



Limited host range in the idiobiont parasitoid *Phymastichus coffea*, a prospective biological control agent of the coffee pest *Hypothenemus hampei* in Hawaii

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Abstract

Phymastichus coffea LaSalle (Hymenoptera:Eulophidae) is an adult endoparasitoid of the coffee berry borer, *Hypothenemus hampei* (Ferrari) (Coleoptera:Curculionidae:Scolytinae), which has been introduced in many coffee producing countries as a biological control agent. To determine the effectiveness of *P. coffea* against *H. hampei* and environmental safety for release in Hawaii, we investigated the host selection and parasitism response of adult females to 43 different species of Coleoptera, including 23 Scolytinae (six *Hypothenemus* species and 17 others), and four additional Curculionidae. Non-target testing included Hawaiian endemic, exotic and beneficial coleopteran species. Using a no-choice laboratory bioassay, we demonstrated that *P. coffea* was only able to parasitize the target host *H. hampei* and four other adventive species of *Hypothenemus*: *H. obscurus*, *H. seriatus*, *H. birmanus* and *H. crudiae*. *Hypothenemus hampei* had the highest parasitism rate and shortest parasitoid development time of the five parasitized *Hypothenemus* spp. Parasitism and parasitoid emergence decreased with decreasing phylogenetic relatedness of the *Hypothenemus* spp. to *H. hampei*, and the most distantly related species, *H. eruditus*, was not parasitized. These results suggest that the risk of harmful non-target impacts is low because there are no native species of *Hypothenemus* in Hawaii, and *P. coffea* could be safely introduced for classical biological control of *H. hampei* in Hawaii.

Keywords Coffee berry borer · Host specificity testing · Non-target · Biocontrol · Endoparasitoid · Scolytinae

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Key message

- *Phymastichus coffea* is an idiobiont adult parasitoid of the coffee pest *Hypothenemus hampei*.
- In host range testing, *P. coffea* parasitized only five *Hypothenemus* spp.
- The parasitism rate was highest and parasitoid development time was shortest in *H. hampei*.
- No Hawaiian native species was parasitized by the parasitoid.
- *Phymastichus coffea* can be introduced safely for biocontrol of coffee berry borer in Hawaii.

Introduction

The coffee berry borer, *Hypothenemus hampei* (Ferrari) (Coleoptera:Curculionidae:Scolytinae), native to Central Africa, is the most damaging insect pest of coffee worldwide, inflicting economical losses of over US \$500 million annually (Vega et al. 2015). In Hawaii, *H. hampei* was first recorded in Kona, Hawaii island, in 2010 (Burbano et al. 2011) and is now widespread throughout all the coffee-growing areas of Hawaii. Coffee is the third largest cash crop in the state of Hawaii, valued at more than \$43 million (USDA-NASS 2018). *Hypothenemus hampei* has had the effect of making coffee farming more intensive and less profitable, which is a major economic challenge to small-scale coffee production like that in Hawaii (Johnson et al. 2020). If left unmanaged, *H. hampei* can damage > 90% of the crop.

Hypothenemus hampei attacks coffee berries when the dry matter content of the endosperm, which increases with age, exceeds 20% (Jaramillo et al. 2005). After finding a suitable berry host, *H. hampei* bores into the coffee fruit through the central disk and excavates galleries where it lays eggs. The offspring develop inside the seeds and feed on the endosperm tissue of the berries (Damon 2000), reducing both coffee yield and quality. *Hypothenemus hampei* feeding damage can also cause premature fall of berries younger than 80 days (Decazy 1990). *Hypothenemus hampei* adults boring into the berry may remain in the 'A' position (Jaramillo et al. 2006) with the abdomen half exposed outside the berry potentially for weeks waiting for the dry matter content to reach 20% (Jaramillo et al. 2005).

Strategies to control *H. hampei* include mechanical, chemical and biological controls (Infante 2018). Sanitation and biological control (using parasitoids, predators and entomopathogenic microorganisms) are the most sustainable, environmentally friendly and widely used non-chemical control methods. The parasitoids, *Cephalonomia stephanoderis* Betrem, *C. hyalinipennis* Ashmead and *Prorops nasuta* Waterston (Hymenoptera:Bethylidae), *Heterospilus coffeicola* Schneideknecht (Hymenoptera: Braconidae) and *Phymastichus coffea* LaSalle (Hymenoptera: Eulophidae), all of African origin, have been introduced in many coffee producing countries, particularly in Central and South America (Klein-Koch et al. 1988; Barrera et al. 1990; Baker 1999; Jaramillo et al. 2005; Portilla and Grodowitz 2018), but none have been released in Hawaii. In Hawaii, the primary methods for controlling *H. hampei* are sanitation (frequent harvests and removal of all left over coffee berries after harvest) and applications of the biopesticide *Beauveria bassiana* (Ascomycota:Hypocreales), an entomopathogenic fungus (Aristizábal et al. 2016). Two generalist predators, *Leptophloeus* sp. and *Cathartus*

quadricollis (Coleoptera:Laemophloeidae and Silvanidae, respectively), occur naturally in Hawaii coffee and have been shown to feed on immature stages of *H. hampei* in overripe and dried berries (Follett et al. 2016; Brill et al. 2020), but are not very efficient in preventing damage in the first place.

Most of the studies on biological control of *H. hampei* have been conducted outside Hawaii, but in similar coffee production systems. In field-cage studies conducted in Mexico and Costa Rica, *P. coffea* proved to be the most promising biological control agent against *H. hampei* with parasitism rates as high as 95% (Espinoza et al. 2009; Infante et al. 2013). To date, *P. coffea* has been released in 12 countries as a classical biological control agent (Bustillo et al. 1998; Damon 2000; Jaramillo et al. 2005; Vega et al. 2015). *Phymastichus coffea* is native to Africa and present in most coffee producing countries on that continent. It is a primary, gregarious, idiobiont endoparasitoid of adult *H. hampei* females with a high capacity for host discrimination (Feldhege 1992; Infante et al. 1994; López-Vaamonde and Moore 1998; Castillo et al. 2004). Two laboratory studies reported that in addition to *H. hampei*, *P. coffea* parasitizes other *Hypothenemus* spp. such as *H. seriatus* and *H. obscurus* (López-Vaamonde and Moore 1998), and *H. eruditus* Westwood and *H. crudiae* (Panzer) (Castillo et al. 2004). However, parasitism of closely related species in the field has not been reported (Escobar-Ramírez et al. 2019). Gravid *P. coffea* females start to search for their hosts immediately after emerging from the adult female host and parasitism occurs within the first hours after emergence (Infante et al. 1994). *Phymastichus coffea* has an extremely short life span as an adult; the longevity of males ranges from 8 to 48 h and females from 16 to 72 h (Vergara et al. 2001; Portilla and Grodowitz 2018). *Phymastichus coffea* generally lays two eggs (into the abdomen, thorax, or between the thorax and abdomen) in an *H. hampei* adult female at the time she is initiating fruit perforation, which causes paralysis and prevents further damage to the coffee berry. The parasitized *H. hampei* usually dies within 4–12 days after parasitism (Infante et al. 1994). The life cycle (egg to adult) of *P. coffea* varies from 30 to 47 days depending on the environmental conditions (temperature and humidity). Females are ~ 1 mm long, whereas males are half that size (LaSalle 1990).

Earlier studies have shown the high host specificity of *P. coffea* and its ability to significantly reduce and regulate *H. hampei* populations (Gutierrez et al. 1998; López-Vaamonde and Moore 1998; Castillo et al. 2004; Rodríguez et al. 2017). Therefore, we decided to consider *P. coffea* as a biological control agent of *H. hampei* in Hawaii. A critical step was to determine its host specificity and assess possible risks to the Hawaii environment though impacts on endemic and other non-target species (Follett and Duan 1999; Messing and Wright 2006). Greatest non-target species impacts from

introduced biological control agents are likely to occur on species closely related to the target pest species (Van Drieseche and Murray 2004), but not always (Messing 2001), and thus, phylogenetically closely and distantly related species should be included in non-target screening efforts. This is an important element of biological control, particularly in Hawaii, where classical biological control may have had significant negative impacts on native species in the past (e.g., Howarth 1991; Henneman and Memmott 2001). While some studies have suggested that this is true (see references in Messing and Wright 2006), a number of carefully crafted field studies of population level impacts on non-target species have suggested that introduced parasitoids have had minimal, or sometimes moderate, impacts on endemic species (Johnson et al. 2005; Kaufman and Wright 2009). Where higher impacts have been detected, they are typically from accidentally introduced parasitoid species, and host insects in disturbed habitats are most susceptible to these impacts (Kaufman and Wright 2011). However, the potential for non-target impacts must be carefully considered, and outcomes of exposures of unintended hosts to prospective biological control agents can provide insights into host range patterns and determinants.

In this paper, we present new insights into the host specificity of *P. coffea*, a prospective biological control agent of *H. hampei* in Hawaii, by testing it against 43 different species of Coleoptera. Non-target testing included Hawaiian endemic, exotic and beneficial coleopteran species. There are currently no records of native Hawaiian *Hypothenemus* spp. except for an old record (1913) of *H. ruficeps* (Swezey 1954), which has never been collected or reported since and is possibly a synonym with the adventive species *H. eruditus* or *H. crudiae* (C. Gillett, unpublished). There are, however, many native species in another scolytine genus, *Xyleborus* (Samuelson 1981; Gillett et al. 2019), which may potentially be impacted by release of an exotic parasitoid against a scolytine pest such as *H. hampei*. We test the hypothesis that *P. coffea* is host specific and will not attack native Hawaiian Scolytinae species.

Materials and methods

Parasitoid, *Phymastichus coffea*

Phymastichus coffea used in this study were obtained from an established stock maintained at the National Coffee Research Center-Cenicafé, Manizales (Caldas) Colombia, which was started from *P. coffea* collected in Kenya and shipped to Colombia in 1996 and has been maintained in colony in large numbers since that time (Orozco-Hoyas and Aristizábal 1996). *Phymastichus coffea* has been mass reared by Cenicafé for field releases on multiple occasions

and the colony receives frequent infusions of field-collected material. *Phymastichus coffea* was shipped from Cenicafé in its larval stage in parasitized *H. hampei* hosts under USDA APHIS PPQ, permit no. P526P-18-00,696 to a certified quarantine insect containment facility managed by the USDA Forest Service at Hawaii Volcanoes National Park, Volcano, Hawaii. Parasitized *H. hampei* were incubated in controlled climate chambers at $25^{\circ} \pm 1^{\circ} \text{C}$, $75 \pm 10\%$ relative humidity and 8:16 h light:dark photoperiod at the quarantine containment facility.

Emerged male and female parasitoid adults were collected using a manual aspirator into a clean glass container. Parasitoids were held for mating and oocyte maturation and provided with 50% (w/v) honey (raw organic) solution for ~2 h before being used in the experiments (López-Vaamonde and Moore 1998). Infante et al. (1994) reported that *P. coffea* does not go through a preoviposition period and exhibits facultative arrhenotokous-type parthenogenesis, where the female parasitizes its host before or after copulation, producing haploid males (Portilla and Grodowitz 2018). Feldhege (1992) reported a preoviposition period of between 5 min and 4 h. The adult parasitoids are very short-lived: males (~8–48 h) and females (~16–72 h) (Vergara et al. 2001; Rojas et al. 2006; Espinoza et al. 2009; Portilla and Grodowitz 2018). The ability to parasitize hosts decreases with age, so it was important to use freshly emerged parasitoids (< 12 h old) in all experiments.

Coffee berry borer, *Hypothenemus hampei*

Field-collected *H. hampei* were used in all no-choice host specificity experiments. *Hypothenemus hampei*-infested coffee berries were collected from coffee trees (*Coffea arabica*) at OK Coffee Farm in Hilo, Hawaii (19.727583, -155.111186, elevation 156 m). These collections were transported in cold boxes to the USDA-ARS laboratory and placed in a custom-made extraction unit lined with tissue paper (Tech wipes 1709/7052, Horizon) to absorb condensation and prevent mold growth. Adult *H. hampei* were collected directly from the infested coffee berries by dissecting the berries or from the extraction unit using an aspirator. All the collected *H. hampei* were provided with artificial diet (modified from Brun et al. 1993) until use in the experiments.

Collection of non-target coleopteran species

The selection of non-target hosts was based on phylogenetic relatedness to the target host, sympatry of target and non-target species, and size. Species commonly occurring in the coffee landscape and species in culture at USDA-ARS in Hilo, Hawaii, were also tested. There are 21 native and 38 non-native scolytine species in Hawaii (Samuelson 1981;

Nishida 2002; Cognato and Rubinoff 2008). Because of the relatively large native scolytine fauna in Hawaii, and their remote or poorly studied habitats, only a subset of these species could be tested for their suitability as hosts to *P. coffea*. Exotic and native scolytine species were collected from coffee and macadamia farms and their surrounding habitats, and from native forests from different islands (Hawaii Island, Oahu, Maui, Molokai and Kauai) in Hawaii (Gillett et al. 2020a). Host specificity tests were conducted with a total of 43 species from seven different coleopteran families including Hawaiian endemic species (several Scolytinae in the genus *Xyleborus* and *Nesotocus giffardi*, a curculionid weevil), exotic pest species (e.g., the scolytines *Hypothenemus obscurus* [tropical nut borer] and *Xylosandrus compactus* [black twig borer], and the curculionids *Sitophilus oryzae* [rice weevil] and *Cylas formicarius* [sweetpotato weevil]), and beneficial species (e.g., a weed biocontrol

agent *Uroplata girardi* from lantana, several coccinellids, and two flat bark beetle predators of *H. hampei*, *Catharus quadricollis* and *Leptophloeus* sp.) (Tables 1, 2, 3, 4). All beetles used in host specificity tests were collected live and later preserved in 75% alcohol or pinned for identification by taxonomists with expertise in the respective taxa. The body size of the collected species ranged from 1 to 7 mm, but the majority of species were similar in size to *H. hampei* which is 1.5–2.0 mm in length. Beetles were collected using Lindgren funnels or bucket or Broca traps baited with denatured ethanol only or ethanol + methanol + ethylene glycol lures or collected directly from infested plant material (fruits, pods, stems, bark and seeds) or reared from infested wood in the laboratory (Gillett et al. 2020b). All non-target testing was conducted at the USDA Forest Service quarantine containment facility at Hawaii Volcanoes National Park, Volcano, Hawaii.

Table 1 Development time and sex ratio of *Phymastichus coffea* in no-choice in vitro non-target host selection screening of *Hypothenemus* species, including *H. hampei* as a control species

Species	Insect status	Total beetles exposed	Development time (days ± SE)	Sex ratio (mean % females ± SE)
<i>Hypothenemus hampei</i> (control)	Exotic/pest	170	32.2 ± 0.5	50.8 ± 0.4
<i>Hypothenemus obscurus</i>	Exotic/pest	80	35.0 ± 0.9	54.8 ± 1.6*
<i>Hypothenemus seriatus</i>	Exotic	60	38.0 ± 1.0	51.1 ± 1.1
<i>Hypothenemus birmanus</i>	Exotic	40	37.0 ± 1.0	57.7 ± 3.8*
<i>Hypothenemus crudiae</i>	Exotic	30	41.0 ± 0.0*	50.0
<i>Hypothenemus eruditus</i>	Exotic	80	–	–

*significantly different from *Hypothenemus hampei* (control), $p < 0.05$

Table 2 Parasitism and parasitoid emergence rates in no-choice in vitro non-target host acceptance screening of *Phymastichus coffea* exposed to various Scolytinae (Hawaii native and non-native) species

Family	Species	Insect status	Total beetles exposed	Parasitism (%) (Mean ± SE)	Parasitoid emergence (%) (Mean ± SE)
Curculionidae:Scolytinae	<i>Xylosandrus compactus</i>	Exotic/pest	80	0	0
	<i>Xylosandrus crassiusculus</i>	Exotic	80	0	0
	<i>Xyleborinus saxeseni</i>	Exotic	80	0	0
	<i>Xyleborinus andrewesi</i>	Exotic	60	0	0
	<i>Xyleborus ferrugineus</i>	Exotic	60	0	0
	<i>Euwallacea fornicatus</i>	Exotic	60	0	0
	<i>Euwallacea interjectus</i>	Exotic	60	0	0
	<i>Hypochryphalus</i> sp.	Exotic	60	0	0
	<i>Chryphalus</i> sp.	Exotic	80	0	0
	<i>Ptilopodius pacificus</i>	Exotic	80	0	0
	<i>Xyleborus molokaiensis</i>	Native	30	0	0
	<i>Xyleborus mauiensis</i>	Native	15	0	0
	<i>Xyleborus simillimus</i>	Native	18	0	0
	<i>Xyleborus hawaiiensis</i>	Native	9	0	0
	<i>Xyleborus lanaiensis</i>	Native	19	0	0
	<i>Xyleborus obliquus</i>	Native	3	0	0
	<i>Xyleborus kauaiensis</i>	Native	35	0	0

Table 3 Parasitism and parasitoid emergence rates in no-choice in vitro non-target host acceptance screening of *Phymastichus coffea* on beneficial Coleoptera species

Family	Species	Insect status	Total beetles exposed	Parasitism (%)	Parasitoid emergence (%)
Chrysomelidae:Cassidinae	<i>Uroplata girardi</i>	Exotic	60	0	0
Coccinellidae	<i>Scymnodes lividigaster</i>	Exotic	40	0	0
Coccinellidae	<i>Rhyzobius forestieri</i>	Exotic	60	0	0
Coccinellidae	<i>Halmus chalybeus</i>	Exotic	40	0	0
Laemophloeidae	<i>Leptophloeus</i> sp.	Unknown	60	0	0
Silvanidae	<i>Cathartus quadricollis</i>	Exotic	80	0	0

Table 4 Parasitism and parasitoid emergence rates in no-choice in vitro non-target host acceptance screening of *Phymastichus coffea* on Hawaiian native and introduced coleopteran species from families and subfamilies other than Curculionidae:Scolytinae

Family	Species	Insect status	Total beetles exposed	Parasitism (%)	Parasitoid emergence (%)
Anthribidae	<i>Araecerus simulatus</i> or <i>A. levipennis</i>	Unknown	6	0	0
Anthribidae	<i>Araecerus</i> sp. near <i>varians</i>	Unknown	15	0	0
Brentidae:Brentinae	<i>Cylas formicarius</i>	Exotic/Pest	80	0	0
Chrysomelidae:Bruchinae	<i>Acanthoscelides macropthalmus</i>	Unknown	10	0	0
Curculionidae:Cossoninae	<i>Phloeophagosoma tenuis</i>	Unknown	8	0	0
Curculionidae:Cossoninae	<i>Nesotocus giffardi</i>	Native	12	0	0
Curculionidae:Curculioninae	<i>Sigastus</i> sp.	Exotic/Pest	6	0	0
Curculionidae:Platypodinae	<i>Crossotarsus externedentatus</i>	Exotic	60	0	0
Dryophthoridae:Dryophthorinae	<i>Sitophilus oryzae</i>	Exotic/Pest	60	0	0
Dryophthoridae:Dryophthorinae	<i>Sitophilus linearis</i>	Exotic	40	0	0
Nitidulidae:Carpophilinae	<i>Carpophilus dimidiatus</i>	Exotic	10	0	0
Nitidulidae:Carpophilinae	<i>Carpophilus zeaphilus</i>	Exotic	60	0	0
Tenebrionidae	<i>Tribolium castaneum</i>	Exotic/Pest	21	0	0
Tenebrionidae	<i>Hypophloeus maehleri</i>	Exotic	60	0	0

No-choice tests

In this study, we used no-choice tests because these would reflect physiological host range and the potential for parasitism in the field more accurately than choice tests (Van Driesche and Murray 2004). Choice tests that include the target host may mask the acceptability of lower ranked hosts, thereby producing false negative results (Withers and Mansfield 2005). Twenty individuals of each test species were placed in a sterilized glass Petri dish (80 mm in diameter) lined with filter paper and immediately afterward four *P. coffea* females (< 12 h old) that had not been exposed to adult hosts prior to the experiments were introduced. Therefore, when ample hosts were available, each replicate consisted of 20 hosts and four parasitoids for a 5:1 host–parasitoid ratio. However, due to difficulties in finding certain species live in adequate numbers, e.g., native scolytine bark beetles, and difficulties synchronizing parasitoid emergence with field collection or emergence from wood of live beetles, the host–parasitoid ratio and numbers of

replicates were adjusted as needed. For example, if only 10 non-target beetles were available for screening, then two replicates each with 5 beetles and 1 parasitoid (maintaining the 5:1 host–parasitoid ratio) were performed. In all non-target host screening tests, *H. hampei* was included as a positive control to confirm parasitoid viability. The host–parasitoid ratio of the *H. hampei* controls was adjusted to match the non-target species in the test, whether it was 5:1 or otherwise. The generalized response of the parasitoids toward target and non-target hosts was also determined for a subset of parasitoids by visual observation and video recording of parasitoid behavior, e.g., any contact with the host by landing on the host or antennation, and/or walking on the host. Host acceptance was noted when the parasitoid adopted a characteristic oviposition position on top the elytra of the host (Lopez-Vaamonde and Moore 1998).

After *P. coffea* exposure, *H. hampei* and all other non-target species were incubated at 25 ± 1 °C, $75 \pm 10\%$ RH and 24:0 (L–D) photoperiod for 72 h. After 72 h, parasitoids and filter paper linings were removed and the beetles were

provided with a small cube (2×2×2 cm) of general beetle diet (FY, unpublished). The beetles were again incubated at the same environmental conditions, but now at 0:24 (L–D). After 10 days, all the remaining diet and frass was removed (without disturbing the parasitized beetles) to avoid fungal contamination. Parasitized beetles typically become paralyzed and eventually die within 4–12 days after parasitoid oviposition. Beetles were held for a total of ~5–6 weeks for parasitoid emergence. Beginning after 25-day incubation, *H. hampei* mummies were inspected daily for adult wasp emergence. Parasitism was assessed based on observation of emergence of parasitoid progeny (F1 adult wasps) from the parasitized beetle, by inspection for exit holes on cadavers or by dissection. Beetles with no exit holes were dissected (by separating the thorax from the abdomen) under a stereomicroscope using fine forceps and entomological pins at 20–100X magnification for evidence of parasitism, i.e., presence of *P. coffea* immature life stages (eggs, larvae or pupae), or unemerged adults. The number of unemerged life stages was recorded for each dissected beetle. After 5–6 weeks of incubation, dead beetle specimens sometimes became very dry and searching for the presence of eggs and early instar larvae was difficult. In such cases, beetles were dissected and examined under a compound microscope at 200X to seek unemerged *P. coffea*. The sex of emerged adult *P. coffea* offspring was determined by examination using a stereomicroscope. In most cases, two parasitoids (one male and one female) emerged per beetle host. To confirm this, the sum of the emerged male and female parasitoids in each replicate was divided by two and compared to the number of parasitized hosts with exit holes. The sex of unemerged parasitoids was not determined. For data on parasitism, life stages, sex ratio and development time, averages were calculated for each replicate (per Petri dish) for each species and used in statistical analysis. Grand means of all the replicates for each of the five *Hypothenemus* species are presented in figures and tables.

Statistical analysis

Parasitism rate was calculated by dividing the number of parasitized hosts by the total number of hosts exposed to the parasitoids. Parasitism included both emerged and unemerged wasps. Emergence rate was calculated by dividing the number of beetles with exit holes by the total number of parasitized hosts (emerged plus unemerged wasps). The sex ratio of the parasitoid progeny was calculated by dividing the number of emerged female parasitoids (F) by the total number of emerged male (M) and female (F) parasitoids $[F / (F + M) \times 100]$. The Shapiro–Wilk test (Shapiro and Wilk 1965; Razali and Wah 2011), numerical approaches (skewness and kurtosis indices) and the normal Q–Q plot-based graphical method were used to check the distribution of the

data and showed that the data were not normally distributed. Generalized linear models (GLM) were therefore used to analyze the data, with appropriate distribution function links. Parasitism and emergence rates of the parasitoids, and the percentage of different life stages (larvae, pupae and adults) in parasitized beetles with unemerged parasitoids were analyzed using GLM with a binary logistic function and sex ratio with a gamma log link function. Developmental time of the F1 offspring (egg to adult) was analyzed using GLM with a negative binomial log link function because data were overdispersed (i.e., variance > mean). Wald Chi-squared approximations are reported. All analyses were performed using IBM SPSS statistics software.

Results

Out of 43 total coleopteran species tested, including 23 scolytines, *P. coffea* oviposited and completed developed only in the target *Hypothenemus hampei* and four other species of *Hypothenemus*: *H. obscurus*, *H. seriatus*, *H. birmanus* and *H. crudiae*. Mean percentages of parasitism and emergence for the *Hypothenemus* spp. tested are shown in Fig. 1. Parasitism ($\chi^2 = 65.13$, $df = 4$, $p = 0.0001$) and emergence ($\chi^2 = 23.20$, $df = 4$, $p = 0.0001$) were significantly higher in *H. hampei* than all other *Hypothenemus*

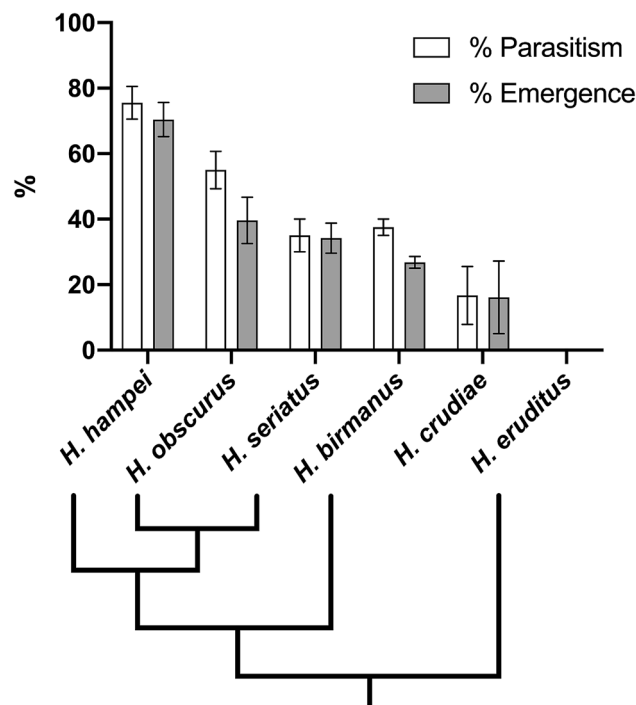


Fig. 1 Percentage parasitism and emergence (mean ± SE) of adult *Phymastichus coffea* parasitoids from *Hypothenemus* spp. The phylogeny below the graph for the species included in the study (except *H. crudiae*) was inferred from Johnson et al. (2018)

species. *Hypothenemus hampei* had the highest percentage emergence of *P. coffea* at 70.4%, whereas *H. crudiae* had the lowest at 16.7% (Fig. 1). In *H. crudiae*, out of five parasitized hosts only one had emergence. Although *P. coffea* only parasitized *Hypothenemus* spp., it did inspect three other non-target scolytine hosts, *Hypothenemus eruditus*, *Xyleborus kauaiensis* and *Xyleborus ferrugineus*, but left hosts without initiating oviposition (i.e., no parasitism found). The phylogenetic relationship of five *Hypothenemus* species included in our tests, extracted from Johnson et al. (2018), is also shown in Fig. 1; *H. crudiae* is not included in the phylogeny because it was not included in Johnson et al. (2018). Both parasitism and emergence in our tests decreased across *Hypothenemus* species with decreasing phylogenetic relatedness to *H. hampei*. *Hypothenemus eruditus*, the most distantly related species from *H. hampei* according to Johnson et al. (2018), was not parasitized (Fig. 1).

Parasitoid development time among the three different *Hypothenemus* spp. did not differ significantly compared with *H. hampei* ($\chi^2 = 0.17$, $df = 4$, $p = 0.997$), but did differ with *H. crudiae* (Table 1). The mean development time of *P. coffea* from oviposition to adult emergence was shortest in *H. hampei* (32.2 ± 0.5 days, mean \pm SE), longest in *H. crudiae* (41.0 ± 0.0 days) and intermediate in the other three *Hypothenemus* spp. (Table 1), which generally agrees with the phylogenetic pattern observed for parasitism and emergence (Fig. 1). The percentage of female versus male *P. coffea* emerging from parasitized *H. hampei* was $50.8\% \pm 0.4$ (mean \pm SE), which was significantly different ($\chi^2 = 27.3$, $df = 4$, $p = 0.0001$) from *H. seriatus* and *H. birmanus* (Table 1). *Hypothenemus eruditus* was not parasitized by *P. coffea* and hence was not included in any statistical analyses.

Parasitized *H. hampei* had the lowest percentage of unemerged parasitoids compared to the other four *Hypothenemus* species (Fig. 1), indicating that *H. hampei* is a superior host for *P. coffea* development. For each parasitized host beetle with unemerged parasitoids, invariably two parasitoids were present, and the parasitoids were of the same life stage (larva, pupa or adult). The frequency of the different life stages for parasitized hosts with unemerged parasitoids differed among *Hypothenemus* species (Fig. 2). Parasitized *H. hampei* had a significantly lower percentage of larval ($\chi^2 = 15.10$, $df = 3$, $p = 0.001$), and higher percentage of adult parasitoids that were unemerged ($\chi^2 = 18.36$, $df = 3$, $p = 0.0001$) compared to the other *Hypothenemus* species. The higher percentage of unemerged parasitoids developing to the adult stage again indicates that *H. hampei* is a superior developmental host than the other *Hypothenemus* spp. The percentage of unemerged pupae found in parasitized *H. hampei* was not significantly different from *H. obscurus*, *H. seriatus* and *H. birmanus*, but *H. crudiae* had a significantly higher percentage of pupae than *H. hampei*

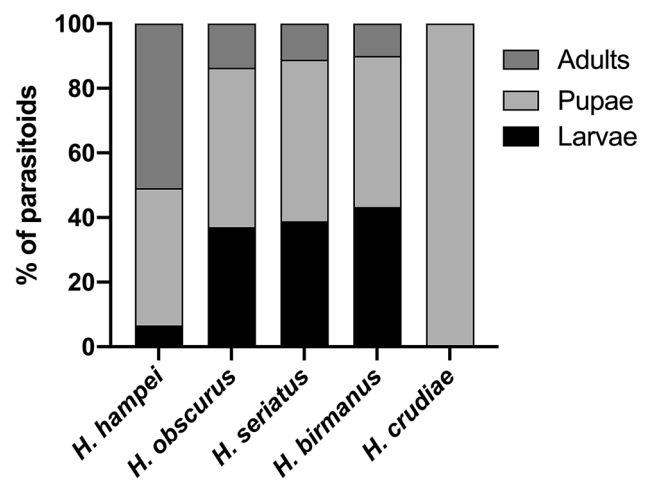


Fig. 2 Fate of unemerged *Phymastichus coffea* parasitoids from parasitized *Hypothenemus* spp. in no-choice in vitro non-target host selection screening. Parasitized *Hypothenemus* beetles with unemerged parasitoids were dissected to identify life stages (larva, pupa, adult)

($\chi^2 = 95.40$, $df = 4$, $p = 0.0001$) (Fig. 2). No eggs were found in any of the parasitized *Hypothenemus* hosts.

Discussion

Phymastichus coffea is a potential biological control agent of *H. hampei* and was brought from Columbia into a quarantine containment facility in Hawaii for host range testing to determine whether the parasitoid might attack non-target species and therefore pose a risk to Hawaiian endemic species. Using no-choice tests, 43 different species of Coleoptera were exposed to *P. coffea* in vitro, including 23 scolytines (six natives, 17 non-native species including *H. hampei*), six beneficial species and 12 other species including one native weevil (*N. giffardi*). Only five species from the genus *Hypothenemus* were parasitized by *P. coffea*, including the two pest species *H. hampei* (coffee berry borer) and *H. obscurus* (tropical nut borer, a macadamia nut pest), and three other exotic species *H. seriatus*, *H. birmanus* and *H. crudiae* (Fig. 1). Thus, *P. coffea* appears to be host specific at the genus level and should pose no harm to endemic species if released in Hawaii coffee for classical biological control of *H. hampei*. Nevertheless, no level of host specificity testing can ensure zero risk to non-target organisms when introducing a natural enemy in a new habitat (Louda et al. 2003).

We observed that once the host and parasitoids were exposed in the Petri dish arena that *P. coffea* inspected *H. hampei* and other *Hypothenemus* spp. hosts by antennation before proceeding to oviposition or rejection. *Phymastichus coffea* did not show any oviposition response to other non-target hosts. This could be dependent on several factors

because parasitoids may search and decide host suitability by using a broad spectrum of different stimuli such as plant–host complex volatiles, host feces volatiles, host sex pheromones, and tactile and visual cues (Chiu-Alvarado and Rojas 2008; Yang et al. 2008). Host habitat and host diet may influence the volatile composition emitted by the potential host insect, which can either deter or attract parasitoids from a distance. To minimize the effect of diet, we provided a general beetle diet to all the field-collected coleopteran hosts during the experiments. Parasitism of non-target hosts in the field may not be the same as our *in vitro* test results because of various factors related to the host's natural habitat. Most of the coleopteran species tested in our study are normally found tunneling in seeds, decomposing wood (under the bark and/or in sapwood) or decaying fruits. This cryptic behavior would likely provide protection from *P. coffea* which is accustomed to searching for *H. hampei* adult females, while they are exposed on the surface of coffee berries.

Phymastichus coffea was attracted to and parasitized only four species of *Hypothenemus* in addition to its target host *H. hampei*. This is consistent with studies reported by López-Vaamonde and Moore (1998), and Castillo et al. (2004). Combining information from our study and previous studies, seven species of beetles are now known to be able to serve as hosts in captive exposure studies for *P. coffea*: *H. hampei*, *H. obscurus*, *H. seriatus*, *Araptus* sp. (Lopez-Vaamonde and Moore 1998), *H. crudiae* and *H. eruditus* (Castillo et al. 2004), in addition to *H. birmanus* (this study). Parasitism of the scolytine *Araptus* sp. seems to be an outlier, but this genus does not occur in Hawaii. Aside from *Araptus*, *P. coffea* appears to be genus specific attacking closely related, but not all *Hypothenemus* species, given that species from closely related genera were not parasitized under no-choice test conditions. In our study, *P. coffea* did not attack *H. eruditus*. We believe that *H. eruditus* may not be a suitable host for the parasitoid because of its small size (≤ 1 mm); *Phymastichus coffea* usually lays two eggs per host (1 male and 1 female), and in such a small host, successful development would be unlikely due to the limited availability of resources within the host. Host size is an important variable on which the survival and growth of parasitoid progeny depends. Females of most parasitoids preferentially lay eggs on larger hosts (Fox and Mousseau 1995). Also, *H. eruditus* is phylogenetically distant from *H. hampei* (Fig. 1) which is addressed below.

Our results also showed that *H. hampei* had the lowest numbers of unemerged parasitoids when compared with the other four *Hypothenemus* species (Fig. 2). The number of larvae and pupae were lower, and adults were higher in parasitized *H. hampei* with unemerged parasitoids. Similarly, in other three *Hypothenemus* spp. (*H. obscurus*, *H. seriatus* and *H. birmanus*) many unemerged parasitoids could not complete their development and died in their larval or pupal

stage with only a few reaching to the adult stage. In parasitized *H. crudiae* with unemerged parasitoids, most apparently could not reach the adult stage. Although the rate of completing the life cycle differed among *Hypothenemus* species, eggs did hatch in all parasitized species. Many factors can be responsible for suitability of the host for parasitoid development (Pennacchio and Strand 2006). Factors such as host physiology (e.g., presence of endosymbiotic bacteria), behavior (e.g., feeding habitat-sequestering secondary metabolites) and ecology (e.g., spatial/temporal overlap) may influence host acceptance by parasitoids and successful development (Desneux et al. 2009). All the non-target species used in the experiments were freshly collected from the field and may have carried toxins (accumulated from plant feeding) that may have interfered with the successful development of immature parasitoids within the hosts due to the ingestion of unsuitable food (e.g., see Desneux et al. 2009).

Phymastichus coffea also did not successfully parasitize any of the non-*Hypothenemus* species tested, including both native (*Xyleborus*) and exotic (*Xyleborinus*, *Xylosandrus*, *Xyloborus*, *Euwallacea*, others) Scolytinae, and other curculionid species from subfamilies other than Scolytinae, including the native weevil, *N. giffardi*. We did not find any *P. coffea* life stages (eggs, larvae, pupae, adults) after dissection in any of the non-*Hypothenemus* non-target species tested (Tables 2, 3, 4). Host specialization is relatively common in parasitic Hymenoptera and can be related to phylogeny, ecology and life histories (Price 1980; Stireman et al. 2006). It appears that at least host phylogeny was an important factor in host selection for *P. coffea* under our laboratory conditions.

Host range of idiobiont parasitoids is typically broader than koinobiont species (Askew and Shaw 1986; Hawkins et al. 1992), and it would hypothetically be reasonable to expect that *P. coffea* would follow this pattern. However, our results show that *P. coffea* was unable to successfully parasitize any species outside of the genus *Hypothenemus* and, even within the genus, was only moderately successful on species even closely related to *H. hampei*. While parasitism of *H. hampei* and subsequent parasitoid emergence was relatively high, both were significantly lower in *H. obscurus* and *H. seriatus*, sister species to *H. hampei*; *H. eruditus*, in a sister clade to the other species (Johnson et al. 2018), had zero parasitism. This demonstrates decreasing susceptibility to *P. coffea* with increasing phylogenetic distance among the *Hypothenemus* spp. exposed to the parasitoids in this study. Among the *Hypothenemus* spp. included in the phylogenetic reconstruction published by Johnson et al. (2018), *H. hampei* is the only species that has undergone a reversal in host range breadth, to become monophagous on coffee, while the other *Hypothenemus* spp. have retained a host generalist biology. *Hypothenemus hampei* has developed a unique association with *Pseudomonas* bacterial endosymbionts to

facilitate detoxification of caffeine, permitting it to exploit *Coffea arabica* seeds as their host (Ceja-Navarro et al. 2015), and potentially other physiological adaptations to its unique host, possibly providing adaptive challenges to parasitoids, and mediating host specificity of *P. coffea*. Messing (2001) questioned the practicality of applying centrifugal phylogeny approaches to selecting species to examine in non-target studies of potential biological control agents, particularly parasitoids. Our results support the predictions of the latter approach, with more distantly related *Hypothenemus* species less susceptible to *P. coffea* attack and more distantly related genera (e.g., *Xyleborus* spp.) not attacked at all. However, Messing (2001) emphasized the fact that interactions between the host insect and its host plant may override host phylogenetic patterns, by providing the stimuli for parasitoids to attack hosts, a consideration which may play a role in this study system. If this is the case, it is possible that *P. coffea* will produce even higher levels of parasitism than recorded in the artificial environment we used in our study, when attacking wild *H. hampei* boring into coffee fruits, producing the full range of cues stimulating parasitism, and lower field parasitism of the non-target *Hypothenemus* spp. included here.

Among all the parasitized *Hypothenemus* species, *H. hampei* had the highest rate of parasitoid emergence. The total developmental time (from egg to adult) of *P. coffea* was shortest in *H. hampei* (32 days); parasitism of *H. crudiae* resulted in the longest developmental time (41 days). Another study reported a similar development time of the *P. coffea* in *H. hampei*, 38–42 days at 23 °C and 66% RH (Rafael et al. 2000). Castillo et al. (2004) reported a *P. coffea* development time of 42.6 days for *H. hampei* and 40 days for *H. crudiae* at 26 ± 2 °C and 70–80% RH. Total developmental time is directly related to the temperature. For example, the total development period of *Diglyphus isaea* (Hymenoptera:Eulophidae) decreased with increasing temperature between 15 and 35 °C and no development was found at 10 and 40 °C (Haghani et al. 2007). Temperature is a critical abiotic factor influencing the physiology and dynamics of insects. Therefore, in this study we selected a temperature for our no-choice assays which reflects the ambient field temperature the insects are expected to experience. In addition to temperature, age of the parasitoids and host play an important role in the subsequent development of parasitoid offspring (Pizzol et al. 2012). Hence, we used uniformly aged parasitoids and hosts throughout our experiments to minimize any impact on host parasitism and parasitoid development.

Phymastichus coffea commonly lays two eggs (a male and a female) per host (López-Vaamonde and Moore 1998). Both male and female develop in a single host, the female in the abdomen and the male in the prothorax (Espinoza et al. 2009). In this study, slightly fewer

male parasitoids emerged as compared to females from parasitized hosts. The proportion of females emerging from *H. hampei* was 50.8% which is consistent with the results obtained by López-Vaamonde and Moore (1998) and Rafael et al. (2000). Likewise, sex ratios of *P. coffea* emerging from *H. obscurus* 54.8%, *H. seriatus* 51.1% and *H. crudiae* 50.0% were consistent with the sex ratio results reported by (López-Vaamonde and Moore 1998; Castillo et al. 2004) of 1.25:1, 1:1 and 1:1 (female–male), respectively, for these species. In our study, the proportion of females emerging from parasitized *H. birmanus* 57.7%, was the highest among all other *Hypothenemus* species tested. The slightly fewer males produced per host in our study could be due to either to some parasitoid's preference to lay one egg per host (Feldhege 1992) or the lower survivorship of male eggs or larvae. Preference to lay female eggs over male can be dependent on several factors such as host quality, host age, immune response, genetic factors, photoperiod and relative humidity, host density or host-related volatile composition (King 1987).

All the above tests were conducted in a quarantine laboratory with no field studies. We conducted no-choice tests because they may provide more accurate and conservative information on host preferences and physiological host range than choice tests because of lower levels of interference due to unexpected responses to multiple host cues (Van Driesche and Murray 2004). Sands (1997) showed that laboratory studies often overestimate the host range of the parasitoid and realized ranges under field conditions may be substantially less than predicted from no-choice tests, but they are necessary to give a worst-case prediction of the number of hosts at risk of being attacked in the field (Avilla et al. 2016). *Phymastichus coffea* attacked other non-target *Hypothenemus* species in our no-choice trials, but this does not necessarily mean that those species will be attacked in the field. For example, an idiobiont braconid wasp, *Bracon hebetor* is reported to parasitize a wide variety of moths within and outside in Phycitinae (Lepidoptera:Noctuidae) in the laboratory, but in the field it is restricted to only larvae of *Plodia interpunctella* (Lepidoptera:Noctuidae) (Antolin et al. 1995). This is because in the field, parasitoids use a spectrum of long- and short-range cues (chemical, visual, vibrational and tactile signals) to locate hosts (Strand and Pech 1995). Chemical cues (infochemicals) can play an important role in host location. A study conducted by Rojas et al. (2006) showed that *P. coffea* can distinguish between *H. hampei*-infested and uninfested coffee berries, and were highly attracted to the dust/frass originating from *H. hampei* infested berries, but showed no response to the dust/frass originated from the closely related non-target host, *H. crudiae*. This behavior depending on plant and host cues suggests that it is very unlikely that *P. coffea* will have any

negative effects on non-target scolytids, or any other beetles, under field conditions.

No biocontrol agents were previously released in Hawaii against *H. hampei*. Two exotic predatory beetles, *Cathartus quadricollis* and *Leptophloeus* sp., are commonly found in overripe and dried coffee berries predated on the immature stages of *H. hampei* (Follett et al. 2016; Brill et al. 2020). Our host testing in quarantine showed that *P. coffea* will not parasitize these beetles and that the beetles did not predate on the parasitoids. Also, these predators attack eggs, larvae and pupae of *H. hampei* in overripe and dried berries (left after harvesting), whereas *P. coffea* only attacks adult female *H. hampei* at an earlier stage of crop maturity. The other four *Hypothenemus* species that were attacked by *P. coffea* have very different field habitats, but might serve as useful transitory hosts for *P. coffea* at times when, or in areas where, *H. hampei* populations are at low densities, such as between coffee seasons. For example, macadamia nut farms are often located close to coffee farms in Hawaii and may provide a year-round source of *H. obscurus*, a pest of macadamia nut. Feral coffee in Hawaii could also serve as a continuous source of *H. hampei* throughout the year.

Phymastichus coffea is a potentially effective biological control agent for *H. hampei* and could be incorporated into existing IPM programs in Hawaii. *Phymastichus coffea* may be simply released and monitored for establishment in a classical biological control program, or it may be mass reared for inundative releases. Currently, trapping and sampling of infested coffee fruits is conducted to monitor *H. hampei* flights and optimize timing of *Beauveria bassiana* applications for control (Aristizabal et al. 2016). After *H. hampei* bores into the coffee berries, it is protected and difficult to control with biopesticides or conventional insecticides. To achieve maximum *P. coffea* parasitism in the field, inundative releases should be made at times when *H. hampei* adults are active (e.g., when trap catches are high or female *H. hampei* are actively boring into fruits) and the coffee crop is at a susceptible stage. Optimal timing of inundative releases may differ for different elevations due to *H. hampei* population dynamics (Hamilton et al. 2019). Studies suggest *P. coffea* may be susceptible to *B. bassiana*, however (Barrera 2005; Castillo et al. 2009; Ruiz et al. 2011), so inundative releases should be timed to avoid *B. bassiana* applications or used in alternation with *B. bassiana* against *H. hampei*. If *P. coffea* is highly effective, then dependence on *B. beauveria* applications could be reduced dramatically.

Author contributions

FY designed methodology, conducted the experiments and wrote the manuscript; PF designed the experiments; PF and MW provided overall project management and manuscript

editing; FY, CG and DH conducted field surveys and collected live beetles for testing. FY, CG and LC identified beetle species; MGJ and PBM reared and supplied *Phymastichus coffea*. All authors read and gave final approval for publication.

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Declarations

Conflict of interest The authors have declared that no conflict of interest exists.

Informed consent Informed consent was obtained from all individual participants included in the study.

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