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A molecular tool to identify *Anastatus* parasitoids of the brown marmorated stink bug

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Abstract

Globally, *Anastatus* species (Hymenoptera: Eupelmidae) are associated with the invasive agricultural pest *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae). In Europe, the polyphagous *Anastatus bifasciatus* (Geoffroy) is the most prevalent native egg parasitoid on *H. halys* eggs and is currently being tested as a candidate for augmentative biological control. *Anastatus bifasciatus* frequently displays behavior without oviposition, and induces additional host mortality through oviposition damage and host feeding that is not measured with offspring emergence. This exacerbates accurate assessment of parasitism and host impact, which is crucial for efficacy evaluation as well as for pre- and post-release risk assessment. To address this, a general *Anastatus* primer set amplifying a 318-bp fragment within the barcoding region of the cytochrome oxidase I (COI) gene was developed. When challenged with DNA of three *Anastatus* species —*A. bifasciatus*, *Anastatus japonicus* Ashmead, and *Anastatus* sp.—, five scelionid parasitoid species that might be encountered in the same host environments and 11 pentatomid host species, only *Anastatus* DNA was successfully amplified. When applied to eggs of the target host, *H. halys*, and an exemplary non-target host, *Dendrolimus pini* L. (Lepidoptera: Lasiocampidae), subjected to host feeding, no *Anastatus* amplicons were produced. Eggs of the two host species containing *A. bifasciatus* parasitoid stages, from 1-h-old eggs to pupae, and emerged eggs yielded *Anastatus* fragments. Confirmation of parasitoid presence with dissections and subsequent PCRs with the developed primer pair resulted in 95% success for 1-h-old parasitoid eggs. For both host species, field-exposed sentinel emerged eggs stored dry for 6 months, 100% of the specimens produced *Anastatus* amplicons. This DNA-based screening method can be used in combination with conventional methods to better interpret host-parasitoid and parasitoid-parasitoid interactions. It will help address ecological questions related to an environmentally friendly approach for the control of *H. halys* in invaded areas.

Introduction

In the field of biological control, molecular diagnostic tools are useful for evaluating population genetics of natural enemies, assessing non-target effects, and accurately identifying biocontrol agents (Bigler et al., 2005; Gariepy et al., 2007). These tools may supplement traditional methods of species identification that, for parasitoids, mainly consist of rearing the natural enemies and dissecting their hosts.

Of recent interest globally is the brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae), and the potential to use parasitoids in a biocontrol strategy for this pest. *Halyomorpha halys* is a polyphagous species of Asian origin that has become highly invasive in North America and Europe following accidental introduction in the 1990s and early 2000s, respectively (Hoebeke & Carter, 2003; Wermelinger et al., 2008; Haye et al.,

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2014a,2014b). A more recent introduction occurred in 2017 in South America (Faúndez & Rider, 2017). In North America and Europe, *H. halys* has caused tremendous damage as an agricultural pest on several economically important host plants, including apple, pear, peach, hazelnut, and maize (Leskey et al., 2012; Maistrello et al., 2017; Bosco et al., 2018).

In its native range, *H. halys* is attacked by a complex of scelionid and eupelmid egg parasitoids, with *Trissolcus japonicus* Ashmead (Hymenoptera: Scelionidae) as the most prevalent species (Yang et al., 2009; Zhang et al., 2017). In Europe, native scelionid species typically associated with other pentatomids attempt to attack *H. halys* eggs, but are unable to complete development (Haye et al., 2015). As such, European *Trissolcus* and *Telenomus* species are not promising candidate agents for biological control of *H. halys*. In contrast, another European egg parasitoid, *Anastatus bifasciatus* Geoffroy (Hymenoptera: Eupelmidae), is the species most commonly found parasitizing *H. halys* eggs, and can complete its development on live *H. halys* eggs. This polyphagous, solitary species has been reared from sentinel and natural field-collected *H. halys* eggs in several European countries (Haye et al., 2015; Costi et al., 2019) and is currently being tested as a biocontrol agent in the field in Switzerland and Italy. Natural field parasitism assessed by rearing is generally quite low (e.g., Costi et al., 2019). However, *A. bifasciatus* induces additional host mortality through oviposition damage (the parasitoid oviposits, the host dies, but parasitoid offspring fails to emerge) and host feeding (the parasitoid feeds on the host fluids, the host dies), which in fact is equivalent to host mortality related to offspring production (Stahl et al., 2019a). In addition to *H. halys*, *A. bifasciatus* successfully parasitizes eggs of other European Heteroptera and Lepidoptera, including several species of conservational concern (Noyes, 2014; Stahl et al., 2018).

Accurate assessment of parasitism of *H. halys* in the field can be difficult. First, species-level identification of the host eggs is challenging, as there are few distinguishing morphological characters in the egg stage (Esselbaugh, 1946; Bundy & McPherson, 2000; Gariepy et al., 2014). Usually, recovered egg masses are found when nymphs have already hatched, which further exacerbates identification efforts. Identifying the associated egg parasitoids after their emergence is similarly challenging. 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 & Mineo, 1970), but 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 can prevent identification via rearing and even dissections are of little use once the parasitoids have disintegrated. To overcome some of these difficulties, a modified DNA barcode approach was developed with general primers for Pentatomidae and Scelionidae in order to evaluate host-parasitoid associations in this system (Gariepy et al., 2014; Gariepy et al., 2019). However, *Anastatus* species were not included in this analysis.

For risk assessment and to evaluate biocontrol efficacy of *A. bifasciatus*, a molecular approach using PCR primers that can detect the presence of *A. bifasciatus* DNA within a host would greatly improve the ability to rapidly and accurately identify *A. bifasciatus* in *H. halys* and other potential hosts, and would provide a more accurate estimate of parasitism level. Once designed, newly developed primers need to be tested in terms of their specificity and sensitivity: if the primers are not specific enough, they can amplify each other, or DNA from species other than the one they were designed for, both of which would lead to false positives (Admassu et al., 2006). 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 designed for and species that parasitize closely related hosts (Gariepy et al., 2007). The sensitivity of a primer set measures which stages of the parasitoid can be detected. It is important to ensure that all developmental stages are detectable, to prevent an underestimation of parasitism level due to false negatives. To gain more information regarding host-parasitoid associations in this system, a set of general PCR primers nested within the DNA barcode regions were developed for *Anastatus*, and evaluated in terms of specificity, sensitivity, and applicability to field samples using the target host *H. halys* and an exemplary non-target host.

Material and methods

Primer design

Publically available DNA sequences for *Anastatus* species associated with pentatomid eggs (generated as part of another research project; TD Gariepy, unpubl.) were retrieved from the DNA barcode of life datasystems (BOLD), project NASTA (Table 1). The 652-bp COI sequences for *Anastatus* were aligned using the Clustal W algorithm (Thompson et al., 1994) in Codon Code Aligner (v.4.0.4). Visual inspection of regions of DNA sequence similarity were used to design primers nested within the DNA barcode region that would amplify all three species of *Anastatus*. Furthermore, *Anastatus* DNA sequences were aligned with representative members of the Scelionidae (BOLD project NSCEL) and Pentatomidae (BOLD project HCNC) to ensure adequate sequence variation

existed within these regions to prevent non-specific amplification of the Pentatomidae and the Scelionidae, as one or more of these species could be present in mixed DNA samples extracted from host eggs.

Primer specificity

The specificity of the primer pair (Ana-361F and HCO-2198) was tested in separate PCR reactions with DNA from three *Anastatus* species, as well as with DNA of five scelionid parasitoids, and 11 pentatomid hosts (Table 2). Amplification of DNA was performed in an Eppendorf Mastercycler Pro PCRs in a 25-µl volume containing 0.125 µl of Taq Platinum, 2.5 µl of 10× PCR buffer, 1.25 µl of 50 mM $MgCl_2$, 0.125 µl of 10 µM dNTPs (Invitrogen), 0.25 µl of 10 µM Ana-361F (5′-ATCACATAG GGGTCCTTCAGTA-3′), 0.25 µl of 10 µM HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al., 1994), 19.5 µl ddH_2O, and 1 µl of template DNA. Thermocycling conditions were as follows: initial denaturation at 94 °C for 1 min, followed by 35 cycles of 94 °C for 40 s, 58 °C for 40 s, and 72 °C for 1 min, and a final extension period of 5 min at 72 °C.

Primer sensitivity

The ability of the molecular markers to detect trace amounts of parasitoid DNA was tested in time trials to determine how soon parasitoid DNA could be detected following oviposition, which developmental stages of the egg parasitoid could be detected, and whether *A. bifasciatus* DNA can be detected in host eggs following emergence (based on trace levels of DNA present in parasitoid frass left in the egg). As *A. bifasciatus* regularly displays host feeding and can display oviposition behavior without egg deposition, host mortality caused by these behaviors are of interest in the assessment of this species as a biocontrol agent. However, they cannot be measured by offspring emergence or with identification of punctured host eggs, particularly in field-collected samples. Therefore, the sensitivity of the primers was tested on *H. halys* eggs that had been probed and subjected to host feeding. Each treatment was repeated 20×.

Table 1 Origin of tested *Anastatus* species

Species	BIN	Origin	n
A. japonicus	ACE0140	Fresh *Halyomorpha halys* egg masses in Asia	37
A. bifasciatus	ACX9265	Sentinel *H. halys* egg masses in Europe	3
Anastatus sp.	ACP8173	Unidentified pentatomid egg mass in Canada	10

Table 2 List of tested parasitoid and host species

Group	Species	n
Eupelmid parasitoids	*Anastatus japonicus* Ashmead	17
	Anastatus bifasciatus (Geoffroy)	11
	Anastatus sp.	6
Scelionid parasitoids	*Telenomus chloropus* (Thomson)	10
	Trissolcus semistriatus (Nees)	10
	Trissolcus japonicus Ashmead	10
	Telenomus podisi Ashmead	11
	Trissolcus euschisti (Ashmead)	4
Pentatomid hosts	*Acrosternum hilare* Say	3
	Banase dimidiadae (Say)	2
	Brochymena quadripustulatus (Fabricius)	3
	Cosmopepla bimaculate (Thomas)	2
	Dolycoris baccarum (L.)	2
	Euschistus variolarius (Palisot)	2
	Halyomorpha halys (Ståhl)	8
	Nezara viridula (L.)	2
	Podisus maculiventris (Say)	3
	Rhaphigaster nebulosa (Poda)	3
	Thyanta acerra McAtee	2

All tests apart from the host-feeding treatment were conducted both with the hosts *H. halys* and *Dendrolimus pini* L. (Lepidoptera: Lasiocampidae), a non-target species recently shown to be a suitable host for *A. bifasciatus*. As a negative control, fresh (<24 h), non-parasitized *H. halys* and *D. pini* eggs were used.

To obtain different stages of the parasitoid, fresh host eggs (<24 h) were exposed at 26 °C to randomly selected *A. bifasciatus* females in 54 × 14 mm Petri dish arenas. Oviposition behavior was observed with a 'Leica MS5' microscope (Leica Biosystems, Nussloch, Germany), probed eggs were separated and stored for different periods at 26 °C to allow the parasitoids to stay in the egg phase (1 h) or develop into an early instar (3 days), late instar (7 days), and the pupal stage (21 days). After the selected time periods the eggs were transferred to 2-ml Sarstedt tubes filled with 95% ethanol. For the probed and host-fed *H. halys* eggs, the parasitoid females were removed from the eggs after observed ovipositor insertion and the end of the first host-feeding event and the eggs immediately transferred into ethanol.

To correct for parasitization behavior without actual oviposition, stored *H. halys* eggs were visually and non-invasively examined for *A. bifasciatus* presence after 7 and 21 days. Only those host eggs containing parasitoid larvae (after 7 days) or pupae (after 21 days) were transferred into ethanol. The presence of earlier developmental stages (as well as all stages in *D. pini*) can rarely be ascertained in

a non-invasive way, so for these categories all host eggs were placed in ethanol. As a control for the presence of *A. bifasciatus*, additional potentially parasitized *H. halys* and *D. pini* eggs placed into ethanol 1 h (egg stage), 12 h (egg stage), 24 h (egg stage), and 3, 7, and 21 days after observed oviposition behavior were dissected. When *A. bifasciatus* presence was confirmed by dissection, the remains of the dissected host and parasitoid were subjected to the same molecular procedures as described below. In addition to the host eggs containing premature *A. bifasciatus* stages, empty eggs were transferred to ethanol <24 h after parasitoid emergence to determine the ability to detect parasitoid DNA from empty eggs.

Application to field-collected samples

As one of the most important applications of the developed molecular marker is the identification of *Anastatus* parasitoids from field-collected samples, field-exposed sentinel eggs of *H. halys* and *D. pini* were stored for ca. 6 months at 26 °C and subjected to the same DNA extraction and amplification procedures as the time trial samples. This included empty eggs (from which *Anastatus* had already emerged), as well as non-emerged eggs from the same egg masses that yielded *Anastatus* adults.

Molecular analysis

Samples were transferred to 1.5-ml Eppendorf vials individually with a clean, sterile paint brush after which 50 µl of digestion buffer (100 mM NaCl, 10 mM Tris:HCL pH 8.0, and 25 mM EDTA) was added. The material was shredded with a pellet mixer (VWR International, Lutterworth, UK) for ca. 1-2 min until homogenized. Previously dissected material used to control for parasitoid presence was not shredded further. The paint brush and mixer tip were washed first in diluted bleach solution, then 70% ethanol, and finally water between each sample to avoid contamination. Fifty µl of a mixture of digestion buffer (48 µl) and proteinase K (0.2 mg ml^{-1}; Qiagen, Hilden, Germany) (2 µl) was added to the homogenate, and vials were incubated for 2–5 h in a 56 °C shaking water bath (type 1083; GFL Gesellschaft für Labortechnik, Burgwedel, Germany) after which 40 µl 6 M NaCl and 140 µl 100% chloroform was added; the samples were mixed for 20 min with a horizontal shaker (HS 501 digital; IKA Labortechnik, Staufen, Germany). After 5 min of centrifuging at 20 000 ***g***, the supernatant water phase with the isolated nucleic acids was transferred into a new 1.5-ml Eppendorf tube. To precipitate the DNA 140 µl of 100% isopropanol was added as well as 2 µl 15 mg ml^{-1} GlycoBlue (Thermo Scientific, Waltham, MA, USA) to stain the forming pellet. The mixture was incubated for 5 min on the shaker and then centrifuged for 20 min at 20 000 ***g***. Subsequently, the supernatant was discarded, and the remaining pellet washed 3× with 150 µl of 70% ethanol (centrifuged for 5 min at 20 000 ***g*** and liquid discarded), after which the DNA pellets were vacuum dried (Concentrator 5301; Eppendorf, Hamburg, Germany) for 1–2 min at 45 °C until all remaining liquid had evaporated. The DNA was resuspended in 20 µl Milli-Q water.

For the PCR, a 25-µl reaction volume was prepared with 2.5 µl 10× reaction buffer + Mg (Roche Diagnostics, Mannheim, Germany), 0.125 µl 10 mM dNTP mix (Fermentas, Waltham, MA, USA), 0.25 µl 10 µM Ana-361F forward primer (5′-ATCACATAGGGGTCCTTCAGTA-3′) and 0.25 µl 10 µM HCO-2198 reverse primer (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′), 20.75 µl ddH_2O, 0.125 µl Taq DNA polymerase (Roche Holding, Basel, Switzerland), and 1 µl template DNA. The PCR was run in an Applied Biosystems Veriti 96-Well Thermal Cycler (Thermo Scientific) with initial denaturation at 94 °C for 1 min, 40 cycles of 40 s annealing at 58 °C, and 1 min extension at 72 °C, followed by 5 min of final extension at 72 °C.

PCR products were visualized with gel electrophoresis, 5 µl of template DNA was blended with 3 µl 6× DNA Gel Loading Dye (Thermo Scientific) and run on a 2% agarose gel with 1× TAE buffer and 0.05 µl ml^{-1} ethidium bromide next to 1 µl 0.1 µg $µl^{-1}$ flanking ladder GeneRuler 100 bp Plus (Thermo Scientific). The results were visualized with a GeneFlash Bio Imaging Gel Documentation System UV/VVIS Gene Flash (Syngene International, Bangalore, India).

Statistical analysis

The number of samples in which *Anastatus* DNA was detected was compared for time trials and field samples with a generalized linear model (GLM) with a binomial distribution using the logit link function, with detection as the dichotomous response variable, and the hosts *H. halys* and *D. pini* as independent variable for each time interval/treatment, in R v.3.2.3 (R Core Team, 2014) and the development environment RStudio (2017).

Results

Primer design

Alignment of the DNA barcode region of the COI gene from public sequences available on BOLD allowed the identification of short nucleotide sequences conserved within the genus *Anastatus*, but with sufficient variation to permit exclusion of members of the families Pentatomidae and Scelionidae (Table S1). This resulted in the development of a unique forward primer, Ana-361F (5′-ATCA CATAGGGGTCCTTCAGTA-3′) that when used in

combination with the universal reverse primer HCO-2198 (Folmer et al., 1994), yields a 318-bp PCR fragment for *Anastatus* spp.

Primer specificity

When challenged with DNA from different *Anastatus* species, the primer combination Ana-361F and HCO-2198 successfully amplified an ca. 320-bp fragment for all *Anastatus* specimens collected from Europe (*A. bifasciatus*, n = 11), China (*A. japonicus*, n = 17), and Canada (*Anastatus* sp., n = 6), whereas no DNA amplification was observed for specimens of Scelionidae and Pentatomidae (Figure 1). No PCR amplicons were observed for *D. pini*, the representative lepidopteran host used in subsequent experiments (data not shown).

Primer sensitivity

Anastatus-specific products were yielded by 45% of *H. halys* and 55% of *D. pini* eggs placed in alcohol 1 h after observed parasitization behavior (Figure 2). Of specimens transferred to ethanol after 3 days, 30 and 35% of *H. halys* and *D. pini*, respectively, were positive for *Anastatus* DNA (Figure 2). For both time intervals the values were not significantly different between host species (binomial GLM, 1 h: $z = -0.631$, $P = 0.53$; 3 days: $z = -0.337$, $P = 0.74$, both d.f. = 1,38). After 7 and 21 days development, *Anastatus* was more frequently detected in *H. halys* samples (95 and 100%) than in *D. pini* samples (70 and 55%) (binomial GLM, 7 days: $z = 1.846$, $P = 0.030$; 21 days: $z = 0.008$, $P<0.001$, both d.f. = 1,38). For both host species, 100% of the tested samples transferred to alcohol <24 h after adult *A. bifasciatus* emergence produced *Anastatus* amplicons (Figure 2).

Dissection of eggs drilled by *A. bifasciatus* (from the same batch as those from which DNA was extracted and tested with the *Anastatus*-specific primers) revealed that 20–56% of the *H. halys* and *D. pini* eggs contained the expected *A. bifasciatus* stages (Figure 3). Upon testing only those eggs that were found to contain *A. bifasciatus*, DNA amplification resulted in 89% (24-h-old eggs) to 100% (young and old larvae, pupae) detection of *Anastatus* (Figure 4).

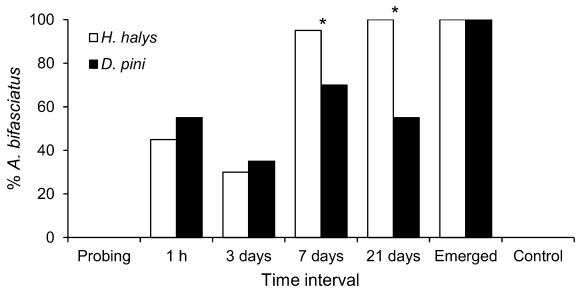

Figure 2 Percentage of *Anastatus bifasciatus* parasitoid DNA detected after various time intervals with the hosts *Halyomorpha halys* and *Dendrolimus pini*. Host eggs were placed in alcohol directly after probing without oviposition (probing), after a development time of the parasitoid of 1 h (egg), 3 days (early instar), 7 days (late instar), and 21 days (pupa), and <24 h after adult parasitoid emergence (emerged) (n = 20 host eggs per time interval). Non-parasitized host eggs served as negative control (control; n = 20). Asterisks indicate significant differences of pairwise comparisons between host species within a time interval (binomial GLM: $P<0.05$).

Application to field-collected samples

In field-exposed sentinel eggs belonging to both host species, parasitoid DNA was detected in empty eggs following parasitoid emergence ca. 5–6 months prior to preservation in ethanol for subsequent molecular analysis. For both host species, all specimens (n = 20 for *H. halys*, n = 10 for *D. pini*) produced *Anastatus* amplicons (Figure 5). In contrast, *Anastatus* was detected in 15% of non-emerged *H. halys* eggs and 58% of non-emerged *D. pini* eggs collected from the same field-exposed sentinel egg masses that

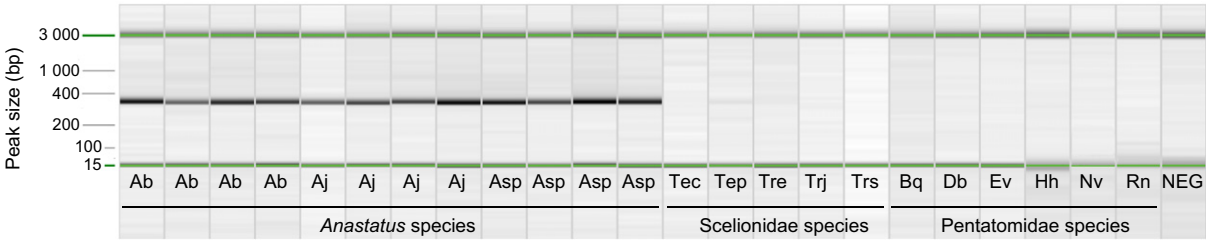

Figure 1 Specificity of *Anastatus* PCR primers (Ana-361F and HCO2198) when challenged with DNA from *A. bifasciatus* (Ab), *A. japonicus* (Aj), and *Anastatus* sp. (Asp), as well as several scelionid parasitoid species (*Telenomus chloropus*, Tec; *Te. podisi*, Tep; *Trissolcus euschisti*, Tre; *Tr. japonicus*, Trj; *Tr. semistriatus*, Trs) and pentatomid species (*Brochymena quadripustulata*, Bq; *Dolycoris baccarum*, Db; *Euschistus variolarius*, Ev; *Halyomorpha halys*, Hh; *Nezara viridula*, Nv; *Rhaphigaster nebulosa*, Rn). Peak size refers to DNA fragment size (no. base pairs) according to a size-ladder on the left of the figure.

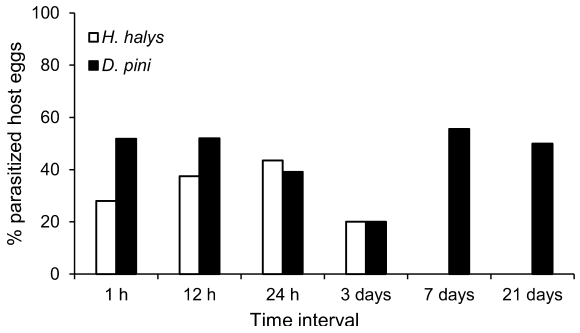

Figure 3 Percentage of dissected eggs containing *Anastatus bifasciatus* after various developmental time intervals in the hosts *Halyomorpha halys* and *Dendrolimus pini*. Samples with a development time of 1 h (egg; n = 25 *H. halys*/27 *D. pini*), 12 h (egg; n = 24/25), and 24 h (egg; n = 23/23), 3 days (early instar; n = 20/20), 7 days (late instar; n = 0/9), and 21 days (pupa; n = 0/9).

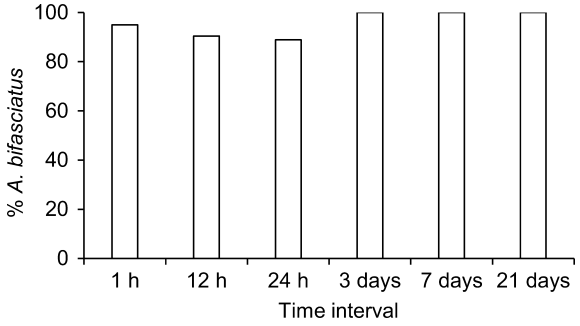

Figure 4 Percentage of *Anastatus bifasciatus* parasitoid DNA detected in the dissected parasitoid stages, with a development time of 1 h (egg; n = 20 host eggs), 12 h (egg; n = 21), and 24 h (egg; n = 18), 3 days (early instar; n = 2), 7 days (late instar; n = 6), and 21 days (pupa; n = 5).

had also yielded *Anastatus* adults (Figure 4). There were no significant differences between the detection levels in the two hosts (binomial GLM, z = −1.92, d.f. = 1,47, P = 0.052).

Discussion

In the present study, a set of PCR primers capable of detecting *Anastatus* species DNA without non-target amplification of scelionid, pentatomid, and Lepidoptera DNA is reported. Although these primers are capable of amplifying the three *Anastatus* species from the present study, their broad applicability to other members with the same genus and/or in the Eupelmidae from other host-parasitoid systems is unknown and should be tested. However, in the current context, the number of species

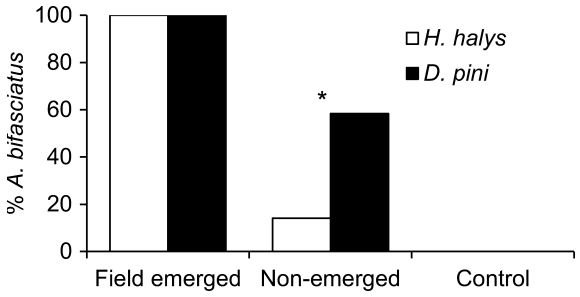

Figure 5 Percentage of *Anastatus bifasciatus* parasitoid DNA detected in the various treatments of the field-collected samples with *Halyomorpha halys* and *Dendrolimus pini* as hosts. Eggs were stored in ethanol for up to 6 months after adult parasitoid emergence (field emerged; n = 20 *H. halys*/10 *D. pini*); non-emerged eggs (n = 113/12) were from the same egg masses from which *A. bifasciatus* had emerged (field emerged eggs). Non-parasitized host eggs served as negative control (control; n = 20).

interactions that may occur within the present host-parasitoid system are limited and therefore the ability of the primers to separate *Anastatus* DNA from pentatomid and scelionid DNA is sufficient for the research questions addressed here.

A total of 57% of host eggs that potentially contained various stages of *A. bifasciatus* (egg, larva, pupa, post-emergence empty egg) produced *Anastatus* amplicons. In contrast, 95% of host eggs that were dissected 1 h post-parasitism and subjected to molecular analysis (following confirmation of the presence of a parasitoid egg) were positive for *Anastatus* DNA. This suggests that the molecular tool is sensitive enough to consistently detect parasitoid eggs, and the comparatively low detection level (57%) in the time trial series was largely the result of parasitoid probing behavior without actual oviposition. Successful oviposition by *A. bifasciatus* cannot be evaluated by visual inspection of the egg, and although probing of the egg can be observed, roughly only half of the eggs are used for oviposition, whereas the other half are exclusively used for host feeding (Konopka et al., 2017; Stahl et al., 2019a).

Recently, trophic interactions between Pentatomidae hosts, including *H. halys*, and their scelionid parasitoids were investigated in North America with a modified DNA barcode approach (Gariepy et al., 2019). Given that the *Anastatus* primers are also within the DNA barcoding region, they could, if proven to be compatible, potentially be used in combination with the primer pair for Scelionidae (SCEL: SCEL-F1/HCO-2198) (Gariepy et al., 2014) to assess multispecies interactions in the Pentatomidae. However, in order to determine species-level interactions, DNA sequencing of both scelionid and *Anastatus* amplicons would be required for species-level resolution (as was

done for scelionids and pentatomids in Gariepy et al., 2019). This type of DNA sequencing approach allows broad applicability of the technique, regardless of geography, as the same primers amplify *Anastatus* from North America, Europe, and Asia, and the DNA barcode library for these species (BOLD project NASTA) would allow species separation based on single-nucleotide polymorphisms in the DNA barcode region. An improved understanding of species interactions is of particular interest, as *T. japonicus* has recently been discovered in Europe, and is established in Switzerland in locations where *A. bifasciatus* is frequently found (Stahl et al., 2019b). Laboratory studies on interactions between *T. japonicus* and *A. bifasciatus* suggest the potential for coexistence, but field data from Europe are needed to confirm this, and to determine whether the two species will compete for target and non-target host resources (Yang et al., 2009; Qiu & Yang, 2010; Konopka et al., 2017).

Recent studies indicate that pre-imaginal mortality of *A. bifasciatus* in *H. halys* is indeed a variable that should not be neglected (Stahl et al., 2019a). However, accurate measurements 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). In the present system, parasitoid-induced mortality can be from host stinging, either with or without oviposition, and it can also be inflicted from host-feeding. Host mortality due exclusively to parasitoid feeding events cannot be detected by dissection (Day, 1994; Cebolla et al., 2018), nor can it be detected with the *Anastatus* primers described here. Although we assume that minute traces of parasitoid DNA would be transferred to the host egg during probing stinging (e.g., calyx fluid) or feeding (e.g., saliva), these are not detectable with the protocol developed here. Perhaps further refinement of the protocol to increase detection sensitivity will allow minute quantities of DNA to be detected.

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). *Anastatus bifasciatus* is known to be an extremely polyphagous parasitoid that attacks and develops in a variety of Hemiptera and Lepidoptera, some of which are of conservational concern (Noyes, 2014; Stahl et al., 2018). With this molecular tool, recovered eggs from the field can be screened for the presence or absence of *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) 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 26 °C nonetheless yielded *Anastatus* 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 *Anastatus* primers are capable of detecting DNA from dry, decaying eggs from emerged and non-emerged eggs. As the PCR primers amplify a fairly short fragment of the COI gene (ca. 320 bp), 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 & McKenna, 2012).

The availability of a molecular tool to detect the presence of *A. bifasciatus* is also very useful in laboratory studies. Oviposition behavior in *A. bifasciatus* may or may not result in the insertion of an egg within a host, and there is no way to determine whether oviposition was successful, based on observation or visual inspection of the probed host egg. 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., 2019a). 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, 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 development occurs.

The utility of the *Anastatus* primers as a molecular tool in both field and laboratory studies will permit the evaluation of a promising biocontrol agent for invasive *H. halys*, and will greatly facilitate pre- and post-release studies on *A. bifasciatus* in Europe. In addition, the fact that these primers amplify at least three species of *Anastatus* associated with the Pentatomidae provides additional flexibility in their application in other geographic areas where these species exist and where there may be interest in evaluating their biocontrol potential for *H. halys*. Although the current application of the primers is to detect presence/absence of *Anastatus*, their utility can be expanded by implementing them in a DNA barcoding approach. DNA sequencing of the resulting 320-bp PCR product, and comparison of sequences with publicly available DNA barcodes (BOLD project NASTA) would allow species separation, which may be of interest in areas where multiple *Anastatus* species co-occur with overlapping host range. 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 invaded areas, and can be used in combination with conventional methods to better interpret host-parasitoid and parasitoid-parasitoid interactions.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Alignment of the COI barcode region for representatives of the Pentatomidae, Scelionidae, and *Anastatus* to illustrate areas of sequence variation.