Entomologia Experimentalis et Applicata

DOI: 10.1111/eea.12541

TECHNICAL NOTE

New PCR primers enhance investigation of host-parasitoid food webs

Victoria G. Pook[1], Kacie J. Athey[2], Eric G. Chapman[2], Stephanie A. Clutts-Stoelb[3] & Michael J. Sharkey[2]*

[1]Department of Horticulture, University of Kentucky, Lexington, KY 40546, USA, [2]Department of Entomology, University of Kentucky, Lexington, KY 40546, USA, and [3]Bluegrass Community & Technical College, University of Kentucky, Lexington, KY 40546, USA

Accepted: 24 November 2016

Key words: Hymenoptera, Lepidoptera, trophic links, molecular detection, green cloverworm, Erebidae, Ichneumonoidea, Chalcidoidea, Tachinidae, Diptera

Introduction

Host-parasitoid interactions provide some of nature's most fascinating examples of co-evolution. However, only a minute fraction of these interactions are known to science and the majority of these are the result of long-term, labor-intensive rearing studies (e.g., Smith et al., 2006; Burns et al., 2008; Janzen et al., 2009; Janzen & Hallwachs, 2011). Rearing studies entail the collection and incubation of potential host arthropods, followed by the documentation of the emergence of an adult host or parasitoid. When successful, these studies produce unequivocal data on host-parasitoid relationships. However, unnatural rearing conditions frequently result in the untimely death of the potential host arthropod, preventing documentation of the adult arthropod that would have emerged. These premature deaths do not occur at random; rather they plague particular taxonomic groups, hindering the collection of any data regarding host-parasitoid relationships in these taxa. For example, in Lee Dyer's studies in Ecuador, ca. 60% of the caterpillars failed to produce an adult moth or parasitoid (LA Dyer, University of Nevada, Reno, NV, USA, unpubl.). Even those taxa in which rearing studies are deemed successful may be under- or overestimating the prevalence of parasitism by neglecting to examine specimens that died during the rearing process.

Over the last decade, molecular techniques have revolutionized the study of host-parasitoid interactions (reviewed in Hrček & Godfray, 2015). DNA-barcoding has enabled accurate identification of species of hosts and species of parasitoids, revealing the prevalence of morphological crypsis in these organisms (Smith et al., 2008, 2013; Janzen & Hallwachs, 2011; Stahlhut et al., 2013; Fernández-Triana et al., 2014). These data have substantially altered our view of food webs, indicating that many species of parasitoids thought to be generalists are, in fact, complexes of cryptic specialists (Smith et al., 2011). In addition, the use of taxon-specific polymerase chain reaction (PCR) primers, first employed in molecular gut content analysis of predators (Symondson, 2002; Sheppard & Harwood, 2005; King et al., 2008), has proven to be effective at detecting the DNA of immature parasitoids within potential host arthropods (Zhu et al., 2004; Traugott & Symondson, 2008; Derocles et al., 2012; Wirta et al., 2014). This technique can be highly sensitive, with one study detecting the presence of a parasitoid in its host just 5 min after oviposition (Traugott & Symondson, 2008).

The design of taxon-specific primers requires a priori knowledge of the parasitoids that are interacting with the potential host arthropods. Therefore, studies employing these techniques are either detecting just one or a few species of closely related parasitoids (Zhu et al., 2004; Traugott & Symondson, 2008) or are investigating food webs that are species-poor (Wirta et al., 2014). In species-poor food webs, it is possible to collect all adult parasitoids in the system and sequence their DNA, enabling the design of primers specific to all the species in the system. In contrast, amplification of DNA from a broad range of parasitoids requires the use of degenerate primers and to date, this has only been successful at the subfamily level. Derocles et al. (2012) designed primers to amplify DNA from the majority of the species of Aphidiinae (Hymenoptera: Braconidae) from within their aphid hosts.

Molecular techniques have the power to reveal host-parasitoid relationships in a highly efficient and cost-

*Correspondence: Michael J. Sharkey, S-225 Ag Sci Ctr N, University of Kentucky, Lexington, KY 40546, USA. E-mail: msharkey@uky.edu

effective manner and will undoubtedly play a significant role in ecological studies in the future. However, at present, the major limitation is the design of PCR primers that amplify a broad range of parasitoids from within their hosts. Here, we show that this can be done using priming sites located in the nuclear 28S rDNA gene. Primers were designed to amplify DNA from either parasitoid wasps (from the superfamilies Ichneumonoidea and Chalcidoidea) or parasitoid flies (from the family Tachinidae) from within lepidopteran hosts. These primers were extensively tested on non-target lepidopteran taxa and were then used in combination with a rearing study to detect the rate of parasitism in the green cloverworm, *Hypena scabra* (Fabricius) (Lepidoptera: Erebidae).

Material and methods

Primer development

Primer design. To design primers that amplify parasitoid DNA within their Lepidoptera hosts, sequences of the D2–D3 region of the nuclear gene, 28S, from Lepidoptera, Hymenoptera (Ichneumonoidea and Chalcidoidea), and Diptera (Tachinidae) were downloaded from GenBank and aligned with MAFFT using default settings on http://mafft.cbrc.jp/alignment/server/ (Katoh et al., 2006). Using Primer3 (Rozen & Skaletsky, 1998), primers were developed for a ca. 450 bp amplicon of Hymenoptera DNA: Hym-28S-F (5′-GTAAACCTGAGAAACCCAA AAGAT-3′) and Hym-28S-R (5′-CCTGAAAGTACC CAAAGCAGTAG-3′), and a ca. 350 bp amplicon of Tachinidae DNA: Tach-28S-F (5′-AAGCCCGATGA ACCTGAATATC-3′) and Tach-28S-R (5′-ATCCTGA ATCTTTCGCATTGTTAATC-3′). The 3′ ends of these primers are conserved across their target taxa, and have multiple mis-matches that are conserved across Lepidoptera. The gene 28S was chosen for this study because it is easy to amplify and provides satisfactory taxonomic resolution of the target taxa, often to the level of genus. The barcoding region of the mitochondrial gene, cytochrome *c* oxidase subunit I, was investigated for potential priming sites but no suitable sites were found, due to the high variability in this gene.

Primer testing. PCRs using DNA extracted from 35 species of Hymenoptera and four species of Tachinidae as template were conducted to confirm that the Hym-28S and Tach-28S primers amplify parasitoid DNA. In addition, the primers were tested on a total of 53 Lepidoptera specimens from 15 families to confirm that they do not amplify lepidopteran DNA. DNA was then extracted from tobacco budworms, [*Heliothis virescens* (Fabricius)] parasitized by *Campoletis* spec. and subsequently amplified by the Hym-28S primer pair. Finally, DNA extractions were performed on whole unparasitized tobacco budworms with the addition of a tachinid tibia and tarsus or only a tachinid tarsus and the Tach-28S primer pair was used to amplify the DNA from these samples. Adult Lepidoptera specimens were collected from the Berea College Forest (37.555°N, 84.238°W) on 23 May 2010. The remainder of extractions used to test the primers was from specimens from multiple prior projects. All DNA extraction and PCRs were conducted as described below and the results are listed in Table S1.

Rearing

On 15 and 17 August 2011, 493 specimens of green cloverworm were collected at University of Kentucky's Spindletop Research Farm, Lexington, KY, USA (38.12°N, 84.50°W). All caterpillars were sorted based on size (small, medium, and large) as an approximation of instar. As parasitoids vary in their instar preference, an equal number of each size of caterpillar was assigned to each of two treatments: immediately frozen at −20 °C (n = 250) or reared (n = 243). Caterpillars were reared in an incubator at 24 °C and L16:D8 photocycle. Specimen survival was checked every 2–3 days. If found dead, the specimen was frozen at −20 °C and rearing was considered to have failed.

Molecular detection of parasitoids

DNA extraction and PCR. DNA was extracted from all specimens in the frozen treatment and from all specimens that died during the rearing process. Total DNA was extracted from crushed whole specimens using DNeasy Blood and Tissue Kits (Qiagen, Valencia, CA, USA) following the standard animal tissue protocol. Each cloverworm DNA extraction was screened for hymenopteran and dipteran parasitoids using the 28S primers. PCRs consisted of 1X Takara buffer (Takara Bio, Shiga, Japan), 0.2 mM of each dNTP, 0.2 mM of each primer, 1.25 U Takara Ex Taq, and template DNA (1–2 μl of total DNA). PCRs were carried out in Bio-Rad PTC-200 (Bio-Rad Laboratories, Hercules, CA, USA). The PCR cycling protocol was 94 °C for 1 min followed by 50 cycles of 94 °C for 50 s, 56 °C for 45 s, 72 °C for 45 s, and a final extension of 72 °C for 5 min. Reaction success was determined by electrophoresis of 10 μl of PCR product in 2% SeaKem agarose (Lonza, Rockland, ME, USA) pre-stained with ethidium bromide (0.1 mg ml^{-1}).

All cloverworm extractions that did not yield a parasitoid sequence with our 28S primers were tested with general primers – COI: LCO-1490 (Folmer et al., 1994), HCO-700me (Breton et al., 2006), or 16S: LR-N-13398/ LR-J-12887 (Simon et al., 1994) – to determine extraction

success, and those that did not amplify with either primer pair were removed from all downstream analyses.

Sequencing. The Advanced Genomics Technologies Center at the University of Kentucky performed the post-PCR processes for sequencing. PCRs that yielded significant product were purified with QIAGEN MinElute PCR purification kit according to the manufacturer's guidelines. Cycle sequencing reactions were carried out in both the forward and reverse directions in an ABI 9700 thermal cycler using the ABI Big-Dye Terminator mix (v.3.0; Applied Biosystems, Foster City, CA, USA).

Identification of parasitoids

Frozen specimens and failed rearings. Parasitoid 28S sequences recovered from green cloverworm DNA extractions were queried against GenBank using BLASTN (Karlin & Altschul, 1990, 1993). The genus of the highest % match for each parasitoid-positive caterpillar is listed in Table S2. Species-level identifications were inferred by combining 28S sequence data with pre-existing knowledge of the species documented as parasitoids of green cloverworm found in studies such as Harper et al. (1983), Yeargan & Braman (1986, 1989), Braman & Yeargan (1991), and Pavuk & Williams (2003).

Successful rearings. Adult parasitoids and their associated cocoons/puparia were identified to the lowest possible taxonomic level using morphology. As with molecular data, the taxonomic resolution of these identifications was then increased with pre-existing knowledge of the species of parasitoids known to parasitize green cloverworm.

Statistical analysis

A z-score test was conducted to assess the difference in proportions of parasitized caterpillars in the frozen and reared treatments. In the frozen treatment, caterpillars that did not have viable DNA (16 of 250) were presumed to have the same parasitism rate as the specimens that did have viable DNA. In the reared treatment, only those that died needed to have their DNA assessed for viability and those that were not viable (28 of 243) were presumed to have the same parasitism rate as the other specimens that died (as opposed to the same rate as the treatment as a whole). A χ^2 goodness of fit test was then conducted on the reared specimens to assess whether the parasitism rates in the specimens that successfully yielded an adult (cloverworm or parasitoid) and those that failed to yield an adult differed from the expected rate (i.e., the rate of the whole treatment).

Results

Primer development

Both the Hym-28S and the Tach-28S primer pairs amplified their target DNA from extractions of hymenopterans and tachinids, respectively. In addition, lepidopteran DNA was not amplified by either of the primer pairs. The Hym-28S primer pair was 100% successful in detecting the DNA of the parasitoid *Campoletis* spec. from within parasitized lepidopterans (*H. virescens*). Likewise, the Tach-28S primer pair always produced a positive PCR result when tested on DNA extractions of a combination of ground up lepidopteran larva and a tachinid tarsus. In addition, the two parasitoid primer pairs are mutually exclusive: the tachinid primers do not amplify hymenopteran DNA and the hymenopteran primers do not amplify tachinid DNA.

Interestingly, three lepidopteran specimens that were collected for non-target testing also tested positive with the Hym-28S primer pair (Table S1). In two of the cases it appears that, unbeknownst to us, these specimens were already parasitized by chalcidoid wasps, lending further support for the efficacy of the primer pair. The third case yielded a positive PCR result from the Hym-28S primer pair but the resulting sequence matched that of a chrysomelid beetle, indicating that although this primer pair does not amplify lepidopteran DNA, there may be other orders of insects for which DNA will be amplified, and therefore sequencing is necessary to determine the identity of the amplified DNA.

Rearing study

Of the 243 reared caterpillars, 20 specimens ended the experiment alive (as a last stage larva inside a spun cocoon, pupa, or adult) and 68 specimens resulted in a live parasitoid (an adult or identifiable parasitoid pupa/puparium). These included Tachinidae, Ichneumonidae, and Braconidae (Microgastrinae and Rogadinae). Those that were identified to species were the braconid primary parasitoids *Cotesia marginiventris* (Cresson) and *Aleoides nolophanae* (Ashmead), the tachinid primary parasitoid *Campylochaeta plathypenae* (Sabrosky), and the ichneumonid hyperparasitoid *Mesochorus discitergus* (Say), all of which are documented parasitoids of the green cloverworm (Yeargan & Braman, 1989; Braman & Yeargan, 1991; Pavuk & Williams, 2003). The remaining 155 specimens (64%) died during the rearing process.

Parasitoid detection through PCR with parasitoid-specific primers

Parasitoids were detected in 90 of 234 specimens of green cloverworm in the frozen treatment and in 34 of the 155 that failed the rearing process. Of the 124 cloverworm extractions that tested positive for parasitoids, 117

produced viable sequences, with the remaining PCR products too weak to sequence. These sequences were queried against the NCBI database and the best hits were noted (Table S2). Importantly, all parasitoid genera found in the rearing study (*Aleoides*, *Cotesia*, *Mesochorus*) were also detected using molecular methods. There was also one additional genus, *Diolcogaster*, that was not successfully reared but whose DNA was amplified from failed rearings (n = 10) as well as frozen specimens (n = 15). The species *Diolcogaster facetosa* (Weed) is a documented parasitoid of green cloverworm (Harper et al., 1983; Yeargan & Braman, 1986; Braman & Yeargan, 1991), so it is likely that the DNA amplified corresponds to this species. Only seven green cloverworm larvae (four frozen, three reared) produced a band with the Tach-28S primers. These sequences could not be matched to any taxon higher than Tachinidae due to a lack of 28S sequence data available for this family (Table S2). It is important to note that PCRs that yield a positive result for parasitoid DNA do not imply successful parasitism and it is therefore only possible to provide a maximum rate of parasitism with a strong likelihood that some of the parasitoids detected would not have completed development.

Statistical analysis of parasitism rates

A two-tailed z-test indicates that the proportion of parasitized larvae in the reared treatment (45%) is not different from that of the frozen treatment (38%) ($z = 1.45$, $P = 0.15$). A χ^2 test demonstrates that the rate of parasitism in the successful rearings (77%) is higher than the expected 45% ($\chi^2 = 36.89$, d.f. = 1, $P<0.01$), whereas the rate of parasitism in the failed rearings (27%) is significantly lower than the expected 45% ($\chi^2 = 21.68$, d.f. = 1, $P<0.01$).

Discussion

The interactions between parasitoid arthropods and their hosts are of great interest to the entomological community and beyond. The organisms involved are highly influential in all terrestrial ecosystems and provide an excellent system in which to study evolutionary and ecological processes (LaSalle & Gauld, 1993). In addition, parasitoids which attack pest arthropods can be employed in integrated pest management programs (Dent, 2000). However, to study these systems, it is imperative to accurately record 'what is parasitizing what', a task which has greatly benefited from the advancement of molecular techniques. These techniques have not only improved the identification of specimens in rearing studies but have also enabled the molecular detection of parasitoids from within their hosts through the use of taxon-specific PCR primers (Hrček & Godfray, 2015).

In this study, we present new PCR primers that amplify the DNA of hymenopteran or dipteran parasitoids from within lepidopteran hosts. Unlike previous studies in which primers are designed to assess specific parasitoids within specific hosts, these primers can be used in the investigation of a broad range of lepidopteran parasitoids. In this study, we used these primers to compare a traditional rearing study with molecular detection of host-parasitoid relationships. We demonstrate that although rearing studies and molecular approaches reveal almost the same trophic links, the resulting estimates of parasitism rates may vary greatly.

The green cloverworm is an example of a lepidopteran that suffers high mortality under artificial rearing conditions. In our experiment, only 88 of the 243 reared caterpillars ended the experiment alive, either as a lepidopteran (20 specimens) or as a parasitoid (68 specimens). Had we only been able to assess parasitism in these specimens, we would have missed one species of parasitoid, the braconid *D. facetosa*, and our estimate of the rate of parasitism would have been 77%. Although this parasitism rate could occur under certain circumstances (e.g., Mitchell et al., 1997), the likelihood of this rate being accurate in this experiment is low. The results of our molecular investigation of parasitism indicate that it is, indeed, false.

After excluding specimens from the frozen treatment that had unviable DNA (6%), the rate of parasitism indicated by molecular data is 38%. In addition, we were able to extract viable DNA from more than 80% of the specimens that died during the rearing process. Combining the data from the successfully reared specimens with those that died during the rearing process indicates that the rate of parasitism in the reared treatment as a whole is 45%, a rate which is not statistically different from that of the frozen treatment (38%). We therefore conclude that the true parasitism rate of the population of green cloverworm in our study is likely to be under 50%, rather than the 77% suggested by the rearing study alone. It is important to emphasize that 45 and 38% are maximum rates with a good chance that a proportion of these positive hits are from parasitoid DNA within eggs that were encapsulated by the green cloverworm and therefore would not have resulted in successful parasitism, further lowering the estimated parasitism rate.

We also found that specimens that were successfully reared were significantly more likely to have been parasitized, whereas specimens that failed were significantly less likely to have been parasitized. There is growing evidence that parasitoids are capable of protecting their hosts

from pathogenic disease (Danneels et al., 2013; Martinson et al., 2014, 2016) and this may be the case here. Protecting their hosts in this way is evolutionarily advantageous since parasitoids can only complete development if their host survives, and our data indicate that parasitized green cloverworm are more likely to survive the unfavorable conditions of artificial rearing than those that are not parasitized.

In summary, we show that the PCR primers developed in this study can be used to detect hymenopteran and dipteran parasitoids within their lepidopteran hosts. These primers enable a much broader assessment of parasitism than those that have been published previously (Zhu et al., 2004; Traugott & Symondson, 2008; Wirta et al., 2014) which, for the most part, amplify DNA from only one or a few closely related parasitoids. Although 28S sequence data are not sufficiently divergent to enable all species-level identifications, it is possible to infer lower taxonomic assignments (often genus), which is valuable information in systems for which there is little or no data regarding host-parasitoid relationships. We therefore promote the use of these primers as an initial screen of populations of lepidopteran larvae to determine which families, subfamilies, and genera of parasitoids are present in the system and the rate at which parasitism occurs. Once this information has been gathered using our 28S primers, researchers could then design primers tailored to their specific study taxa to identify species (e.g., using the barcode region of COI).

Although it is possible that these primers could be used to investigate parasitization in other insect orders, it is difficult to predict to which taxa they may be best suited. For example, we found that though the Hym-28S primer pair does not amplify DNA from specimens of Tenthredinidae, another family of Hymenoptera, they did amplify DNA from a chrysomelid beetle. We therefore promote these taxon-specific primers as a new molecular tool in food-web studies but emphasize that testing of the potential host arthropod is imperative if alternative taxa are the focus of the study.

Acknowledgements

We thank Clint Patterson, Berea College Forester, for permitting us to collect specimens in the Berea College Forest. Funding for this research was provided by Hatch projects KY008041 and KY008065 (to MJS). The information reported in this paper (No. 16-08-071) is part of a project of the Kentucky Agricultural Experiment Station and is published with the approval of the Director.

References

Braman SK & Yeargan KV (1991) Reproductive strategies of primary parasitoids of the green cloverworm (Lepidoptera: Noctuidae). Environmental Entomology 20: 349–353.

Breton S, Burger G, Stewart DT & Blier PU (2006) Comparative analysis of gender-associated complete mitochondrial genomes in marine mussels (*Mytilus* spp.). Genetics 172: 1107–1119.

Burns JM, Janzen DH, Hajibabaei M, Hallwachs W & Hebert PDN (2008) DNA and cryptic species of skipper butterflies in the genus *Perichares* in Area de Conservacion Guanacaste, Costa Rica. Proceedings of the National Academy of Sciences of the USA 105: 6350–6355.

Danneels EL, Formesyn EM, Hahn DA, Denlinger DL, Cardoen D et al. (2013) Early changes in the pupal transcriptome of the flesh fly *Sarcophaga crassipalpis* to parasitization by the ectoparasitic wasp, *Nasonia vitripennis*. Insect Biochemistry and Molecular Biology 43: 1189–1200.

Dent D (2000) Insect Pest Management. CAB International, Wallingford, UK.

Derocles SA, Plantegenest M, Simon JC, Taberlet P & Le Ralec A (2012) A universal method for the detection and identification of Aphidiinae parasitoids within their aphid hosts. Molecular Ecology Resources 12: 634–645.

Fernández-Triana JL, Whitfield JB, Rodriguez JJ, Smith MA, Janzen DH et al. (2014) Review of *Apanteles sensu stricto* (Hymenoptera, Braconidae, Microgastrinae) from Area de Conservación Guanacaste, north-western Costa Rica, with keys to all described species from Mesoamerica. ZooKeys 383: 1–565.

Folmer O, Black M, Hoeh WR, Lutz R & Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3: 294–299.

Harper JD, McPherson RM & Shepard M (1983) Geographical and seasonal occurrence of parasites, predators, and entomopathogens. Southern Cooperative Series Bulletin 285: 7–19.

Hrček J & Godfray HCJ (2015) What do molecular methods bring to host-parasitoid food webs? Trends in Parasitology 31: 30–35.

Janzen D & Hallwachs W (2011) Joining inventory by parataxonomists with DNA barcoding of a large complex tropical conserved wildland in north-western Costa Rica. PLoS ONE 6: e18123.

Janzen DH, Hallwachs W, Blandin P, Burns JM, Cadiou J et al. (2009) Integration of DNA barcoding into an ongoing inventory of complex tropical biodiversity. Molecular Ecology Resources 9: 1–26.

Karlin S & Altschul SF (1990) Methods for assessing the statistical significance of molecular sequence features by using general scoring schemes. Proceedings of the National Academy of Sciences of the USA 87: 2264–2268.

Karlin S & Altschul SF (1993) Applications and statistics for multiple high-scoring segments in molecular sequences. Proceedings of the National Academy of Sciences of the USA 90: 5873–5877.

Katoh K, Kuma K, Toh H & Miyata T (2006) MAFFT version 5: improvement inaccuracy of multiple sequence alignment. Nucleic Acids Research 33: 511–518.

King RA, Read DS, Traugott M & Symondson WOC (2008) Molecular analysis of predation: a review of best practice for DNA based approaches. Molecular Ecology 17: 947–963.

LaSalle J & Gauld ID (1993) Hymenoptera and Biodiversity. CAB International, Wallingford, UK.

Martinson EO, Wheeler D, Wright J, Mrinalini, Siebert AL & Werren JH (2014) *Nasonia vitripennis* venom causes targeted gene expression changes in its fly host. Molecular Ecology 23: 5918–5930.

Martinson EO, Martinson VG, Edwards R, Mrinalini & Werren JH (2016) Laterally transferred gene recruited as a venom in parasitoid wasps. Molecular Biology and Evolution 33: 1042–1052.

Mitchell ER, Hu GY & Okine JS (1997) Diamondback moth (Lepidoptera: Plutellidae) infestation and parasitism by *Diadegma insulare* (Hymenoptera: Ichneumonidae) in collards and adjacent cabbage fields. Florida Entomologist 80: 54–62.

Pavuk DM & Williams CE (2003) Simultaneous parasitism of field-collected green cloverworm, *Hypena scabra* (Lepidoptera: Noctuidae) larvae by endoparasitoids and an entomopathogenic fungus. Great Lakes Entomologist 36: 122–128.

Rozen S & Skaletsky H (1998) *Primer3* on the WWW for general users and for biologist programmers. Bioinformatics Methods and Protocols: Methods in Molecular Biology (ed. by S Misener & SA Krawetz), pp. 365–386. Humana Press, Totowa, NJ, USA.

Sheppard SK & Harwood JD (2005) Advances in molecular ecology: tracking trophic links through predator-prey food-webs. Functional Ecology 19: 751–762.

Simon C, Frati F, Beckenbach A, Crespi B, Liu H & Flook P (1994) Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Annals of the Entomological Society of America 87: 651–701.

Smith MA, Woodley NE, Janzen DH, Hallwachs W & Hebert PDN (2006) DNA barcodes reveal cryptic host-specificity within the presumed polyphagous members of a genus of parasitoid flies (Diptera: Tachinidae). Proceedings of the National Academy of Sciences of the USA 103: 3657–3662.

Smith MA, Poyarkov N & Hebert PDN (2008) COI DNA barcoding and amphibians: take the chance, meet the challenge. Molecular Ecology Resources 8: 235–246.

Smith MA, Eveleigh ES, McCann KS, Merilo MT, McCarthy PC & Van Rooyen KI (2011) Barcoding a quantified food web: crypsis, concepts, ecology and hypotheses. PLoS One 6: e14424.

Smith MA, Hallwachs W, Janzen DH & Blanco Segura R (2013) DNA barcoding a collection of ants (Formicidae) from Isla del Coco, Costa Rica. Florida Entomologist 96: 1500–1507.

Stahlhut JK, Fernández-Triana J, Adamowicz SJ, Buck M, Goulet H et al. (2013) DNA barcoding reveals diversity of Hymenoptera and the dominance of parasitoids in a sub-arctic environment. BMC Ecology 13: 2.

Symondson WOC (2002) Molecular identification of prey in predator diets. Molecular Ecology 11: 627–641.

Traugott M & Symondson WOC (2008) Molecular analysis of predation on parasitized hosts. Bulletin of Entomological Research 98: 223–231.

Wirta HK, Hebert PDN, Kaartinen R, Prosser SW, Várkonyi G & Roslin T (2014) Complementary molecular information changes our perception of food web structure. Proceedings of the National Academy of Sciences of the USA 111: 1885–1890.

Yeargan KV & Braman SK (1986) Life history of the parasite *Diolcogaster facetosa* (Weed) (Hymenoptera: Braconidae) and its behavioral adaptation to the defensive response of a lepidopteran host. Annals of the Entomological Society of America 79: 1029–1033.

Yeargan KV & Braman SK (1989) Life history of the hyperparasitoid *Mesochorus discitergus* (Hymenoptera: Ichneumonidae) and tactics used to overcome the defensive behavior of the green cloverworm (Lepidoptera: Noctuidae). Annals of the Entomological Society of America 82: 393–398.

Zhu Y-C, Dowdy AK & Baker JE (2004) Molecular differentiation of two strains of the parasitoid *Anisopteromalus calandrae* (Hymenoptera: Pteromalidae) using specific PCR primers. Journal of Entomological Science 39: 1–10.

Supporting Information

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

Table S1. Specimens used for testing the parasitoid 28S primers and their PCR test results (positive and negative results indicated + and − signs, respectively). Adult Lepidoptera specimens were identified using the identification engine on BOLD using their COI sequences (last column; GenBank numbers KY020352-KY020399). Please note that specimens HD-493, HD-523, and HD-498 tested positive for hymenopteran DNA, indicating in two cases parasitization by the parasitoid listed in the species column. In the third case, the DNA amplified is from a chrysomelid beetle.

Table S2. Cloverworm extractions yielding parasitoid DNA sequences (GenBank accession numbers KY034141-KY034257).