng/µL used in PCR.

Detection of specificity of primers

The specificity of the primer pair (TCYF and TCYR) was tested in separate PCR reactions with DNA from two *Trissolcus*, two *Anastatus* parasitoids, and *H. halys* hosts to distinguish the specific parasitoid. Amplification of DNA was performed in an Applied Biosystems™ Veriti™ Thermal Cycler, 96-Well (Thermo Fisher Scientific, US) in the same method as described in "Genomic DNA extraction, PCR amplification and sequencing"

Parasitoid detection over time

The ability to determine whether an empty or unmerged host egg has been parasitized by *Trissolcus* or *Anastatus* wasps can be a valuable tool in determining the success of these parasitoids as a biological control agent. To evaluate the ability of the primers to sensitively detect specific parasitoid DNA in parasitized BMSB eggs, the primers were tested on following samples: (1) 1 h parasitized eggs, (2) 12 h parastitized eggs, (3) 24 h parasitized eggs, (4) 48 h parasitized eggs, (5) 72 h parasitized eggs, (6) 5 d parasitized eggs, (7) 10 d parasitized eggs, and (8) after emergence (empty eggs) previously parasitized by *Trissolcus* or *Anastatus* sp.

Results

Morphological identification

The specimens of *Anastatus* and *Trissolcus* spp. recovered from *H. halys* egg masses in field survey are fully in line with the species' identical morphological characteristics presented by Peng et al. (2020), Talamas et al. (2017), and Tortorici et al. (2019), respectively. Acropleuron of *A. japonicus* is paler than mesoscutum and posteromedial part of mesoscutum has bright metallic luster, and acropleuron is also variably distinctly though noticeably paler (brown to orangish or yellow) than dark mesoscutum over at least about posterior half (Peng et al. 2017, 2020) (Fig. 1a, b). Inner margin of the orbital furrow of *T. japonicus* is smooth and the orbital furrow expands at its intersection with the malar sulcus, however, *T. cultratus* lacks a well-developed orbital furrow near the malar sulcus. Clypeus of *T. japonicus* contains four setae but

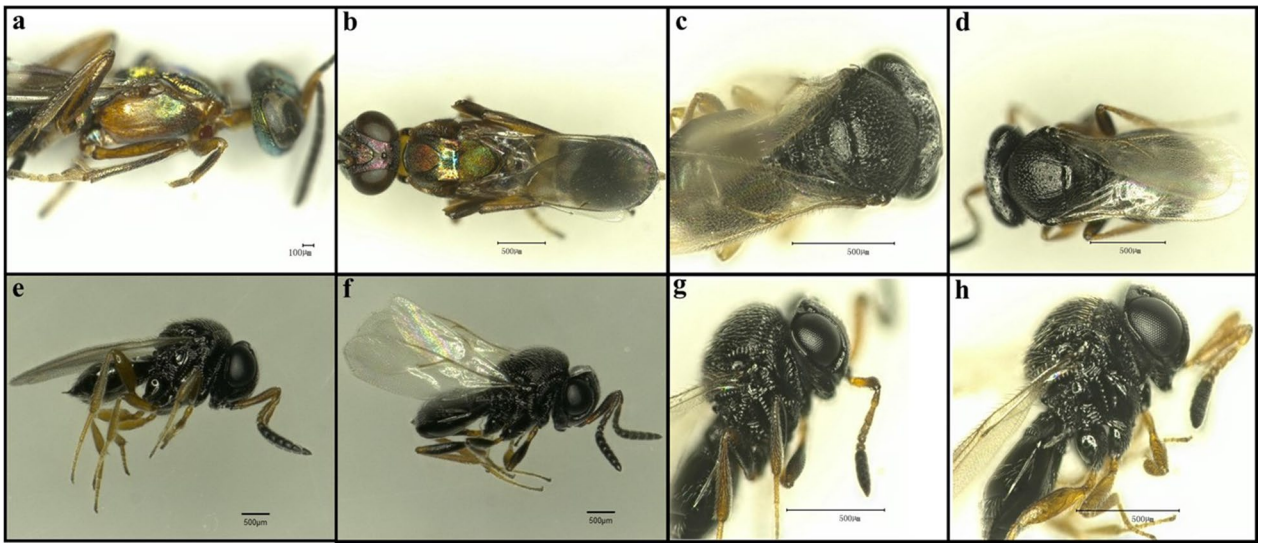

Fig. 1 Morphological characteristics of egg parasitoids: lateral view (**a**) and dorsal view (**b**) of *Anastatus japonicus*; dorsal view of *Trissolcus cultratus* (**c**) and *T. japonicus* (**d**); lateral view of *T. cultratus* (**e**, **h**) and *T. japonicus* (**f**, **g**)

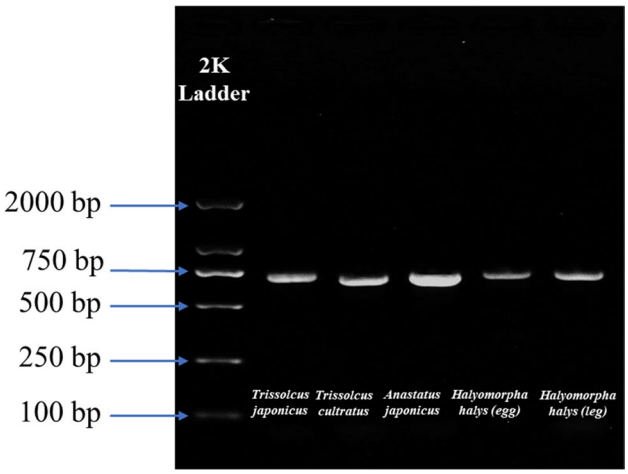

Fig. 2 PCR amplifications of the four species DNA of *Trissolcus japonicus*, *T. cultratus, Anastatus japonicus* and *Halyomorpha halys* using universal forward LCO1490 and reverse primers HCO2198. The leftmost well contains 2 µL of Trans2K® DNA Marker (Transgen, Beijing, China)

T. cultratus group have two clypeal setae (Talamas et al. 2015, 2017) (Fig. 1c–h).

Species-specific primer design

Collected specimens were preliminary identified via universal primer pairs forward primer LCO1490 and reverse primer HCO2198 which gave approximately 700 bp bands for all tested specimens (Fig. 2). Species specific primers for *T. japonicus*, *A. japonicus*, and *H. halys* were gained through literatures, however for *T. cultratus* we designed and analyzed in this study (Table 1). When all primers were tested against their specific species DNA as template, PCR amplification got positive results however against each other negative results were found (Table 2). Sequencing (Sangon Biotech Co., LTD; Shanghai, China) and BLAST analyses of PCR products confirmed that the amplified DNA matched the specific insect COI gene (Table 3). No non-target amplification of DNA was observed at any of the annealing temperatures assayed.

Primer sensitivity

After challenging the novel primers with *T. cultratus* DNA in amounts of 50, 25, 2.5, 0.025, 0.0025 and 0.00025 ng/µL in a 20 µL PCR reaction, the primers produced the expected 340-bp band length (Fig. 3). Bright, recognizable bands were produced in samples containing 0.0025 ng or greater than 0.0025 ng of DNA in a 20 µL reaction volume, and the sample containing 0.00025 ng of DNA showed a positive, faint band receiving less of template DNA in a 20 µL reaction did not produce bands bright enough to be reliably visualized under our current transillumination conditions.

Primer specificity

We developed species-specific markers (TCYF and TCYR) to amplify a 340-bp portion of parasitoid DNA for *T. cultratus*. Primer specificity was assessed in separate PCR tests using the DNA from all Beijing locations where parasitoids were collected. DNA was successfully amplified from all of the tested *T. cultratus* samples and

Table 2 Insect specimens evaluated by PCR in this study

n[a]	Sample type and specimen identification[b]	Location	Collection year	Universal primer pairs	Tj Species Specific PCR	Tc Species Specific PCR	Aj Species Specific PCR	BMSB Species Specific PCR
30	Field collected samples							
	Trissolcus japonicus	Beijing, CHN	2021	+	+	−	−	−
	Trissolcus cultratus	Beijing, CHN	2021	+	−	+	−	−
	Anastatus japonicus	Beijing, CHN	2021	+	−	−	+	−
	Halyomorpha halys	Beijing, CHN	2021	+	−	−	−	+
30	Laboratory reared samples							
	Trissolcus japonicus	Beijing, CHN	2021–23	+	+	−	−	−
	Trissolcus cultratus	Beijing, CHN	2021–23	+	−	+	−	−
	Anastatus japonicus	Beijing, CHN	2021–23	+	−	−	+	−
	Halyomorpha halys	Beijing, CHN	2021–23	+	−	−	−	+

The identification and origin of all insect samples bioassays along with their collection year and PCR results

Tj: *Trissolcus japonicus*, Tc: *Trissolcus cultratus*, Aj: *Anastatus japonicus*, BMSB: Brown marmorated stink bug; *Halyomorpha halys*

[a] Number of analyzed samples. [b]Field samples were collected using BMSB egg pasted on cards. [+] Positive PCR amplification. [−] No PCR amplification

Table 3 Results of comparing PCR product sequence with basic local alignment tool (BLAST) in NCBI of three egg parasitoids and their host specimens collected in Beijing, China

n	Species	NCBI BLAST		
		Query cover (%)	Identity (%)	Accession number
1	*Trissolcus japonicus*	99%	99.47%	MH919759.1
2	*Trissolcus cultratus*	99%	100%	MN615601.1
3	*Anastatus japonicus*	93%	97.49%	MZ433261.1
4	*Halyomorpha halys*	100%	100%	MK779997.1

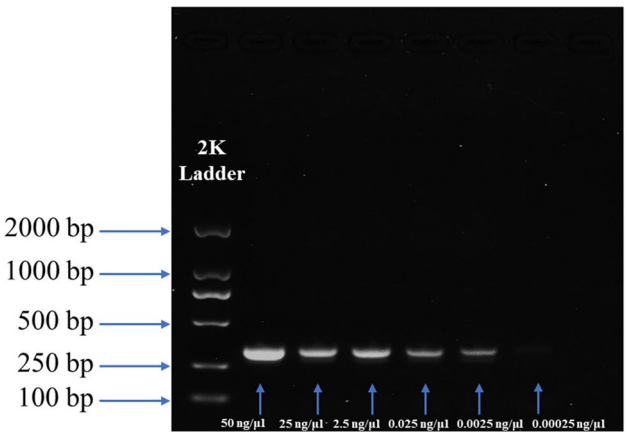

Fig. 3 Sensitivity of the novel *T. cultratus* specific PCR using TCYF and TCYR primers. Total DNA amounts used in PCR were diluted to 50, 25, 2.5, 0.025, 0.0025 and 0.00025 ng/µL in a 20 µL reaction. The leftmost well contains 2µL of Trans2K® DNA Marker (Transgen, Beijing, China)

no fragments were amplified from other parasitoids and host DNA (Fig. 4). Our results showed that this marker could be used to identify *T. cultratus* at any stage.

PCR validation on parasitized eggs

Further validation of all specific primers was performed by detecting the parasitoid egg inside the host eggs at different time periods. The results showed that parasitoid eggs were detectable when they were laid and developed within host eggs for 1 h. For parasitoids eggs laid after 1–24 h, our PCR assays showed less than or around 80% of detection of parasitoid DNA. For parasitoid eggs laid after 48 h up to emergence, our PCR detection was ranged from 80 to 100% (Fig. 5).

Discussion

Recent studies indicate that pre-imaginal mortality of parasitoids in *H. halys* eggs is indeed a variable that should not be neglected (Stahl et al. 2019c). However,

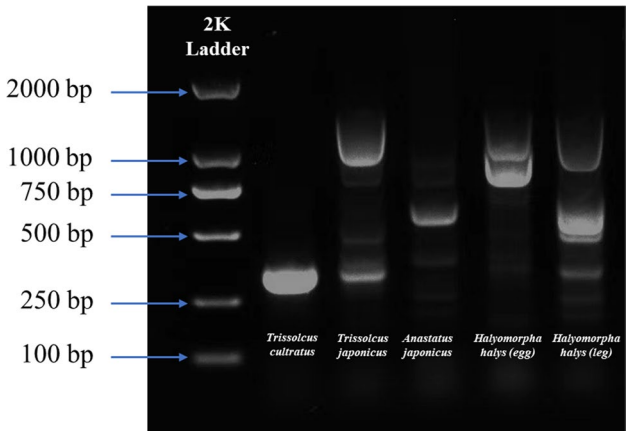

Fig. 4 Specificity of the novel *T. cultratus* species specific PCR using TCYF and TCYR primers. Total DNA amounts used in PCR were in a 20 µL reaction and primers were tested against four different DNA samples. The leftmost well contains 2µL of Trans2K® DNA Marker (Transgen, Beijing, China)

accurate measurements of parasitism with dissections are often exacerbated by the possibility that parasitoid development has not yet reached a detectable stage, or that dead parasitoids have decayed beyond the ability to detect or identify them (Ratcliffe et al. 2002). A large-scale comparison of parasitism estimates based on dissection, rearing, and molecular techniques in another host-parasitoid system has shown that molecular techniques provide more accurate estimates of parasitism and parasitoid species composition, whereas rearing and dissection tend to underestimate parasitoid-induced mortality (Gariepy et al. 2008).

Detection of parasitoid DNA within a host does not necessarily indicate parasitoid survival and associated host mortality. However, it does indicate that attack of a given host has occurred. This is extremely valuable as a non-target risk assessment tool, as detection of parasitoid DNA clearly demonstrates the potential of a parasitoid to use a given host species as a resource (Gariepy et al. 2008). Moreover, assessment of host-parasitoid associations with molecular tools would be very helpful to understand whether an exotic host insect acts as 'evolutionary traps' for indigenous parasitoids when the exotic host (i.e. *H. halys*) is attacked and oviposited but the parasitoid (i.e. *Telenomus podisi* Ashmead) fails to complete development (Abram et al. 2014).

A total of three species were recovered from the exposed BMSB eggs, namely, *Trissolcus japonicus*, *T. cultratus* and *Anastatus japonicus*. *Anastatus* wasps are known to be a polyphagous parasitoid that attacks and develops in a variety of Hemiptera and Lepidoptera, some of which are of conservational concern (Stahl et al. 2018b). With the molecular identification tool, recovered eggs from the field can be screened for the presence or absence of *Trissolcus* or *Anastatus* species, and trace amounts of DNA can even be detected following parasitoid emergence. This forensic-style approach, screening of empty egg masses for traces of parasitoid DNA,

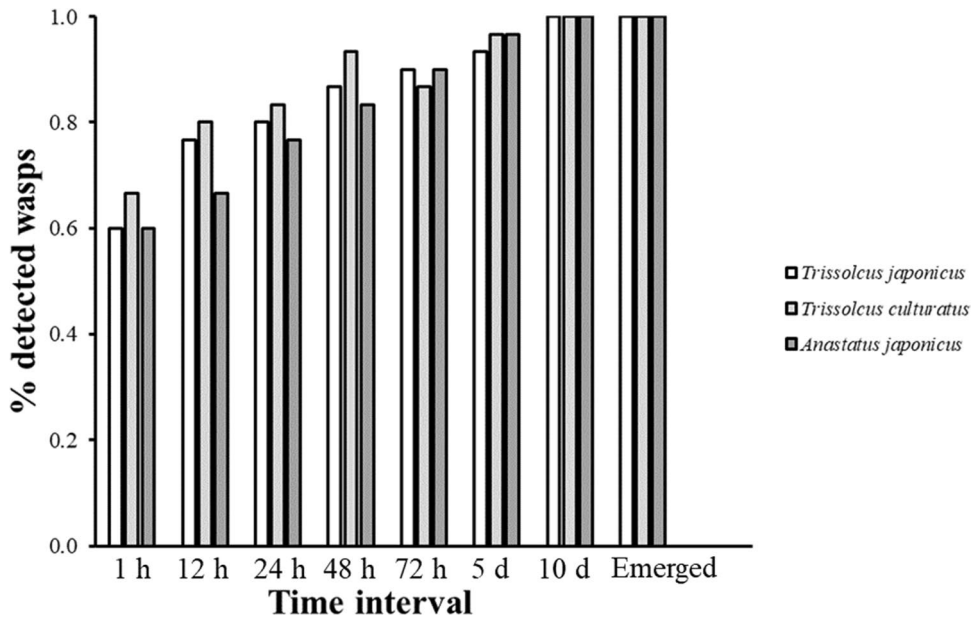

Fig. 5 Percentage of *Trissolcus japonicus*, *T. cultratus* and *Anastatus japonicus* parasitoid DNA detected after various time intervals within the host eggs of *Halyomorpha halys*. Host eggs were placed in alcohol directly after a developmental time of the parasitoid for 1 h, 12 h, 24 h, 48 h, 72 h, 5 days, 10 days, and after emergence of adult parasitoid (n = 20 host egg clusters per time interval)

can provide valuable information regarding the ecological host range and impact of a parasitoid on non-target species, which is often a decisive factor in pre-and post-release assessments of biocontrol agents (Gariepy et al. 2008, 2019). In the present study, field-exposed sentinel egg masses which had been stored for several months at 4 °C nonetheless yielded *Anastatus* and *Trissolcus* PCR products, despite the fact that storage conditions were less than ideal in terms of preserving DNA and preventing degradation. Although prolonged exposure under field conditions may result in quicker degradation of DNA, this clearly demonstrates that the primers used in this study are capable of detecting DNA from dry, decaying eggs regardless of emerged and non-emerged eggs. As the PCR primers amplify a fairly short fragment of the COI gene, it can be considered a 'mini-barcode', which is more likely to be detectable after longer periods of storage in comparison to longer fragments (Hajibabaei and McKenna 2012).

The availability of a molecular tool to detect the presence of *Trissolcus* and *Anastatus* sp. is also very useful in laboratory studies. Oviposition behavior of these parasitoids may or may not result in the insertion of an egg within a host. There is no way to determine whether oviposition was successful, based on observation or visual inspection of the probed host egg (Stahl et al. 2019c). As such, results from laboratory experiments can be difficult to interpret, as they rely primarily on offspring emergence, which can take several weeks under regular conditions and several months if diapause has been induced, during which time the host and/or parasitoid may be subjected to increased mortality (Konopka et al. 2017; Stahl et al. 2018a). This delay between observed attack and confirmation of parasitism is time consuming and may not provide accurate results based on mortality experienced in rearing. In contrast, DNA-based molecular tools can provide a rapid, accurate assessment of parasitism within < 24 h following observed oviposition. However, it is important to note that molecular detection of parasitism does not necessarily reflect successful parasitism, and molecular assessment requires destructive sampling, which therefore prevents the measurement of successful parasitoid development within a given host species. However, a combination of rearing and molecular analysis of separate samples would facilitate a more accurate representation of parasitism events, and an indication of whether successful parasitism or development occurs.

Our species-specific primers for *T. cultratus* produced no amplications in other tested parasitoid species and BMSB tissue samples. This expands the applicability of our PCR assay, as it can be used to determine if eggs were parasitized by *T. cultratus* without inspecting the emerged adults. For field studies, this could offer insight to the success of *T. cultratus* in parasitizing *H. halys* eggs. The utility of the *Trissolcus* and *Anastatus* primers as a molecular tool in both field and laboratory studies will permit the evaluation of a promising biocontrol agent for *H. halys*, and will greatly facilitate pre and post-release studies in China and other invaded countries. Overall, the development and application of such a tool will help address ecological questions related to a reduced risk, environmentally friendly approach for the control of *H. halys* in both original and invaded areas, and can be used in combination with conventional methods to better interpret host-parasitoid and parasitoid-parasitoid interactions.

Considering the agricultural importance of the *Trissolcus* and *Anastatus* wasps, the ability to quickly identify parasitized BMSB eggs is an important tool for present and future research. In the different regions of China as well as Switzerland and Italy, efforts are currently underway to use *Trissolcus* or *Anastatus* wasps for augmentative releases to mitigate crop damage from BMSB (Stahl et al. 2019b; Mi et al. 2022). Surveying of released parasitoid species is especially important to assess the ability of the parasitoid species to overwinter, field release efficiency, the time frame in which parasitism tends to occur, and the identity of the parasitoid in the absence of the emergence (Herlihy et al. 2016; Moraglio et al. 2020). In all these studies, a rapid identification method can facilitate the speed and accessibility of research results to make a evidence-based decision in a bological control context.

In summary, our DNA-based method is both an accessible and reliable way to identify *Trissolcus* and *Anastatus* species, promising biocontrol agents for BMSB. With ongoing biological control efforts, the established range of the *Trissolcus* and *Anastatus* wasps will likely increase, and so will demand for identification for the study of the parasitoid's capacity to overwinter, field parasitism rate, and potential non-target effects on ecologically important pentatomids (Jentsch 2017). In this scenario, the quick and inexpensive nature of PCR assay will be a valuable tool for understanding of the host-parasitoid associations in any biological control programme developed for sustainable management of BMSB. Further work could aim to (i) create a multiplex PCR protocol combining both universal primers and species-specific primers, dramatically reducing the workload of the method; (ii) optimize on the existing molecular method for these parasitoids' haplotypes and thereby assessing Hymenoptera biodiversity at different geographical locations; (iii) and/or develop multiplex or quantitative PCRs for

simultaneous detection and identification of different COI sequences or *Trissolcus* and *Anastatus* species.

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Availability of data and materials
The data is available from corresponding author on reasonable request.

Declarations

Competing interests
The authors declare no conflicts of interest.

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