Cocoa agroecosystems and the cocoa pod borer, *Conopomorpha cramerella*. A review

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Keywords: integrated pest management; Southeast Asia, Papua New Guinea, nomenclature morphology, DNA, host range, artificial diet, sterile insect technique,

Abstract

Cocoa is the livelihood of many smallholders along the wet tropics. Cocoa production in South East Asia and the Pacific is under threat from the cocoa pod borer, *Conopomorpha cramerella*. Affected areas can lose up to 50% of their crop production. In this review, we appraise the identity and distribution of recorded *C. cramerella.* We also review the different forms of pest management, including cultural, chemical and biological control, promoting opportunities for future research in several areas, while highlighting more recent discoveries, or novel tools that warrant research consideration. We conclude that despite the challenges for pest management in cocoa agroecosystems, there is scope for new and novel strategies to be developed including entomopathogenic endophytes and the sterile insect technique, that may improve management on a wider scale. Ultimately, however, we propose that any new, or novel CPB IPM practices should be practical, and readily integrate, or complement existing pest management strategies.

Introduction

The cocoa tree, *Theobroma cacao* (Malvaceae) originated from South America (Zarrillo *et al.*, 2018) and is now grown largely across wet tropical areas of America, Africa and Asia (Monteiro *et al.*, 2009; ICCO, 2019). Cocoa beans, extracted from the cocoa pod of the tree, are an important cash crop across these regions (Monteiro *et al.*, 2009; Hulme *et al.*, 2018).

The butter and powder produced from cocoa beans are used mainly in chocolate manufacture (Babin, 2018). The demand for cocoa continues to grow, particularly with increased demand for

chocolate with high cocoa content and rising popularity of processing in origin countries (Hawkins and Chen; ICCO, 2019). Globally, chocolate confectionery is estimated to be worth over US$120billion (ICCO, 2019).

In 2016 world cocoa bean production was estimated at 4.467 million tonnes, harvested from 10.197 million hectares from over 50 countries (FAOSTAT, 2018). Africa produces almost 70% of world production, followed by 16% in the Americas and 15% in Asia, with Oceania producing just over 1% (FAOSTAT, 2016). Almost 90% of production comes from about 5 million smallholdings of under 2 hectares, where management is generally minimal (World Cocoa Foundation, 2014).

In Southeast Asia and Pacific regions, cocoa production is under threat by *Conopomorpha cramerella* (Snellen) (Lepidoptera: Gracillariidae), a micro-Lepidoptera commonly known as the cocoa moth or cocoa pod borer (Bradley, 1986; Shapiro *et al.*, 2008). The first reported incidence of *C. cramerella* attacking cocoa was in the 1840s in Indonesia (then known as Sulawesi) (Toxopeus et al., 1983). It became a major pest of cocoa in Southeast Asia in the mid-1980's (Bradley, 1986; Keane, 1992; Posada *et al.*, 2011) and since early this century, is considered the main pest threat for cocoa production in Indonesia, the Philippines and Malaysia (Posada *et al.*, 2011). In Papua New Guinea *C. cramerella* was not considered a pest until an outbreak in 2006, in the East New Britain Province. Two eradication programs were attempted but were unsuccessful (Yen *et al.*, 2010; Curry *et al.*, 2015). *Conopomorpha cramerella* is now found in most coastal cocoa growing provinces of PNG including the islands

of New Ireland and Bougainville and the mainland Provinces of Madang, East Sepik and East New Britain. In 2011, *C. cramerella* was detected at a single commercial plantation in far north Queensland, Australia, and was declared as eradicated by 2013 (Diczbalis, 2013). The literature has not reported its spread to any other major cocoa producing regions of the world.

In situations of severe infestation, cocoa yield has reportedly been reduced by 60-84%, with dry bean quality also affected (Posada *et al.*, 2011). Cocoa crop loss due to *C. cramerella* in Asia early this century was estimated at US$500 million/annum (Posada *et al.*, 2011).

A range of methods have been utilised to manage this pest, however it is generally accepted that several tactics are required to obtain effective pest management. Existing tools include frequent harvesting of cocoa pods and the use of pesticides. New and emerging technologies for this pest are currently under development, but most are still some way off and require further investment. Further, we still know very little about the population genetics of this pest. Taxonomic and genetic resolution is crucial to improve management of this pest (REF).

In this critical analyses, we attempt to draw together the literature on the identification and management of *C. cramerella*, highlighting the discrepancies, and the gaps in our knowledge, while suggesting areas of current and future research that require attention in order to manage this pest effectively and sustainably. Obtaining clarity around the biology, behaviour and identification of CPB is essential, with the ability to culture *C. cramerella* key in facilitating in depth studies of the pest, sustainable and effective pest management, and offer the possibility for the mass-rearing of parasitoids of this pest, and indeed for use in sterile insect technique programs, for which there is current investment, largely form the FAO/IAEA.

Cocoa Agroecosystems

Cocoa monoculture production has often been associated with expensive higher inputs and improved genetic material (Andres et al., 2016). Traditionally cocoa was grown within the rainforest environment but as production intensified full-sun monocultures were advised and became widespread. Full sun production was initially thought to be higher yielding and have reduced pest and disease problems, however in practice, these problems as well as soil erosion increased, resulting in decline in productivity over time (Andres *et al.*, 2016).

Management of disease and pests is a challenge for many growers, particularly in Africa and Asia, due to socio-cultural factors, poverty, limited education and availability of information on farming practices as well as restricted access to finance or improved cultivars (ICCO, 2012; Babalola *et al.*, 2017). Many cocoa producers offset the risk of loss from pests and disease in cocoa by diversifying into various other crops as a source of income (Babalola *et al.*, 2017). In parts of the world farmers are dependant on government supplied inputs including improved varieties of cocoa seedlings, fertilisers and even the application of pesticides (Andres *et al.*, 2016; Babalola *et al.*, 2017; Babin, 2018). Cocoa plantations in many places are declining in productivity due to the age of the trees, soil degradation, in addition to pest and disease pressure, and in some circumstances farmers do not have the means to replant (Babalola *et al.*, 2017), and/or may not have the incentive due to several factors such as alternative crops that don't suffer the same pest pressure. Smallholder cocoa plantation holders may also make a business decision to cut down rainforest to plant additional cocoa trees because it is more profitable to do so, as opposed to attempting to rehabilitate their existing cocoa plantings.

Combined with degraded soils, and without the knowledge or the means to improve them, growers may cut down rainforest in order to increase productivity and yields (REF).

Effect of cocoa pod borer on cocoa ecosystems

CPB has had significant effects on cocoa production across its distribution. The low effectiveness and high cost of attempted control measures was one factor in the demise of cocoa production in Malaysia from a peak of 247,000 tonnes in 1990 to about 1,000 tonnes in 2017 (Lee, 2013). CPB has also played a significant role in the decline of production in Indonesia, where cocoa bean production has fallen to almost half of the supply seven years ago (2012/2013 410,000 tonnes to 220,000 tonnes in 2018/19; https://www.statista.com/statistics/497882/production-of-cocoa-beans-in-indonesia/ accessed 27 May 2019 from about 600,000 tonnes in 2010/11 to an estimated 240,000 tonnes in 2017/18 (Neilson, 2007; ICCO, 2012).

In 2015/16 Papua New Guinea produced 36,000 tonnes of beans valued at K140 million Simatab (2007), with agricultural export earnings ranking third after oil palm and coffee. Production has not recovered from a peak of 48,000 tonnes in 2010/11 since the incursion of the CPB in 2006 and the subsequent failure to eradicate the pest. The Gazelle Peninsula of East New Britain Province in Papua New Guinea was until 2008 the most important cocoa-growing region of PNG, producing about 20,000 tonnes or 54% of national production. By 2012 annual production from East New Britain Province had fallen by over 80% to approximately 4,000 tonnes due to CPB, rampasan and poor crop management that allowed CPB losses to reach catastrophic levels (Curry et al., 2015).

Taxonomic Identification

Nomenclature

Conopomorpha cramerella (Snellen) is a Gracillariid moth of about 10-14 mm wingspan. Its importance as a major cocoa pest is recognised with the moniker of cocoa pod borer (CPB). This pest has previously been known as *Gracilaria cramerella* by Snellen who originally described the species from Java (Indonesia) (Bradley, 1986). A junior synonym of *C. cramerella* is *Acrocercops heirocosma.* This species was described from Port Darwin, Australia by Meyrick in 1910 and was synonymised by Bradley (1985) when he also transferred the nominal taxon from *Acrocercops* to *Conopomorpha* (Bradley, 1985; De Prins and De Prins, 2018).

Similar species include the leaf-mining pest of litchi (*Litchi chinensis*) which was previously erroneously recorded as *A. cramerella*. One Indian paper refers to *C. cramerella* as the litchi fruit borer (Bhatia *et al.*, 2000).

Adult Morphology

The adults are small delicate moths with narrow wings of 10-14mm wingspan. Bradley (1986) redescribed *C. cramerella*. Like most Gracillarids, the narrow wings are feathered at the tornus of the forewing and along the costa and hind margin of the hind wing. The forewing of *C. cramerella* has a pale orange patch in the distal third with a number of small metallic spots towards the tornus, whilst the basal two thirds has an ochreous brown base overlaid with 6 irregularly spaced white lines. This patterning forms the basis of the forewing for *C. cramerella* and its closely related allies. Bradley (1986) gives detailed descriptions of adults of *C. cramerella*

188 (Fig. 1) and these three closely related new species from the Pacific and Asia (Nepal to Taiwan)
189 (viz *C. oceanica, C. sinensis* & *C. litchiella*). The most conspicuous morphological differences of
190 the three described taxa are summarised below. Other morphological differences, perhaps less
191 observable, are also present (Bradley, 1986) but only the most diagnostic are referred to here.

192

193 **Figure 1.** Adult *Conopomorpha cramerella* (Snellen) (Lepidoptera: Gracillariidae).

194 *Conopomorpha oceanica* can be distinguished from *C. cramerella* using forewing
195 colour/patternation with *oceanica* having fifth and sixth lines of white zig-zag pattern
196 converging on costa to form an angle and not separated as in *C. cramerella.* Orange apical wing

spot in *C. oceanica* is separated by a thicker (sometimes twice as thick) falcate white line than in *C. cramerella* (Bradley, 1986). This species may also be differentiated using the adult genitalia. The aedeagus of the male has a minute sclerotized thorn-like cornutus mid length, a feature absent on the other taxa prescribed here (Bradley, 1986).

Conopomorpha sinensis can be distinguished from the other three species by the purple black hind wings and the presence of scattered white scales on the fore and hind wings – absent from the other taxa (Bradley, 1986). This species may also be differentiated using the adult genitalia. The distal part of the male sacculus has 12-15 stout setae in *C. sinensis* and if present, then numerous fine short setae present in the other three taxa (Bradley, 1986).

Conopomorpha litchiella is very similar to *C. cramerella* but only has five not six white lines on the forewing (not always obvious) and also the white lines in the middle of the wing appear to be 'doubled' or thickened in width, through the appearance of a very fine brownish line subdividing each line (Bradley, 1986). This species can be better differentiated using the genitalia (Bradley, 1986). Male valvae simple, elongate and with a ventrally directed 'prong' (this character is lacking in *C. sinensis, C. cramerella* & *C. oceaninca*) (Bradley, 1986).

The gender of adult moths can be ascertained using a relatively simple field assay as outlined by Posada *et al.* (2011). The last female segment is narrow, compressed laterally and has whitish scales whilst the last segment of the male is broader and black.

Larval Morphology

Only passing reference to the larval morphology of any of the *Conopomorpha* species has been made in the literature. Li (2011) in (Hu *et al.*, 2011) described and illustrated a new species of

Conopomorpha, C. fluegella from China. Li provided (scant) larval and pupal descriptions and life history table and illustrations for this species, which is a temperate specialist on its Euphorbiacious host. Most references to larvae usually pertain to the damage to cocoa pods (Froggatt, 1938; Gende *et al.*, 2007) whilst providing little taxonomic insight into the pest.

Pupal Morphology

Limited references to pupal morphology exist in the literature other than contextual references. Posada *et al.* (2011) provide a methodology of sexing pupae in the lab and field and provide some morphological characters of the pupa that are representative of *C. cramerella*. They showed that (putatively – no vouchers recorded) that *C. cramerella* pupae are about 7.2mm long including the antennae and about 4.6 mm without. A number of pupal characteristics are described for *C. cramerella* recognise the different positions of the genital slits as one of several features that distinguish the sex of the pupae (Posada *et al.*, 2011).

Molecular Identification

DNA analyses of Conopomorpha species

Thirteen species of *Conopomorpha* (Gracillariidae: Ornixolinae) are currently recognised (including *C. heirocosma*, a junior subjective synonym of *C. cramerella*), nine of which are recorded in Australasia, and four, three and two species recorded in the Oriental, Afrotropical and Palaearctic Regions respectively (De Prins and De Prins, 2018). Phylogenetic relationships among *Conopomorpha* species have not been comprehensively reported. However, in the context of pest species management of this genus, investigation of these relationships is crucial to allow genetic identification of morphologically similar species across life stages, delimitation

239 of cryptic taxa, and for understanding host-plant and predator relationships. Available
240 molecular reports of *Conopomorpha* are limited to few species included in high level
241 systematics of Gracillariidae (Kawahara *et al.*, 2011; Kawahara *et al.*, 2017), broader ecological
242 analyses of lepidopteran host plant specificity (Kawakita *et al.*, 2010; Sam *et al.*, 2017),
243 predator-prey analysis (Meng *et al.*, 2014), and population genetics of the pest species
244 *Conopomorpha cramerella* (Shapiro *et al.*, 2008); see later).

245 DNA barcode analysis (Hebert *et al.*, 2003) used as an independent genetic means to test alpha-
246 taxonomic hypotheses (Gopurenko *et al.*, 2015) would be beneficial to investigation of
247 *Conopomorpha*, where there is a need to test for suspected cryptic pest species presence
248 (Muhamao and Tan, 1987; Valenzuela *et al.*, 2014), and for development of species diagnostics
249 to improve identification of Gracillariidae moth ecologies across life stages (Shapiro *et al.*, 2008;
250 Craft *et al.*, 2010; Brito *et al.*, 2016). DNA barcode campaigns for analysis of genetic distances
251 within and among described taxa at mitochondrial encoded 5' cytochrome *c* oxidase subunit I
252 (COI) gene have been reported for focal groups within Gracillariidae (Lees *et al.*, 2014; Brito *et*
253 *al.*, 2016), but these have not been inclusive of *Conopomorpha*. An exhaustive search of the
254 Barcode of Life Data systems (Ratnasingham and Hebert, 2007) and GenBank
255 (www.ncbi.nlm.nih.gov) sequence repositories indicates public availability of 137 DNA barcode
256 equivalent COI sequences attributed to *Conopomorpha* species and several unidentified taxa in
257 the genus (last searched 07/Aug/2018; Supplementary Table S1XXX). Summary analyses of
258 phenetic and phylogenetic relationships among these DNA barcode sequences using MEGA X
259 (Kumar *et al.*, 2018) are reported here.

Over 77% of the available sequences are placed to *C. cramerella*; the remainder of sequences are sparsely representative of three other described *Conopomorpha* species (*C. heliopla*; *C. litchiella*; *C. sinensis*) and four unidentified (putative) species attributed to *Conopomorpha* (Table 1XXX). Barcode Index Numbers (BINs) (Ratnasingham and Hebert, 2013) auto-assigned to sequence clusters in the BOLD repository (for objectively delimiting molecular operational taxonomic units [MOTU's] in the database) are each supportive of a single *Conopomorpha* species description (Table 1XXX). Similarly, query searches of *Conopomorpha* sequences at NCBI using the BLAST tool did not match to multiple species at > 95% similarity. Comparisons of unweighted genetic distances among *Conopomorpha* sequences estimated here using MEGA X (Kumar *et al.*, 2018) indicate all identified and putative species, with the exception of *C. litchiella*, exhibit a DNA barcode gap between the maximum observed intraspecific distance (< 0.76% sequence difference) and the range of minimum distances to nearest neighbour species (1.99 – 9.04%). *C. litchiella* comprises two genetically shallow DNA barcode groups (< 0.55%) that minimally differ by 6.38 %. Maximum Likelihood (ML) phylogenetic analysis of the *Conopomorpha* sequences (Fig. 2) supports species monophyly in all cases except for *C. litchiella* which is sorted as two geographically distinct sequence clades (Vietnam and India) that are poorly resolved with respect to several other *Conopomorpha* species. Further taxonomic and DNA sequence inquiry of *C. litchiella* is required, preferably including type locality specimens (Serdang, West Malaysia) and broader sampling, to determine if this genetic division of *C. litchiella* as two divergent non-sister clades is a result of taxonomic mis-identification at one of the clades, or is 1st evidence of historical population genetic structure in the species and or cryptic species presence in its distribution. Similarly, future availability of

voucher specimen DNA barcode records currently lacking from ten of the fourteen recognised *Conopomorpha* species are essential for improved understanding of diversity in this genus and will assist in determining identities of four MOTU's recorded to the genus but evidenced as genetically distinct in the available *Conopomorpha* phylogeny (Fig. 2).

Interestingly, two of the unidentified *Conopomorpha* species (BIN:ACY9663 & BIN: ACP2572) sampled sparsely from Madang PNG (Sam *et al.*, 2017) are genetically closest (< 1.99% and 5.48 % minimum sequence difference respectively) to BIN:AAA4000 containing all identified *C. cramerella* sampled across much of the species range. The close genetic similarity of these MOTUs to *C. cramerella* indicates they are either representative of divergent mitochondrial DNA lineages within the species or are potentially its nearest sister taxa. In either case, the absence of these MOTUs from earlier extensive genetic analysis of *C. cramerella* sampled mainly from plantations of introduced cocoa (*Theobroma cacao*) across much of the Indo-Malay Archipelago and PNG (Shapiro *et al.*, 2008), indicates these two MOTUs may not be using introduced cocoa plant as a host.

The population genetic analysis by Shapiro *et al.* (2008) examined sequences of independent mitochondrial COI and nuclear EF-1 loci from cocoa borer moths (across life stages), and was primarily motivated by a need to genetically determine if more than one species of *Conopomorpha* was affecting cocoa plantations in the regions. Total genetic diversity among sampled moths was evidenced as exceedingly shallow at both genes (0.3 % and 0.7% at COI and EF-1 respectively) and most sample locations were dominated by presence of a geographically widespread haplotype/allele at each locus. Evidence of geographic or host associated genetic structure was inconclusive, indicating it unlikely that allopatric and or sympatric evolved host

304 specialisation was present in the sampled range. Earlier contentions of cryptic species or
305 biotype complex presence in the cocoa pod borer moth were not supported by the genetic
306 evidence, which collectively identified *C. cramerella* as the sole *Conopomorpha* pest affecting
307 cocoa plantations in the sampled regions. Whether this extends beyond the sampled range
308 reported by Shapiro *et al.* (2008) remains to be determined.

309 Evidence of shallow genetic diversity and a maternal population genetic bottleneck among *C.*
310 *cramerella* sampled across the 5,000 km east – west transect indicated the species likely
311 underwent a demographic and or range expansion following growth of the plantation industry
312 (Shapiro *et al.*, 2008). Subsequently much of the species current distribution among cocoa
313 plantations likely contains only a subset of the species natal genetic diversity. Shapiro *et al.*
314 (2008) were unable to identify the source provenance of *C. cramerella*, but noted such
315 information will be critical for survey of source specific taxa that have co-evolved with the moth
316 and are potentially useful for its biocontrol.

317 The sample strategy used by Shapiro *et al.* (2008) provided limited opportunity to identify use
318 of native host(s) by *C. cramerella*. Current database records (De Prins and De Prins, 2018) list 19
319 host plants in four families used by the species; however this list is likely to be upwardly revised
320 following broader integrative taxonomic surveys incorporating DNA barcoding. For example,
321 Sam *et al.* (2017) reported additional host plants including (Euphorbiaceae) *Ryparosa javanica*,
322 and (Fabaceae) *Maniltoa psilogyne* used by genetically identified *C. cramerella* sampled from
323 Madang PNG. Similar DNA barcode based identifications will help to improve knowledge of the
324 diversity of host use by the various *Conopomorpha* species; this is especially the case when

surveys include morphologically ambiguous, immature or degraded specimens that may otherwise be difficult to identify to species (Gopurenko *et al.*, 2013).

Table 1. DNA barcode sequences attributed to *Conopomorpha* species at BOLD and or GenBank sequence repositories, and total number (N) available. *source references of deposited sequences as reported at repositories. Sequence BINs as reported at BOLD except where unavailable (NA). Maximum intra-specific (D_{intra}) and minimum nearest neighbour (D_{NN}) genetic distances estimated as percent (unweighted) sequence differences, using MEGA X (Kumar *et al.*, 2018).

Species	*source	N	BIN	D_{intra}	D_{NN}	sampled countries
Conopomorpha ?	*9*	10	AAI5875	0.46	2.41	PNG
Conopomorpha ?	*9*	2	AAN5037	0.61	2.41	PNG
C. cramerella	*2,5,6,7,8*	106	AAA4000	0.40	1.99	Malay Archipelago & PNG
C. heliopla	*3*	4	AAY7068	0.76	9.04	Australia
C. litchiella (#1)	*5*	2	AAW1281	0.23	6.38	Vietnam
C. litchiella (#2)	*1*	4	NA	0.55	5.34	India
C. sinensis	*1, 4,8*	7	ACD5201	0.32	6.02	India (& N2 PNG? Valenzuela)
C. sp.	*6*	1	ACP2572	-	5.26	PNG
C. sp.	*6*	2	ACY9663	-	1.99	PNG

*source:

1: Choudhary, J.S., Srivastava, K. and Nath,V. (unpublished, direct submission to GenBank)

2: Hayden,J.E., Kawahara,A.Y., Xiao,L. and Somma,L.A. (unpublished, direct submission to GenBank)

3: Hebert *et al.* (2013)

4: Jayanthi Mala, B.R., Kamala Jayanthi,P.D., Shabarish, P.R., Sudhagar, S., Raghava,T., Nagaraja, T. & Reddy, P.V.R (unpublished, direct submission to GenBank)

339 *5*: Kawakita et al. (2010)

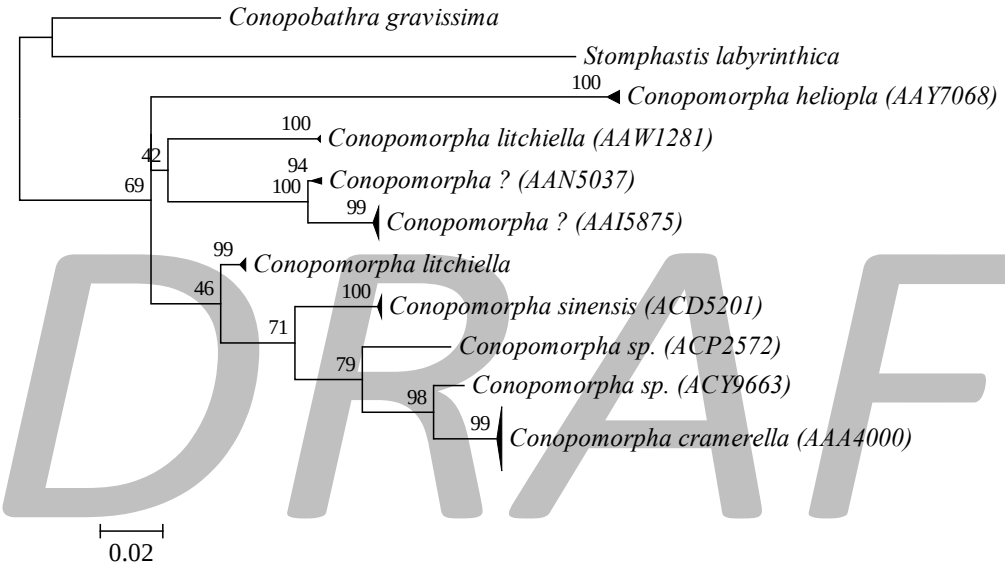
340 *6*: Sam et al. (2017)

341 *7*: Shapiro et al. (2008)

342 *8*: Valenzuela,I., Hamilton,A.J. and Roush,R (unpublished, direct submission to GenBank)

343 *9*: International Barcode of Life (unpublished, direct submission to GenBank)

344



345

346 **Figure 2.** Phylogenetic relationships among COI sequences attributed to *Conopomorpha* (refer

347 Sup. Table S1), and associated BOLD BINS (in parentheses, where available). Phylogeny

348 reconstructed by Maximum Likelihood (ML) as implemented in MEGA X (Kumar *et al.*, 2018)

incorporating a GTR+G+I nucleotide substitution model (4 category; G = 0.2980; I = 0.2552), and using a Subtree-Pruning-Regrafting (level 5) heuristic (& very weak branch swap filter) for extensive search of tree space. Available *C. cramerella* sequences (N=106) pared down to N =10 unique haplotypes to reduce ML search space. All terminal species clades collapsed; ML clade supports estimated by bootstrap replication (N =1000). The ML tree was outgroup rooted using related (Gracillaridae: *Parectopa* group) taxa: *Conopobathra gravissima* and *Stomphastis labyrinthica*.

Distribution

Distribution & Diversity

Appendix 1 shows the 13 currently recognised species of *Conopomorpha* based on literature records (De Prins and De Prins, 2018). Importantly more than half the species of *Conopomorpha* are known only from temperate climates and are not known to infest cocoa and its allies and will not be dealt with further (this group includes *C. antimacha, chionosema, chionochtha, cyanopsila, euphanes, flueggella, habrodes*). Records of the genus indicate this genus originates in Australasia (See appendix 1). Molecular data (see further: molecular diagnostics section) suggests the genus is most diverse in Papua New Guinea (Gende, 2012) and potentially Australia as well. The inclusion of three morphological and molecular 'biotypes' of CPB (Valenzuela, 2011(draft), 2012) collected via pheromone trapping in PNG & Indonesia, strongly indicate potential cryptic species or genera. Valenzuela (2011) indicates that one of the biotypes sampled from another unstated study on taun, *Pometia pinnata* from Ohu, PNG may be from the genus *Conopomorphina*, a genus, as recognised by Valenzuela herself, as not being

370 recorded from Southeast Asia or the Pacific. *Conopomorphina* is only currently recognised from

371 subtropical and temperate southern Africa (De Prins and De Prins, 2018).

372 Host range

373 Rambutan (*Nephelium lappaceum*) is considered the primary host of *C. cramerella* and cocoa a

374 secondary host (Bradley, 1986) with the majority of the recorded hosts from the plant families

375 Sapindaceae and Malvaceae (De Prins and De Prins, 2018) (Table 2).

376 **Table 2.** Known host plants of *Conopomorpha* spp.

			Locality			
			SE Asia/Pacific	SE Asia	SE Asia	New Zealand
			Conopomorpha spp.			
Hosts	**Family**	**Common**	***C. camerella***	***C. litchiella***	***C. sinensis***	***C. cyanospila***
Nephelium lappaceum	Sapindaceae	rambutan	Primary*			
Nephelium litchi	Sapindaceae		TRUE*	TRUE*		
Nephelium longana	Sapindaceae				TRUE*	
Nephelium mutabile	Sapindaceae	pulasan	TRUE*			
Nephelium malainse	Sapindaceae		TRUE*			
Pometia	Sapindaceae		TRUE*			
Pometia pinnata	Sapindaceae	island lychee	TRUE*			
Litchi sinensis	Sapindaceae	litchi	TRUE*	TRUE*	TRUE*	
Dimocarpus longan (syn Nephelium longana)	Sapindaceae	longan	TRUE*	TRUE*	TRUE*	
Alectryon excelsum	Sapindaceae					TRUE*
Theobroma cacao	Malvaceae	Cocoa	Secondary*	TRUE*	TRUE*	
Cola acuminata	Malvaceae		TRUE*			
Cola nitida	Malvaceae		TRUE*			
Cola sp.	Malvaceae	kola	TRUE*			
Swietenia sp.	Meliaceae		TRUE*			
Cynometra cauliflora	Fabaceae	nam-nam, namu-namu, Maluku, Manado	TRUE*			
Senna tora	Fabaceae			TRUE*		
Senna obtusifolia	Fabaceae			TRUE*		
Syzygium cumini	Myrtaceae					TRUE*

* De Prins, J. & De Prins, W. 2018. Global Taxonomic Database of Gracillariidae (Lepidoptera). World Wide Web electronic publication (http://www.gracillariidae.net) [29/03/2019]. Green shading depicts the primary host plant family and blue shading depicts the secondary host plant family.

It is interesting to note that in Latin America, the native host range of the cocoa plant, that it is relatively unaffected by insect pests compared to areas where cocoa has been introduced (Babin, 2018). Indeed, globally most pest and disease problems on cocoa are new encounters rather than coevolved species. An exception is *P. palmivora*.

Life cycle

In tropical areas *C. cramerella* can breed continuously with one month per generation (Day, 1989). Eggs are laid singly anywhere on the host surface, preferentially on rough and uneven surfaces including in cocoa, the pod furrows and ridges and hatch after 5-9 days. Eggs are translucent, the shell being whitish when laid, with fertile eggs soon turning a bright orange colour, while infertile eggs are white to very pale yellow and often have a collapsed appearance. The hatched larvae do not roam far, but tunnel directly down into the pod near where they hatched until they reach the sclerotic layer. The larvae then tunnel along the layer until it finds a weak point that it can penetrate. The larvae then feed on the funicle, pith and pulp (mucilage) surrounding the beans but also directly on the beans until fully grown (Day, 1989). The larval stage takes 14-18 days to complete 4-6 instars. The larvae then tunnel out through the pod wall, leaving an easily identifiable exit hole, to pupate (Lim, 1992). The larvae produce a thread and use this to swing to a preferred or favourable site, such as the green leaves of a cocoa tree, green or dried leaf litter on the ground, leaves of plants in the

undergrowth, and even on the surface of the host plant cocoa pods. It takes the larvae upto 40 minutes to weave a cocoon, typically on a relatively smooth surface with curved or angular edges. The final instar larvae remains in the woven cocoon and begins to moult after 3 – 4 days. Pupation lasts 6-8 days before adult emergence.

The moths emerge from the pupae and are most active at night; mating and egg-laying takes place at this time. A female can normally produce up to 200 eggs in her lifetime given the right conditions. Adult longevity is generally about one week, but they can live up to 30 days. (Lim, 1992). The female produces a sex pheromone and 'calls', or attracts the males. Adult pairs mostly mate three nights after emergence with females mating up to four times in a lifetime (Lim and Pan 1986 as cited by Lim 1992). Adults tend to rest during the day on the underside of branches and leaves.

Spread

The adult moth is reported to be a weak flier but is capable of covering longer distances particularly if assisted by wind (Lim, 1992; Chaidamsari, 2005; Rosmana *et al.*, 2010). The adult has been observed, when exposed to pesticides, cultural practices by humans, or stress from overcrowding or lack of a suitable food source to fly directly upwards, above the cocoa canopy, where the wind is able to disperse it from its present location. The main form of spread is during the larval stage inside the pod, if they are transported a distance prior to processing (Chaidamsari, 2005). Very wet or very dry seasons reduce CPB numbers, rainfall between 100-200mm per month were the ideal moisture conditions (Lim, 1992).

Incidence

Very wet or very dry seasons reduce CPB numbers, with rainfall between 100-200mm per month thought to be the ideal moisture conditions (Lim, 1992). Although there are no published studies on pest incidence and density across its range, anecdotal evidence suggests for example that numbers are higher in Indonesia than Papua New Guinea. It is feasible that climatic and other factors such as natural enemies, availability of hosts, food, and management practices all influence CPB incidence.

Damage

Ripe cocoa pods are produced continuously, with two seasonal peaks, 5-6 months after flowering (Chaidamsari, 2005). As *C. cramerella* can complete its lifecycle in a month the number of *C. cramerella* can rapidly increase during the season if left untreated.

Investigations have found that *C. cramerella* is more attracted to older pods that are closer to ripening which could be due to volatiles generated by or the greater nutritional value in the ripening pod (Day, 1989; Azhar and Long, 1996). However, *C. cramerella* can attack pods aged from 3 months. Pods attacked at an earlier age tend to show signs of more extensive damage, whereas beans ready for harvest can be found in more mature pods, despite *C. cramerella* damage. Once inside the pod, larvae feed on the placenta and pulp that supply nutrients to the beans (Valenzuela *et al.*, 2014). The interruption of bean development causes clumping of beans and hardening of the pod resulting in harvesting and processing difficulties (Day, 1989; Valenzuela *et al.*, 2014). The tunnelling also causes premature yellowing and hardening of the pod which often results in premature harvesting and reduced quality of beans (Day, 1989).

441 The scale of yield loss caused by *C. cramerella* depends on a variety of factors including
442 infestation severity (Valenzuela *et al.*, 2014), timing of pest attack (Day, 1989), cocoa variety
443 and weather (Day *et al.*, 1995; McMahon *et al.*, 2009). The age of the pod and the number of
444 larvae per pod affects the level of damage (Day, 1989). However, the percentage of pods
445 attacked does not always correspond to yield loss as some beans in the pods can still be
446 recovered; in some instances 60% infestation of pods can still result in negligible yield losses
447 (Day, 1989). Yield loss estimates are sometimes measured by opening a number of pods and
448 classifying the pods into groups based on the range of damage (Valenzuela *et al.*, 2014). Hidden
449 costs include increased handling of the pods, and the increased difficulty in removing the beans
450 from the pods (Day, 1989).

451 The yield losses attributed to *C. cramerella* vary from 20% to total crop loss (Bradley, 1986; Day,
452 1989; Alias *et al.*, 1999). Yield losses also depend on the methods of bean extraction used after
453 pest attack. The highest levels of pest infestation occur when the least pods are available at the
454 lowest part of the cropping cycle (Day, 1989). However, the greatest proportion of the annual
455 yield losses occur during peak harvests, and it has been proposed that this is where control
456 strategies should be targeted (Day, 1989).

457

458 ## Interactions with pathogens

459 There are a range of pathogens that attack cocoa across all regions it is grown around the world
460 (Bailey and Meinhardt, 2016). Pathogens are estimated to reduce global cocoa yields by at least
461 20% annually however under the right conditions are able to completely destroy a crop (Ploetz,

2016). The most significant pathogens are *Phytophthora* species, including the ubiquitous *P. palmivora* and *Phytophthora megakarya* in West Africa, that together cause losses of 20-30% (Bailey and Meinhardt, 2016). *Moniliophthora roreri* and *M. perniciosa* are major pathogens in the Americas, Cacao swollen shoot virus disease in West Africa and vascular-streak dieback (VSD) *Ceratobasidium theobromae* in SE Asia (Ploetz, 2016). Through breeding efforts, many varieties now have partial resistance to these diseases (Monteiro *et al.*, 2009; Bailey and Meinhardt, 2016).

However, there have been no published reports of a relationship is between CPB and disease causing pathogens. Soilborne inoculum can be introduced onto and spread around the pods by visiting insects including ants and beetles (Konam and Guest, 2004). Research on plastic sleeving found, used to manage CPB infestation, was also found to reduce the rate of disease incidence, although once infection occurred rates of disease progression were affected by the type of sleeving used (Rosmana *et al.*, 2010). Research is required to determine if CPB contributes to disease spread and infection.

Management of cocoa pod borer

Cocoa pod borer is difficult to control as the moth is sparsely distributed, nocturnal, and the larval stage is spent inside the cocoa pod (Rosmana *et al.*, 2010). The lack of an effective lure also hinders trapping, and the possibility of using mating disruption to manage the pest. A range of methods have been utilised to manage this pest, however it is generally accepted that

several tactics are required to obtain effective pest management. Currently available management tools for *C. cramerella* include breeding and introduction of tolerant cocoa varieties, good cultural practices such as regular harvesting of mature pods, good hygiene, maintaining soil and tree health and pesticides. Other management tactics that are under development, or require research investment include resistant (as opposed to tolerant) varieties, sterile insect technique, entompathogenic fungi (including endophytic) and bacteria, improved pheromone lures and biological control.

Cultural

Good farming practices including harvesting each fortnight, fertiliser application and heavy and light pruning, depending on the season are among the cultural control methods that may assist in pest management in cocoa plantings (McMahon *et al.*, 2015).

Harvesting

A recommended practice is frequent harvesting or 'regular complete harvesting' to collect the cocoa beans, both healthy and pest infested (Lim, 1992; Wood *et al.*, 1992). This reduces the opportunity for *C. cramerella* to complete development (Lim, 1992; Wood *et al.*, 1992), as once the beans have been extracted the pods can be used as mulch, destroying the immature stages of the pest (Babin, 2018). It is recommended that infested, or discarded pods are buried at least 20cm under the soil to prevent the CPB adults eclosing successfully.

Another practice aimed at eradicating new infestations is 'rampasan', stripping all pods from a plantation at a time of the year when there is only a low number of pods in order to disrupt, or

break the pest life cycle (Chaidamsari, 2005). This strategy however deprives smallholder farmers of their primary source of income for up to one year.

Alternative Hosts

A pest management practice, particularly common in area wide management approaches, is the removal of alternative host plants from areas surrounding the crops. In many systems, cocoa is frequently grown in areas that contain several native and/or planted hosts that may also be harvested by growers, and are sometimes not managed, meaning removal of these hosts is often impractical. Further, given cocoa is the only known host of CPB that produces 'fruit' year round, the short survival time of adults, and no diapause, dormancy or overwintering by any stage, removal of alternative hosts is likely to have little impact.

Pruning

Pruning allows the cocoa tree to focus resources on the cocoa pods and increases the size and number of pods (Govindaraj & Jancirani, 2017). Pruning high branches also ensures that pods grow lower down the tree and are easier to harvest. Further, this practice provides a suitable environment for predators and parasitoids of *C. cramerella* (ICCO, 2017). CPB prefers to lay eggs on cocoa pods higher in the trees, with egg numbers increasing on the pods the higher in the tree they are located (Lim, 1992). Therefore, regular pruning will provide an improved environment for the management of CPB, as well as improve cocoa yield.

The balance of shade is important in cocoa plantations, as shade increases humidity and encourages natural enemies and prevents the soil drying out but can also increase some pest and diseases (Conservation Alliance, 2013). In Papua New Guinea, for example, it is

recommended that shade and cocoa? trees are pruned such that 25% of incident sunlight is intercepted by shade, 50% of sunlight reaches the cocoa canopy, 15% reaches the branches with 10% reaching the ground, or soil surface Reference?.

The labour intensive actions of cocoa management e.g. regular harvesting, regular pruning and removal and burial of infested pods are incompatible with existing smallholder farming systems, values and livelihoods (Curry et al., 2015). Such high input cropping systems not only require practical knowledge and upskilling, but a change in lifestyle that provides position, identity and a moral order, and which is therefore highly resistant to change (Curry et al., 2015). One of the disadvantages in many cocoa systems is that many farmers do not have access to the appropriate resources to appropriately manage the cocoa trees. Poor pruning practices can often increase disease incidence, and in some areas farmers may only make the effort to prune when cocoa prices are high (Andres *et al.*, 2016). Given the multiple benefits of pruning, consideration needs to be given about how this practice can be encouraged further and taken up by growers.

Shade trees

Shade trees in cocoa plantations often provide multiple ecosystem services including nitrogen fixation, and soil stability, protective hedgerows, fodder trees, cocoa yield stability, secondary income such as coconuts or timber and plant material used in medicines or natural remedies (Andres *et al.*, 2016). Cocoa grown under shade provides also better working conditions for farmers. Tropical areas are highly susceptible to climatic variability including heat waves,

546 drought and floods and shade grown cocoa is more resilient to these events (Andres *et al.*,
547 2016). In coffee systems it was found the shade trees can buffer temperature fluctuations by as
548 much as 5 °C, as well as adding carbon and nitrogen to the soil (Beer *et al.*, 1997). In areas
549 under cocoa, *Gliricidia* sp.and coconut are the most common shade trees. Neither are
550 alternative hosts for *C. cramerella* which is an important consideration if shade trees are not
551 managed. Usually smallholders will also plant rambutan in PNG, which is a host for *C.*
552 *cramerella*, and these trees can grow very large and are typically unmanaged, but do provide an
553 alternate source of income. Other non-host options include banana's (*Musa* sp.) which are
554 effective when cocoa is young, i.e. less than 2 years and *Leuceana* sp.

555 Biological Control
556 There are a range of biological control agents that reportedly attack *C. cramerella* (Lim, 1992),
557 although few provide adequate control by themselves, or have not been pursued to their full
558 potential. Noyes (1991) stated that at least 24 species of parasitoids of *C. cramerella* had been
559 identified in Southeast Asia and that *Ceraphron aquinaldoi* Dessart and a new species of
560 *Ooencyrtus* sp. was released into Sabah. *Ooencyrtus* sp. is a prepupal parasitoid and was
561 believed to be promising (Noyes 1991). Since predator–prey ratios decreased with increasing
562 land-use intensity, farmers that have the traditionally diversified agroforestry systems will
563 typically face fewer pest problems (Klein *et al.*, 2002)

564 In Malaysia in 1982 the native egg parasitoid *Trichogrammatoidea bactrae fumata* Nagaraja
565 was found to be attacking *C. cramerella* (Lim, 1986) and is now considered to be an effective
566 biocontrol agent for managing this pest (Alias *et al.*, 1999). *T. bactrae fumata* can be
567 successfully mass-reared on the rice moth, *Corcyra cephalonica* Stainton, and can reduce crop

losses caused by *C. cramerella* from 58% to 6% with field parasitism of up to 54% (Lim and Chong 1987 in Lim 1992).T. *bactrae fumata* is able to parasitize *C. cramerella* regardless of pod age however mass releases are thought to be best timed when a majority of pods are ripening (Azhar and Long, 1996). Despite the effectiveness, the high cost of rearing large numbers of parasitoids and inconsistency in the yield of parasitoids from the rice moths meant that growers had little interest (Alias et al., 1999). Further, although more effective than chemical control (Lim and Chong 1987) the cost of regular augmentative releases was not favoured by growers. Improvements in the cost-effectiveness and labour requirements for mass-rearing and release could make this an attractive option for growers (Alias *et al.*, 1999). The lack of a suitable artificial diet to rear *C. cramerella* has also likely limited the ability to mass-rear, and release other parasitoids, for the biological control of this pest.

Augmentoriums, as used by largely smallholder horticultural growers in Hawaii to manage tephritid fruit flies (Klungness *et al*., 2005), is an approach that might be useful against *C. cramerella*. By placing infested pods into a screened augmentorium, growers will be able to encourage the exit of parasitoids, while retaining the pest inside the augmentorium. This could be an alternative to the mass-rearing and release of parasitoids, while encouraging those parasitoids that persist locally in the environment, however requires investigation.

Conservation biological control could be important for augmentative release, or if augmentoriums are used, ensuring that released and locally persisting natural enemies are encouraged through the provision of selective plants that provide sugar, nectar, alternative prey and pollen (i.e. SNAP) (Gurr *et al.*, 2017). There is considerable evidence to support the

use of selective plants, that encourage and support beneficial insects, while having a neutral or negative effect on the pest (Landis, et al., 2000).

The age of the pod is an important factor in the success of this parasitoid. Releases of parasitoid when the pods are higher lead to higher survival of *C. cramerella* larvae (Azhar and Long, 1996).

The host and geographic origins of *C. cramerella* have not be determined which continues to hinder efforts to find effective natural enemies (Shapiro *et al.*, 2008). Population genetic studies are therefore key, however lacking for this pest, and further research in this area is warranted to improve biological control options.

Bacteria, entomopathogenic fungi and nematodes

There are numerous agents, other than insect natural enemies, listed as potential for use in cocoa systems for CPB management (Bong *et al.*, 1999). In Indonesia, several types of *Bacillus thuringiensis* (*Bt*) toxins were screened for control of *C. cramerella* (Santoso *et al.*, 2004). Laboratory assays showed that eight of the 12 Cry1 proteins tested caused 50% mortality of *C. cramerella* larvae reared on an artificial diet (Santoso *et al.*, 2004). In Indonesia, *Bt* insecticides applied in field trials showed significant reductions in *C. cramerella* infestation and increases in yield (Senewe *et al.*, 2013). These results are encouraging and provide a sustainable and ecologically friendly option that merits further investigation.

An isolate of *Beauveria bassiana* was found on *Clenia celia* (Coleoptera: Cerambicydae) and was investigated as a potential biocontrol agent of *C. cramerella.* The funus caused high mortality of larvae and pupae (40%-100%) (Bong *et al.*, 1999). However, *B. bassiana* tends to be generalist in nature. Several studies have shown that pheromone-baited stations laced with fungal spores

where male moths are attracted, pick up the spores on their bodies, and then depart to mate and horizontally infect females can be very effective for target specific dissemination of the spores (REF). When sprayed on seedlings or flowers, *B. bassiana* establishes as a fungal endophyte of the cocoa tree (Posada and Vega, 2005; Posada *et al.*, 2010) and could be a suitable option for the management of *C. cramerella*, and indeed other cocoa pests (Posada *et al.*, 2010).

There is a single study which looks at the nematode *Steinernema carpocapsae*, a species that is capable of persisting on the pod surface in both the dry and wet seasons and penetrating the pod (Rosmana *et al.*, 2010). The nematode was trialled by applying as a spraying 3 times at intervals of 10 and 20 days and sleeving the pods with plastic sleeving resulted in a synergistic result of totally healthy pods (Rosmana *et al.*, 2010).

The use of these agents to manage *C. cramerella* is a promising avenue to pursue, however we are not aware of the commercial use of any against this pest, and is certainly an area worth further attention.

Ants

In many tropical agroecosystems including cocoa and also coffee, ants can be an important component of pest management (Philpott and Armbrecht, 2006).

Ants are also considered a biological control agent of mirids, especially in shade grown cocoa (Beilhe *et al.*, 2018). Ants have also been shown to be effective biological control agents in Australia (Forbes and Northfield, 2017).

Cocoa black ants, *Dolichoderus thoracicus* Smith (Hymenoptera: Formicidae) are common in cocoa and coconut intercropping in Malaysia, with the nests found in the coconut crown (Saripah and Azhar, 2007). The presence of cocoa mealybug and their honeydew which is a food source is a key part of population build up of cocoa black ants in a cocoa monoculture (Saripah and Azhar, 2007). An abundance of *D. thoracicus* was associated with significant reductions in pod damage where the ants were present but not across the entire plantation (See and Khoo, 1996). The augmentation of black ants into artificial nests can be effective, especially if nests are disturbed during harvesting or pruning, with the presence of these ants in large numbers shown to be effective in controlling cocoa pod borer (Saripah and Azhar, 2007; Anshary *et al.*, 2011). The establishment costs for artificial nests are labour intensive but the cost is considered lower then ongoing chemical costs (Saripah and Azhar, 2007). In a five year trial of augmenting ants in a small plantation the percentage of good (undamaged) pods went from 35% to 75% (Saripah and Azhar, 2012).

However, ants can provide both ecosystem services and disservices (Wielgoss *et al.*, 2014). While ants may decrease pest herbivory and fruit damage, this can be contrasted with increased mealybug density and phytopathogen dissemination (Konam and Guest, 2004; Wielgoss *et al.*, 2014). Notably, there is evidence that tent building ants are passive vectors of *Phytophthora* (Konam & Guest, 2004), but there is no such evidence for arboreal ants. A greater understanding of these ecological relationships and how we might manipulate, or minimise the detrimental impacts of ants requires further studies in these agroecosystems.

651

652 ***Ecosystem services***

653 Around the world cocoa is often grown in agroforestry or intercropping In PNG and other
654 countries, intercropping is common in cocoa (Oladokun, 1990; Osei-Bonsu *et al.*, 2002; Curry *et*
655 *al.*, 2015).

656 Agroforestry around cocoa plantations has been found to protect cocoa trees from heat stress,
657 to enhance soil fertility and control soil erosion. Multi-year controlled trials have found that
658 although monoculture (fun sun) cocoa had higher numbers of cocoa beans, the additional
659 income from other crops meant that the agroforestry systems were far more productive from
660 the farmers' point of view (Andres *et al.*, 2016; Armengot *et al.*, 2016). In agroforestry systems
661 farmers are also partially protected from price shock in the highly variable international cocoa
662 market (Andres *et al.*, 2016). Although the bean yield can be higher in full sun production in the
663 short term, over time increased damage by pests and diseases results in an eventual decline in
664 yield (Andres *et al.*, 2016).

665

666 ***Semiochemical***

667 A female sex pheromone was developed to attract male *C. cramerella* (Beevor *et al.*, 1986;
668 Pereira and Cabezas, 2005; Huang *et al.*, 2017; Vanhove *et al.*, 2019). Trials in Malaysia using
669 sex pheromone baited traps to mass trap males found that damage to cocoa pods decreased
670 over a wide area (Beevor *et al.*, 1993). Mating disruption trials were also shown to reduce
671 mating of females (Tay and Sim 1989, Alias et al. 2004). However, work subsequently halted as

672 the lures were considered inefficient and uneconomic. The failure of previous attempts to
673 manage *C. cramerella* using sex pheromone has also been attributed to the possibility of the
674 existence of more than one strain of *C. cramerella* that behaves differently to the pheromone
675 blend (Beevor et al. 1993, Matlick 1998). More recently a new cost-effective product was
676 developed and tested as a attract-and-kill technology which resulted in better than pesticides in
677 reducing the number of CPB infested pods (Vanhove *et al.*, 2019). This product is not yet
678 commercially available.

679

680 Preliminary studies in PNG and elsewhere have shown that the trap type influences capture,
681 however there is little published literature developing, or comparing traps. A study in Sabah,
682 Malaysia compared three pheromone (100g)-baited (Beevor et al. 1986) trap types, Delta,
683 Pherocon V scale, and Pherocon 1C (Tre´ce´, Salinas, CA) in cocoa fields (Zhang et al 2008). The
684 authors showed that the Delta traps were more effective than Pherocon V scale traps.

685 Continued work in this space is warranted and should include improving the male pheromone,
686 developing a female sex pheromone and assessing the effectiveness of different trap types
687 under a range of conditions. Understanding whether there are differentially responding *C.*
688 *cramerella* populations will be core to developing a lure, or lures to use in integrated pest
689 management (IPM).

690 ***Chemical Control***

691 Insecticides have been widely used to manage this pest, particularly in high input - high output
692 systems (Gotsch, 1997; Bateman, 2009), however resistance to commonly used products has

been recorded (Alias *et al.*, 1999; Bhatia *et al.*, 2000). The effectiveness of pesticides against adults is hampered by the high amount of immigration into the sprayed area (Day *et al.*, 1995), while the larvae live inside the pod, where they are not exposed to insecticide spraying (Day, 1989; Shapiro *et al.*, 2008). Targeting the adult stage by observing behaviour patterns including where the adults were likely to be 'resting' at certain times of day, is an approach that has been reported but this was also countered by the high rate of immigration (Day *et al.*, 1995).

Despite the problems with the use of pesticides, frequent use is reported in some areas with up to biweekly applications at a huge cost (Perry *et al.*, 2018). The potential of botanical insecticides have been investigated utilising a plant family commonly found throughout South East Asia. The Zingiberaceae includes commonly cultivated plants such as turmeric and ginger.

Cc larvae leave the cocoa pod to pupae, sometimes on the surface of the pod but most commonly on leaves or in the left litter around the tree. Targeting the pupal stages which is the life stage found outside the pod. Some compounds of essential oils applied to cc pupae were found to cause adult deformaties with later resulted early death (Perry *et al.*, 2018)

Control was typically calendar spraying which lead to the development of resistance as well as environmental and health problems (Adu-Acheampong *et al.*, 2015; Babin, 2018).

To reduce the cost of chemicals, farmers in some countries still source the cheaper but unapproved older products or product mixtures, incorrect rates and unsuitable timing (Adu-Acheampong *et al.*, 2015). Another reason why pesticide use is high is that until recently there was often no action thresholds for pests of cocoa (Adu-Acheampong *et al.*, 2015). In Ghana the government run mass spraying program was implemented to boost cocoa yields as individual

farmers often did not own spraying equipment, yet problems included timing relied on when the spraying teams were available as opposed to spraying when needed as well as the ecological consequences of blanket spraying large areas (Adu-Acheampong *et al.*, 2015).

Biopesticides are another safer option, however have been little investigated for CPB. There is a project currently underway to identify whether the extracts of several native tree species in PNG are effective for CPB control. Preliminary studies suggest that four may be promising.

Mechanical Control

Placing sleeves over cocoa pods, to prevent the adult moth from ovipositing, has shown some potential, however timing is crucial. In Indonesia, a field trial showed that only 50% of the sleeved pods were protected from *C. cramerella* and that this was attributed to timing (Rosmana *et al.,* 2010). Placing sleeves on the pods earlier can reduce *C. cramerella* infestation by 85-100%. However, this can be confounded by Phytophthora pod rot, or wilt, i.e. physiological death of the pod. Plastic bags have typically been used as sleeves, however the environmental issue these cause has led to investigations of biodegradable forms (Babin, 2018).

Varietal Resistance

There is no cocoa variety that has been identified as resistant to *C. cramerella*. The currently suggested strategy is to plant a mix of varieties, including highly susceptible varieties which may act as a trap crop and concentrate oviposition to a smaller area (McMahon *et al.*, 2009; Babin, 2018). Smallholder farmers commonly grow locally collected seeds, which has led to a large genetic diversity of cocoa (McMahon *et al.*, 2015; Babin, 2018) in addition to varieties distributed from government genetic development programs (Eskes, 2011).

735 Investment in the development of varieties resistant to diseases has probably received the
736 most research attention over the past three decades, and to date has not produced a cultivar
737 with complete resistance to any cacao insect pest (Babin, 2018). Cocoa varieties that have
738 shown some resistance, or tolerance to *C. cramerella,* have higher larval mortalities in the
739 cocoa pod and thus reduced yield losses (Day, 1989). Factors such as pod-surface smoothness,
740 timing of pod development and pod hardness impact the breeding success of the *C. cramerella*
741 (Teh *et al.*, 2006).

742 The diversity of cocoa currently planted in smallholdings is a resource for local selection and
743 hybrid crossing for pest/disease resistance and improved yield and quality (McMahon *et al.*,
744 2015). However, the importance of testing developed genotypes under local conditions in the
745 first instance is integral when deciding upon which cultivars to recommend to local growers
746 (McMahon *et al.*, 2015). Cocoa varieties respond differently to pest attack at different climates.
747 Pod hardness is one aspect of varietal resistance that has been explored to reduce CPB
748 populations. The pod hardness is a partial resistance trait that did not necessary prevent attack
749 but lower numbers of larvae emerging from the pod reduced total CPB populations (Teh *et al.*,
750 2006).

751 Some work has been done on developing better varieties. An ACIAR funded project in Indonesia
752 is one example of working with the growers to select the best traits (McMahon *et al.*, 2009;
753 McMahon *et al.*, 2015). Later results showed that location affects varietal yield and pest
754 resistance (McMahon *et al.*, 2015).

Plant breeding programs often have multiple goals including improving yield, quality, pest and disease tolerance as well as plant architecture to make management easier (Monteiro *et al.*, 2009). Survival of *C. cremerella* in the pod is associated with the sclerotic layer hardness and thickness and is a criteria considered in the selection of CPB resistant varietal development.

Sterile Insect Technique

The sterile insect technique is a widely accepted method of managing a variety of insects pests (Hendrichs *et al.*, 2007; Teal *et al.*, 2007). SIT involves mass rearing and exposure of the male pest species to irradiation to induce sterility. The sterile males are then released into the field pest population where they mate with the wild females which then lay non-viable eggs (Knipling, 1955). The sterile insect technique (SIT), and inherited sterility are currently being explored as an option to manage *C. cramerella*. SIT involves mass rearing and exposure of the male pest species to irradiation to induce sterility (Knipling, 1955). The sterile males are then released into the field pest population where they mate with the wild females which then lay non-viable eggs. Another form of SIT, particularly useful in Lepidoptera, is Inherited sterility which is especially pronounced in the first filial (F1) generation following the exposure of the parents to substerilizing doses of ionizing radiation. This method involves rearing the F1 generation in the field, thereby reducing costs. Total insect population control by SIT is an area-wide top down approach to pest control usually funded and managed by governments or industry organisations. Although managed outside individual farms AW-SIT requires the cooperation of all stakeholders to ensure efficiency and success (Hendrichs *et al.*, 2007). This technique relies on knowledge of the physiology and ecology of each pest species as well as the

ability to mass produce large numbers of insects (Hendrichs *et al.*, 2007). Unfortunately the technology developed for many other SIT programs does not directly transfer easily to other species such as *C. cramerella*, however by following the process that these programs used a program could be created with considerably less effort than what was needed to develop SIT programs such as with the Tephritid fruit flies (Hendrichs *et al.*, 2007).

Larval Diet

The ability to rear insects on artificial diets is the basis of many successful biological control and area wide management programs (Awang *et al.*, 2006; Cohen, 2015). Artificial diets are used for both laboratory rearing to study the lifecycle of a species to determine how best to manage that pest, and for mass rearing for SIT and biological control programs (Cohen, 2015). All insect diets require species specific mixtures of amino acids, carbohydrates, lipids, vitamins and minerals (Chapman, 2012). If any of these components are missing or insufficient the insects may grow but not fully complete their lifecycle (Pritam, 1977; Chapman, 2012; Cohen, 2015).

Several years work (commencing in the late 1990's) on an artificial diet for *C. cramerella* has been completed in Malaysia (Furtek *et al.*, 2001), and Indonesia (Santoso *et al.*, 2004). Prior to this, all work on Gracillariidae species was based on wild specimens (Awang *et al.*, 2006). Awang *et al.* (2006), in the first focused diet development work, tested 50 different diet formulations to rear CPB. Of these many diets tested, only one could they get full life cycle development from egg through to adult, but less than 1% of eggs became adults. It was thus not surprising that colonies could not be sustained. Although many diet formulations allowed the larval stages

798 to develop, the inhibiting step was the production of a cocoon, which was suggested a result of
799 a lack of key nutrients, and possibly the conditions in the artificial rearing environment (Awang
800 *et al.*, 2006). The latter option, which includes good sanitation of the rearing environment, staff
801 training and work flow processes are often key components that are overlooked when insect
802 mass rearing is attempted, and which all impact on the reared insects quality (Schneider, 2009).

803 Previous formulations have contained immature cocoa pulp, cocoa placenta, egg yolk,
804 cellulose, starch, casein, yeast, choline chloride, i-inositol, Vanderzant vitamins, flaxseed oil,
805 agar and water, but the actual amounts of each component in the diets was not given (Awang,
806 2006). A new proposed diet formulation and preparation protocol for rearing CPB will not
807 adopt fully the traditional Lepidopteran based formulation e.g. Awang *et al.* (2006). Instead it
808 will have a further two approaches. The first, a diet composition simplified to a yeast, sugar,
809 cocoa bean powder, antimicrobials, agar, wheat germ oil, and water formulation, and the
810 preparation protocol involving mixing all ingredients at one time in a blender as Chang (2009)
811 uses for their fruit fly larval diet . The second will be the comparative slaughter (or whole
812 carcass) technique to formulate an artificial diet for CPB. This is based on the hypothesis that
813 the composition of an insects body reflects its nutritional needs (Rock and King, 1967), and uses
814 the profile of the various nutrients in a target insects body as a template. It is then compared to
815 the nutrient composition of the target insects natural feed. Although this method has been
816 discussed by Cohen (2015), it has not been widely adopted by entomologists developing
817 artificial diets for insects, even though it has been a standard method for animal scientists to
818 determine various domesticated animals and birds energy and protein requirements (Blaxter,
819 1967).

However, the most effective diet developed may be a combination of the three strategies mentioned above. In this way, a diet can be formulated that is best suited to the nutritional requirements of *C. cramerella*, leading to biological parameters, such as higher fecundity, fertility and body weight that indicate greater insect fitness (Woods 2019).

Conclusions

In this critical analysis we have drawn together the literature on the identification and management of *C. cramerella*, highlighting the discrepancies, and the gaps in our knowledge, while suggesting areas of current and future research that require attention in order to manage this pest effectively and sustainably. Underpinning this is the need to develop an artificial diet that is both cost-effective and has minimal waste, with the ability to culture *C. cramerella* a priority to facilitate sustainable and effective pest management, and offer the possibility for the mass-rearing of parasitoids of this pest, and also for use in sterile insect technique programs. Further studies resolving the identification of the CPB attacking cocoa and other host plants, including native hosts, including population genetic studies, will permit greater clarification around whether management practices targeting non-cocoa hosts will be effective. Understanding and conserving natural enemies of *C. cramerella* in agroecosystems is an area that has received little attention. Similarly, endophytic entomopathogenic fungi is an exciting area that has not yet realised it potential.

As *C. cramerella* can readily disperse via the wind or via human-assisted transport of infested pods, or other material, it would benefit if pest management extended to area wide

management, rather than at the farm level, to minimise the movement of fertile females into managed areas. Socio-economic factors also need to be considered, and vary between countries, but understanding these complexities would aid a landscape-level approach to managing this serious pest of cocoa. Ultimately, *C. cramerella*, although a major pest in most cocoa producing areas in SE Asia and the Oceania regions, is one of several pests and diseases impacting in these regions, and any pest management activity needs to consider this in their approach to managing this devastating pest.

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Supplementary Table S1.

DNA barcode equivalent COI sequences of *Conopomorpha* specimens available at GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and or BOLD (http://www.boldsystems.org/) repositories (last searched 27/February/2019). Except where not reported (N/R), specimen sequences listed by accession (GenBank) and or by process ID (BOLD). Terminal clustering of similar sequences to discrete molecular operational taxonomic units listed by BOLD:BIN (Ratnasingham and Hebert, 2013). Taxonomic description (species level) as reported for specimen sequence at repository(s), and indicating BOLD:BIN (in parentheses) for undefined species. National Center for Biotechnology Information (NCBI) taxonomy identity numbers associated with GenBank accession records lacking a formal taxonomic description. Notes

column (footnoted) indicate instances where sequence suppressed at repository and or assigned to species based exclusively on BOLD:BIN association with identified taxa.

GenBank accession	BOLD process ID	BOLD: BIN	Taxonomic description	NCBI notes
KY323236	FRUT636-13	BOLD:AAA4000	*Conopomorpha cramerella*	
KY323184	FRUT637-13	BOLD:AAA4000	*Conopomorpha cramerella*	
KY323123	FRUT678-14	BOLD:AAA4000	*Conopomorpha cramerella*	
KY323290	FRUT681-14	BOLD:AAA4000	*Conopomorpha cramerella*	
KJ657669	GBGL14622-14	BOLD:AAA4000	*Conopomorpha cramerella*	
KJ657670	GBGL14623-14	BOLD:AAA4000	*Conopomorpha cramerella*	
EU644601	GBGL6017-09	BOLD:AAA4000	*Conopomorpha cramerella*	
EU644600	GBGL6018-09	BOLD:AAA4000	*Conopomorpha cramerella*	
EU644599	GBGL6019-09	BOLD:AAA4000	*Conopomorpha cramerella*	
EU644598	GBGL6020-09	BOLD:AAA4000	*Conopomorpha cramerella*	
EU644597	GBGL6021-09	BOLD:AAA4000	*Conopomorpha cramerella*	
EU644596	GBGL6022-09	BOLD:AAA4000	*Conopomorpha cramerella*	
EU644595	GBGL6023-09	BOLD:AAA4000	*Conopomorpha cramerella*	
EU644594	GBGL6024-09	BOLD:AAA4000	*Conopomorpha cramerella*	
EU644593	GBGL6025-09	BOLD:AAA4000	*Conopomorpha cramerella*	

EU644592	GBGL6026-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644591	GBGL6027-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644590	GBGL6028-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644589	GBGL6029-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644588	GBGL6030-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644587	GBGL6031-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644586	GBGL6032-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644585	GBGL6033-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644584	GBGL6034-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644583	GBGL6035-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644582	GBGL6036-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644581	GBGL6037-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644580	GBGL6038-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644579	GBGL6039-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644578	GBGL6040-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644577	GBGL6041-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644576	GBGL6042-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644575	GBGL6043-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644574	GBGL6044-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644573	GBGL6045-09	BOLD:AAA4000	*Conopomorpha cramerella*

EU644572	GBGL6046-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644571	GBGL6047-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644570	GBGL6048-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644569	GBGL6049-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644568	GBGL6050-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644567	GBGL6051-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644566	GBGL6052-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644565	GBGL6053-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644564	GBGL6054-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644563	GBGL6055-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644562	GBGL6056-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644561	GBGL6057-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644560	GBGL6058-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644559	GBGL6059-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644558	GBGL6060-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644557	GBGL6061-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644556	GBGL6062-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644555	GBGL6063-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644554	GBGL6064-09	BOLD:AAA4000	*Conopomorpha cramerella*
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EU644552	GBGL6066-09	BOLD:AAA4000	*Conopomorpha cramerella*
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EU644549	GBGL6069-09	BOLD:AAA4000	*Conopomorpha cramerella*
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EU644543	GBGL6075-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644542	GBGL6076-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644541	GBGL6077-09	BOLD:AAA4000	*Conopomorpha cramerella*
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EU644535	GBGL6083-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644534	GBGL6084-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644533	GBGL6085-09	BOLD:AAA4000	*Conopomorpha cramerella*

EU644532	GBGL6086-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644531	GBGL6087-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644530	GBGL6088-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644529	GBGL6089-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644528	GBGL6090-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644527	GBGL6091-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644526	GBGL6092-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644525	GBGL6093-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644523	GBGL6094-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644522	GBGL6095-09	BOLD:AAA4000	*Conopomorpha cramerella*
EU644521	GBGL6096-09	BOLD:AAA4000	*Conopomorpha cramerella*
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EU644514	GBGL6103-09	BOLD:AAA4000	*Conopomorpha cramerella*
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EU644512	GBGL6105-09	BOLD:AAA4000	*Conopomorpha cramerella*

EU644511	GBGL6106-09	BOLD:AAA4000	*Conopomorpha cramerella*		
EU644510	GBGL6107-09	BOLD:AAA4000	*Conopomorpha cramerella*		
EU644524	GBGL6108-09	BOLD:AAA4000	*Conopomorpha cramerella*		
GU816413	GBGL8970-12	BOLD:AAA4000	*Conopomorpha cramerella*		
HQ824808	GBMIN37980-13	BOLD:AAA4000	*Conopomorpha cramerella*		
HQ824806	GBMIN37981-13	BOLD:AAA4000	*Conopomorpha cramerella*		
HQ824804	GBMIN37982-13	BOLD:AAA4000	*Conopomorpha cramerella*		
HQ824809	GBMIN38042-13	BOLD:AAA4000	*Conopomorpha cramerella*		
HQ824807	GBMIN38043-13	BOLD:AAA4000	*Conopomorpha cramerella*		
HQ824805	GBMIN38044-13	BOLD:AAA4000	*Conopomorpha cramerella*		
HM900592	EPNG1066-10	BOLD:AAA4000	*Conopomorpha cramerella*	895236	1
GU816414	GBGL8971-12	BOLD:AAW1281	*Conopomorpha litchiella*		
N/R	GRANO147-11	BOLD:AAW1281	*Conopomorpha litchiella*		2
KX150515	GBGL28574-19	BOLD:ADR5157	*Conopomorpha litchiella*		
KX150516	GBGL28575-19	BOLD:ADR5157	*Conopomorpha litchiella*		
KX150517	GBGL28576-19	BOLD:ADR5157	*Conopomorpha litchiella*		
KX150518	GBGL28577-19	BOLD:ADR5157	*Conopomorpha litchiella*		
KF392883	ANICY269-11	BOLD:AAY7068	*Conopomorpha heliopla*		
N/R	LOTSG613-12	BOLD:AAY7068	*Conopomorpha heliopla*		
N/R	MCCAA2989-12	BOLD:AAY7068	*Conopomorpha heliopla*		

N/R	LSM1156-11	BOLD:AAY7069	*Conopomorpha heliopla*		2
KU578041	GBMIN81224-17	BOLD:ACD5201	*Conopomorpha sinensis*		
HQ824810	GBMIN37979-13	BOLD:ACD5201	*Conopomorpha sinensis*		
HQ824811	GBMIN38041-13	BOLD:ACD5201	*Conopomorpha sinensis*		
KX150507	GBGL28578-19	BOLD:ACD5201	*Conopomorpha sinensis*		
KX150508	GBGL28579-19	BOLD:ACD5201	*Conopomorpha sinensis*		
KX150509	GBGL28580-19	BOLD:ACD5201	*Conopomorpha sinensis*		
KX150510	GBGL28581-19	BOLD:ACD5201	*Conopomorpha sinensis*		
KY323139	FRUT677-14	BOLD:ACP2572	*Conopomorpha* sp. (ACP2572)	1963046	
KY323244	FRUT679-14	BOLD:ACY9663	*Conopomorpha* sp. (ACY9663)	1963047	
N/R	WANSP351-15	BOLD:ACY9663	*Conopomorpha* sp. (ACY9663)		
HQ946837	EPNG2125-10	AAN5037	*Conopomorpha* sp. (AAN5037)	962558	1
N/R	MINE124-12	AAN5037	*Conopomorpha* sp. (AAN5037)		
HQ946836	EPNG2124-10	BOLD:AAI5875	*Conopomorpha* sp. (AAI5875)	961210	1
HQ946838	EPNG2126-10	BOLD:AAI5875	*Conopomorpha* sp. (AAI5875)	961210	1
N/R	MINE042-08	BOLD:AAI5875	*Conopomorpha* sp. (AAI5875)		
N/R	MINE043-08	BOLD:AAI5875	*Conopomorpha* sp. (AAI5875)		
N/R	SMLM947-14	BOLD:AAI5875	*Conopomorpha* sp. (AAI5875)		2
N/R	SMLM952-14	BOLD:AAI5875	*Conopomorpha* sp. (AAI5875)		2
N/R	SMLM981-14	BOLD:AAI5875	*Conopomorpha* sp. (AAI5875)		2

N/R	SMLM988-14	BOLD:AAI5875	*Conopomorpha* sp. (AAI5875)	2
N/R	EPNG8963-15	BOLD:AAI5875	*Conopomorpha* sp. (AAI5875)	2
N/R	SMLM993-14	BOLD:AAI5875	*Conopomorpha* sp. (AAI5875)	2

Notes:

1. GenBank accession suppressed due to lack of tentative taxonomic identity - identified here based solely on BOLD:BIN association with described taxa .

2. BOLD specimen record lacking taxonomic identity - identified here based solely on BOLD:BIN association with described taxa.

Reference

Ratnasingham, S., Hebert, P.D.N., 2013. A DNA-Based Registry for All Animal Species: The Barcode Index Number (BIN) System. PLOS ONE 8, e66213.

Appendix 2

Draft Scientific paper of DNA barcode work

Title: DNA barcode analysis of cocoa pod borer moths in cacao plantations of Papua New Guinea

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Abstract

The "cocoa pod borer" (CPB) moth is the most significant pest affecting cacao (*Theobroma cacao* L.) plantations in Papua New Guinea and in South East Asia. The taxonomic identity of CPB is attributed to *Conopomorpha cramerella* (Snellen), a micro Gracillariid moth species endemic to equatorial Australasia and IndoMalaya which has evolved a strong host preference for introduced cacao plants. Potential cryptic species diversity in CPB has been speculated but not validated by earlier genetic research, though sampling for those efforts was limited. The potential presence of multiple species diversity in CPB is of concern to agencies developing species specific lure and control tools for management of the pest. Here we used DNA barcoding to identify species diversity of CPB obtained from affected cacao fruit sampled from three geographically widespread provinces in PNG, at the eastern periphery of the pest's distribution. Specimen DNA barcodes were compared to online reference sequences of *C. cramerella* and other species to provide matched species identities.

Our results genetically identified over 94% of 177 sampled specimens to *C. cramerella*; levels of genetic diversity among these *C. cramerella* are depauperate, and in the East New Britain and Bougainville provinces, are fixed for a common and widespread haplotype reported earlier as prevalent across the species distribution.

Ten specimens were not genetically matched to *C. cramerella*. Of these, DNA barcodes of the Olethreutine moth *Thaumatotibia zophophanes* was recorded from East Sepik and East New Britain provinces. In addition, DNA barcodes of one unidentified *Conopomorpha* species recorded from East New Britain and six other unidentified moth species were detected but lacked matching online sequence references for their comparative identification.

In summary, DNA barcoding indicated the vast majority of CPB sampled from three cacao producing provinces were identified to *C. cramerella*, with a minor but diverse component of other moth species, some of which may require taxonomic description. The extent to which this minor assemblage of moth species impact upon cacao plantations in PNG (and potentially elsewhere) remains to be determined.

Keywords: *Conopomorpha cramerella*, *Theobroma cacao*, cytochrome *c* oxidase subunit I (COI) gene

Introduction

Production of cacao (*Theobroma cacao* L.; Malvaceae) beans from plantations in Papua New Guinea (PNG) and South East Asia is significantly affected by damage to fruit caused by larvae of the "cocoa pod borer" (CPB) moth *Conopomorpha cramerella* Snellen, 1904 (Gracillariidae). The moth is endemic to equatorial locations in Indomalaya and Australasia, where it is associated with at least twelve native host plants mainly within Sapindaceae (De Prins and De Prins, 2018) including several economically important fruiting species such as *Nephelium lappaceum* L. ("Rambutan"). Evidence of a shift to cacao as the optimal preferred host by *C. cramerella* (Gende, 2012) is surprising, given that cacao is a new world species and was 1st introduced as a cash crop to South-East Asia during the 16th century (Day 1985). Pest status of this moth was 1st noted at the earliest South East Asian cacao plantations established in central Java and Sulawesi (Indonesia) during the mid to late 19th Century (Mumford 1986). Substantial CPB damage to emergent plantations in the Philippines were reported from 1936 (Uichanco 1936), and in East Malaysia from 1980 (Shao 1982). In PNG the CPB pest was identified among plantations in East New Britain and East Sepik provinces during 2006, and subsequently over the following five years was observed more broadly as a plantation pest in most cacao producing provinces in the country (Gende 2012).

The rapid appearance of CPB among geographically distant cacao plantations across the Malay Archipelago and to the eastern provinces of PNG suggests its movement has been facilitated by progressive regional expansion of the cacao industry and likely aided by broad scale transfer of infected plant stocks among plantations (Gende 2012). This hypothesis is indirectly supported to some extent by population genetic analysis of CPB conducted by Shapiro *et al*. (2008) who reported evidence of shallow mitochondrial and nuclear genetic diversity among CPB and a general lack of genetic structure among plantations sampled across much of the pest's distribution. Shapiro et al. (2008) argued this multi-locus genetic pattern was similar to that frequently evidenced in the introduced ranges of exotic species, where genome wide genetic diversity is lost through serial founder events as a species progressively moves into new territory. Earlier suggestion of cryptic species diversity among CPB based on allozyme analysis of a Malaysian population (Rita and Tan 1987) was not substantiated by genetic evidence reported by Shapiro et al (2008) which unequivocally showed presence only of

diminished population level genetic variation in the widespread *C. cramerella* pest. Despite the broad regional scope of the genetic survey conducted by Shapiro et al (2008), location sampling in that study was low (average < 7 samples per site). Subsequently, the possibility of undetected CPB species diversity remains to be tested using larger sample surveys of CPB directly affecting cacao crops.

DNA barcode (Hebert et al., 2003) analysis of nucleotide sequence variation within and among taxonomically described species can be used as an independent genetic means to test alpha-taxonomic hypotheses (Gopurenko et al., 2015). Where pre-existing DNA barcodes are available for representative taxa, the method also provides a powerful diagnostic genetic tool for species identification of morphologically ambiguous specimens. Here we used DNA barcoding of the mitochondrial encoded 5' cytochrome *c* oxidase subunit I (COI) gene to identify species diversity of CPB raised from affected cacao fruit sampled from three geographically widespread provinces in PNG, at the periphery of the species eastern distribution. For comparative purposes, we included DNA barcode equivalent sequences from earlier genetic analysis of CPB reported by Shapiro et al. (2008) and all available reported barcode equivalent sequences available at other *Conopomorpha* species.

Methods

Sampling Locations

Field sampling occurred throughout a range of sites in the three main cocoa production provinces of PNG; East New Britain (where the 2006 outbreak was first recorded), East Sepik and the Autonomous Region of Bougainville (Bougainville) (Fig. 1).

Fig. 1. Map of Papua New Guinea showing the three major cocoa growing provinces that were sampled for CPB.

In each province where sampling occurred, a range of methods were employed to survey for *C. cramerella*. These included pheromone trapping, sweep netting, fruit collection, and leaf inspection.

i) Pheromone trapping

Two types of traps were utilised, a modified Biotrap that had four 2cm diameter holes cut out of the lids, (BioTrap Australia Pty Ltd, Victoria, Australia) and a UNI-Trap (AlphaTrap, Oregon, USA). These were baited with *C. cramerella* lures (Supplier details) and contained a 1cm$_2$ dichlorvos-impregnated strip. Traps were placed randomly (singly) in fruiting cocoa trees (all provinces), fruiting rambutan trees (East Sepik only), native forest (ENB only) and native rainforest (East Sepik only), for at least 24h and up to 14 days. Traps were checked daily or weekly/fortnightly and adult *C. cramerella* and some of the bi-catch were collected and stored.

ii) Sampling infested cocoa pods

In each sampling location, *C. cramerella* infested cocoa pods were harvested from the tree, split open using a knife, or machete and carefully inspected for the presence of an insect. Pods that displayed the typical uneven ripening were targeted. Larvae were collected and stored as described below.

iii) Leaf inspection

Leaf litter and leaves on host and non-host plants were visually inspected for the presence of C. cramerella pupae in all cocoa plantations. Pupae were collected and stored as described below.

iv) Sweep netting

Regular sweeping of leaves around and within the cocoa canopy yielded some CPB and a range of other cocoa pests. This activity was typically 5 minutes in duration in places where traps were deployed or fruit collected, but was limited in some instances by the degree to which the cocoa canopy was closed over thus restricting (any) movement.

Curation of Samples

Immature stages were placed in 99% ethanol, while adults were either micropinned and dried or placed in ethanol. Ethanol preserved larvae and pinned adult specimens were received and curated at the biological collections unit in NSW DPI (Orange Agricultural Institute). All

specimen records and associated sequences were reported to the Barcode of Life Data Systems [BOLD] (Ratnasingham and Hebert, 2007) repository under project "Cocoa pod borer in Papua New Guinea" (project code: CPBNG), refer Appendix 3 Supplementary Table S1.

DNA barcoding

Individual specimens were non-destructively digested overnight at 55 °C in 330 µl aliquots containing 310 µl of buffer ATL (with 100% ethanol additive) and 20 µl of Proteinase K (600 mAU/ml) as provided in DNeasy®, Blood & Tissue Kit (Cat ID: 69506; QIAGEN). Prior to digestions, specimens where cleared of ethanol by overnight evaporation. DNA extraction of 250 µl of each digestion was done using volume adjusted reagents and recommended protocols provided in GenElute™ 96 well tissue genomic DNA extraction kit (G1N9604; Sigma-Aldrich). Final DNA eluted to 170 µl was used as template (2 µl) in PCR reactions (15 µl). Semi-automated liquid handling protocols for PCR preparation and sequence picking followed that reported in Gopurenko *et al*. (2013). PCR targeted a 658 bp portion of the 5' mitochondrial cytochrome *c* oxidase I (COI) gene using primers LepF1 and LepR1 (Hebert *et al*. 2004) and in < 3 % of samples using primer AMbc0F1m (Mitchell 2015) as a substitute for LepF1 when initial PCRs failed. Forward and reverse primers were 5' tailed with directional 17 mer M13 vector sequence to facilitate outsourced bi-directional Sanger sequencing at the Australian Genome Research Facility (AGRF, Brisbane node).

Bi-directional sequence chromatograms were quality checked and assembled using Lasergene SeqMan Pro ver. 8.1.0 (DNASTAR Inc., Madison, WI, USA) against reference COI sequence of *C. crammerella* (GenBank accession: HQ824804). Primer truncated consensus sequences were exported in FASTA format and aligned using BioEdit ver. 7.0.5.3 (Hall 1999). Sequence haplotypes in the alignment were listed using FABOX ver. 1.35 (Villesen 2007). Haplotype exemplars were queried for best match to existing COI accessions at NCBI and Barcode of Life Data systems [BOLD] (Ratnasingham and Hebert, 2007) sequence repositories (online searches: 01 Aug. 2019) using *BLASTN* and *Identification Engine* search tools respectively. Haplotype sequences were conservatively treated as unmatched if < 98 % similar to reported accessions; in these cases, the taxonomy of the nearest matching accession was cautiously noted as the closest comparative reference to the query. Haplotypes matched (> 98% sequence similarity) to reported *C. crammerella* accessions were further typed where 100% matched to

C. crammerella COI haplotypes (A- F) reported by Shapiro *et al*. (2008) or typed as novel where not matched.

Genetic distance relationships among COI haplotypes were inferred by Neighbour-joining (NJ) tree construction implemented in MEGA X (Kumar et al., 2018) incorporating a Kimura 2 parameter substitution model to accommodate ts/tv nucleotide rate variation, and bootstrap replication (N=10,000 replicates) for obtaining significance of clades. Unknown nucleotide sites were excluded from pairwise estimates used in NJ analysis. Barcode Index Numbers (BINs) (Ratnasingham and Hebert, 2013) auto-assigned to sequence clusters in the BOLD repository (for objectively delimiting molecular operational taxonomic units [MOTU's] in the database) were noted where earlier reported sequence accessions included in genetic analyses lacked an attributed species binomial.

Genealogical relationships among *C. crammerella* haplotypes (detected here and reported earlier by Shapiro et al., 2008) were inferred as a parsimony network using TCS ver. 1.21 (Clement et al. 2000) using a 95% limit imposed on haplotype connections. Summary genetic statistics reporting percent sequence differences among terminal taxa (uniform rates among sites) and regional population estimates of haplotype (h) and nucleotide (π) diversity (Nei 1987) were estimated using DnaSP ver. 5.0 (Librado and Rozas, 2009). DnaSP was also used to conduct Tajima's *D* test for evidence of deviation from nucleotide neutrality among all identified *C. cramerella* sequences.

Results

Sampling of cacao plantations in PNG provided 190 specimens for DNA barcode analyses, and included 33 specimens from East Sepik province, 99 specimens from East New Britain province, and 58 specimens from Bougainville province.

DNA barcodes from 177 of 190 specimens were successfully amplified and sequenced. All primer truncated barcode sequences were 658 bp sequence length except at specimens ww26741, ww26793 and ww27790 (refer Appendix 3, Supplementary Table S1) containing only 399 -599 bp of quality sequence. Twelve different haplotypes were present among the DNA barcodes (Appendix 3, Supplementary Fig. S3A). Three haplotypes present among 167

specimens were genetically matched (> 99% sequence similarity) at GenBank and BOLD to *Conopomorpha cramerella* accessions (Appendix 3, Supplementary Fig S3B). Levels of sequences difference among the 167 genetically identified *C. cramerella* specimens were minimal (maximum pairwise distance < 0.31 %) and most specimens (96.7%) shared a common haplotype (Supplementary Figure S3B). This haplotype was reported earlier by Shapiro et al. (2008) as "haplotype CO-A" and evidenced as the most frequent mitochondrial COI lineage observed among *C. cramerella* sparsely sampled across South-East Asia. Of the remaining *C. cramerella* in our sample, one specimen from East Sepik shared "haplotype CO-B" reported by Shapiro et al. (2008), and four specimens from East Sepik had a novel haplotype ("haplotype CO-novel"). Four other *C. cramerella* haplotypes identified by Shapiro et al. (2008) were absent from the current sample. Collectively, the estimates of haplotype and nucleotide diversity of the 157 *C. cramerella* specimens were shallow at 0.059 and > 0.0001 respectively; East Sepik province contained the most genetically diverse *C. cramerella* population in PNG, evidenced with haplotype and nucleotide diversity of 0.305 and 0.00048 respectively among 29 specimens. In contrast, no sequence diversity was observed among the 157 *C. cramerella* specimens collectively sampled from Bougainville and East New Britain provinces, where "haplotype A" was ubiquitous. Tajima's *D* test statistic for the entire PNG sample was negatively shifted from expectations of neutrality (D = -1.169), suggestive of the effects of a demographic bottle-neck and or selective sweep event(s), however the test was not significant (P > 0.10).

Ten remaining specimens had DNA barcodes unrelated to *C. cramerella* (Appendix 3, Supplementary Table S2 and Figure S3A). Specimen ww27790 sampled from East New Britain differed from *C. cramerella* haplotypes by > 11.1% but was matched at 99.2% similarity to DNA barcodes (N=10) of an unidentified species of *Conopomorpha* from Madang PNG (unpublished DNA barcodes under BOLD BIN:AAI5875). Three specimens (East New Britain & East Sepik provinces) comprising two closely related haplotypes were genetically identified (> 99.5% similarity) to the Olethreutine moth *Thaumatotibia zophophanes* (Tortricidae). Six remaining specimens from Bougainville & East New Britain provinces, each with unrelated singleton haplotypes, could only be ambiguously matched (96.8 - 91.9% genetic similarity) to sequences of various lepidoptera and in one specimen, to orthoptera, the latter likely sampled as an incidental by-catch. Excluding the orthoptera specimen, the presence of up to seven moth species in the current sample other than *C. cramerella*, suggests farmed cacao plants in PNG

may host a broader diversity of pest moth fauna than previously reported, albeit at low frequencies (< 6 % of barcoded specimens) relative to the abundant *C. cramerella* pest.

Discussion

DNA barcoding of 177 CPB sampled from cacao plantations in three PNG provinces identified *Conopomorpha cramerella* as the prevalent pest species in the sample. An additional seven moth species detected by DNA barcoding of the CPB sample collectively represented less than six percent of the barcoded sample (see later).

Genetic diversity among the dominant *C. cramerella* pest in the sample was depauperate and similar in haplotype content to an earlier report by Shapiro et al (2008) of diminished genetic diversity among *C. cramerella* sampled across much of the species distribution. Our DNA barcode results identified three closely related haplotypes among *C. cramerella*, two of which were reported as common by Shapiro et al. (2008), and a third novel haplotype present only at low frequency in East Sepik province plantations. The presence of this minor novel haplotype indicates additional low frequency population genetic diversity in the distribution of *C. cramerella* will likely emerge through more intensive sampling of the species in affected cacao plantations. Interestingly the presence of a single fixed common haplotype at the two eastern island provinces in PNG where most of our sampling was conducted indicates the eastern periphery of the species distribution in PNG is far more genetically depauperate than elsewhere. Similar negative but non-significant neutrality test outcomes for our total CPB sample was also reported by Shapiro et al. (2008) in their multi-region analysis of the species where comparative neutrality testing of two independent loci indicated low genetic diversity across the species distribution was likely caused by demographic founder events as the species progressively moved east with the cacao industry, rather than through selective sweep processes for a favoured genotype of the species. The extremely low level of nucleotide diversity evidenced in our mitochondrial DNA barcode sample of *C. cramerella* is insufficient to allow meaningful statistical tests of population gene flow among sampled provinces. Future population genetic analysis of this species may better benefit from analysis of more polymorphic loci such as microsatellites and or genome wide sequence analysis of single nucleotide polymorphisms.

The presence of up to seven additional CPB pest moth species evidenced here, albeit at low levels of abundance, is significant for future CPB research and management. The taxonomic uncertainty concerning CPB moths has persisted for several decades, largely driven by taxonomic confusion of the main pest *C. cramerella* relative to its closely related congeners, and to some extent, by inaccuracy of field diagnosed CPB. The interim taxonomic report by Bradley (1986) clarified descriptions of *C. cramerella* and three previously unrecognised congeneric species, all of which are "*remarkably similar in wing pattern and coloration*" and partially sympatric in distribution. Bradley refereed to these four species as within a species-complex due to the subtlety of their morphological differences, though only one of which (*C. cramerella*) was known to use cacao as a host plant. Most importantly, Bradley (1986) provided key features to separate *C. cramerella* from *C. litchiella* Bradley, the latter species being an economically important pest of litchi (*Litchi chinensis*, Sapindaceae) and widely misreported as *C. cramerella* following an incorrect listing in India by Fletcher (1916).

Potential multiple species diversity among CPB was noted by Rita and Tan (1987) based on allozyme analysis of CPB raised from cacao pods in Malaysia. Multiple species diversity in CPB was however not evidenced in the broader distribution of the pest examined by Shapiro et al. (2008) whose sequence based genetic evidence of two independent loci indicated presence only of population level genetic variation in the widespread *C. cramerella* pest. Unpublished analysis of three putative CPB "biotypes" trapped using CPB lures in East New Britain during 2009 were genetically identified to *C. cramerella* and two unrelated and undefined species (refer [I. Valenzuela comm.], pp 28, Gende 2012), however these CPB "biotypes" were trapped outside of cacao plantations, so pest status of the two unidentified species is unclear.

An additional contributing factor affecting CPB species identifications concerns operations-based identifications of moth larvae present in cacao fruit. For example, *Thaumatotibia zophophanes* genetically identified in the current sample from a larva and two reared adults has an adult morphology readily distinguishable from *C. cramerella*. In contrast, differences among larvae of the two species are less obvious and their presence in cacao fruits may be

potentially misidentified in CPB surveys as the common pest *C. cramerella* by handlers lacking specialised taxonomic expertise. The facility of DNA barcoding to resolve identification of specimens to species at ambiguous life stages has been reported elsewhere. This facility is particularly profitable in cases where there is available a DNA barcode library inclusive of available genetic diversity of the focal taxonomic group being examined, with well-defined genetic separations among taxonomically described species (deWaard et al., 2010; Gopurenko et al., 2013; Fletcher et al., 2016).

Conclusions

DNA barcoding of larvae and adult moths reared from larvae indicate the vast majority of CPB sampled from three cacao producing provinces in PNG are *C. cramerella*, with a minor but diverse component of other moth species. Several of these minor pest moths are not matched to existing DNA barcode records, or in one case, matched to an undefined *Conopomorpha* species distantly related to *C. cramerella*. All of these minor moth species require taxonomic description beyond the scope of this report. The extent to which this minor assemblage of moth species impact upon cacao plantations in PNG (and potentially elsewhere) remains to be determined, though the current report indicates they are likely to be less abundant pests of cacao, relative to that seen for *C. cramerella*.

Acknowledgements

This work was funded by Australian Centre for International Agricultural Research (SRA project: HORT/2018/114). Sample preparations and laboratory tasks in Australia were provided by Ros Mirrington and Sujeewa Rathnayake at respective Orange and Wagga Wagga Agricultural Institutes (NSW DPI).

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Appendix 3.

Supplementary Table S1. Cocoa borer moth sample information and associated DNA barcode records deposited at BOLD (http://v4.boldsystems.org) under project "Cocoa pod borer in Papua New Guinea" (project code: CPBNG). Specimen sample ID's at NSW DPI collections associated with BOLD process ID's linked to DNA barcode records. DNA barcode sequence length in base pairs (bp) as indicated except where sequence unavailable (0). Best matched specimen identifications using BOLD ID genetic search engine, or based on morphology* where DNA barcodes unavailable. General location information and specific sample site co-ordinates (decimal) as indicated.

Sample ID	Process ID (BOLD)	bp	Identification	Province	Location	Latitude	Longitude
ww26701	CPBNG001-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.23093	155.556
ww26702	CPBNG002-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.23093	155.556
ww26703	CPBNG003-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.23093	155.556
ww26704	CPBNG004-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.23093	155.556
ww26705	CPBNG005-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.23093	155.556
ww26706	CPBNG006-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.23093	155.556
ww26707	CPBNG007-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.23093	155.556
ww26708	CPBNG008-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.23093	155.556
ww26709	CPBNG009-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.23093	155.556
ww26710	CPBNG010-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.23093	155.556
ww26711	CPBNG011-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.23093	155.556
ww26712	CPBNG012-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.23093	155.556
ww26713	CPBNG013-19	658	*Conopomorpha cramerella*	Bougainville	Tinputz	-5.59942	154.997
ww26714	CPBNG014-19	658	*Conopomorpha cramerella*	Bougainville	Tinputz	-5.59942	154.997
ww26715	CPBNG015-19	658	*Conopomorpha cramerella*	Bougainville	Tinputz	-5.59942	154.997
ww26716	CPBNG016-19	658	*Conopomorpha cramerella*	Bougainville	Tinputz	-5.59942	154.997
ww26717	CPBNG017-19	658	*Conopomorpha cramerella*	Bougainville	Tinputz	-5.59942	154.997
ww26718	CPBNG018-19	658	*Conopomorpha cramerella*	Bougainville	Tinputz	-5.59942	154.997
ww26719	CPBNG019-19	658	*Conopomorpha cramerella*	Bougainville	Tinputz	-5.59942	154.997
ww26720	CPBNG020-19	658	*Conopomorpha cramerella*	Bougainville	Tinputz	-5.59942	154.997
ww26721	CPBNG021-19	658	*Conopomorpha cramerella*	Bougainville	Tinputz	-5.59942	154.997
ww26722	CPBNG022-19	658	*Conopomorpha cramerella*	East Sepik	Suanum	-3.68931	143.841
ww26723	CPBNG023-19	658	*Conopomorpha cramerella*	East Sepik	Suanum	-3.68931	143.841
ww26724	CPBNG024-19	658	*Conopomorpha cramerella*	East Sepik	Suanum	-3.68931	143.841
ww26725	CPBNG025-19	658	*Conopomorpha cramerella*	East Sepik	Suanum	-3.68931	143.841
ww26726	CPBNG026-19	658	*Conopomorpha cramerella*	East Sepik	Suanum	-3.68931	143.841
ww26727	CPBNG027-19	658	*Conopomorpha cramerella*	East Sepik	Suanum	-3.68931	143.841
ww26728	CPBNG028-19	658	*Conopomorpha cramerella*	East Sepik	Suanum	-3.68931	143.841
ww26729	CPBNG029-19	658	*Conopomorpha cramerella*	East Sepik	Suanum	-3.68931	143.841
ww26730	CPBNG030-19	658	*Conopomorpha cramerella*	East Sepik	Suanum	-3.68931	143.841
ww26731	CPBNG031-19	658	*Conopomorpha cramerella*	East Sepik	Suanum	-3.68931	143.841
ww26732	CPBNG032-19	658	*Conopomorpha cramerella*	East Sepik	Suanum	-3.68931	143.841
ww26733	CPBNG033-19	658	*Conopomorpha cramerella*	East Sepik	Suanum	-3.68931	143.841
ww26734	CPBNG034-19	658	*Thaumatotibia zophophanes*	East Sepik	Suanum	-3.68931	143.841
ww26735	CPBNG035-19	658	*Conopomorpha cramerella*	East Sepik	Suanum	-3.68931	143.841
ww26736	CPBNG036-19	658	*Conopomorpha cramerella*	East Sepik	Suanum	-3.68931	143.841
ww26737	CPBNG037-19	658	*Conopomorpha cramerella*	East Sepik	Suanum	-3.68931	143.841
ww26738	CPBNG038-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.2399	155.564
ww26739	CPBNG039-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.2399	155.564
ww26740	CPBNG040-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.2399	155.564
ww26741	CPBNG041-19	399	*Conopomorpha cramerella*	Bougainville	Arawa	-6.2399	155.564
ww26742	CPBNG042-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.2399	155.564
ww26743	CPBNG043-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.2399	155.564
ww26744	CPBNG044-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.2399	155.564

ww26745	CPBNG045-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.2399	155.564
ww26746	CPBNG046-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.2399	155.564
ww26747	CPBNG047-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.2399	155.564
ww26748	CPBNG048-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.2399	155.564
ww26749	CPBNG049-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.23108	155.557
ww26750	CPBNG050-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.23108	155.557
ww26751	CPBNG051-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.23108	155.557
ww26752	CPBNG052-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.23108	155.557
ww26753	CPBNG053-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.23108	155.557
ww26754	CPBNG054-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.23108	155.557
ww26755	CPBNG055-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.23108	155.557
ww26756	CPBNG056-19	658	*Conopomorpha cramerella*	Bougainville	Buka	-5.35739	154.68
ww26757	CPBNG057-19	658	*Conopomorpha cramerella*	East Sepik	Wewak	-3.49211	143.488
ww26758	CPBNG058-19	658	*Conopomorpha cramerella*	East Sepik	Wewak	-3.49211	143.488
ww26759	CPBNG059-19	658	*Conopomorpha cramerella*	East Sepik	Wewak	-3.49211	143.488
ww26760	CPBNG060-19	658	*Conopomorpha cramerella*	East Sepik	Wewak	-3.49211	143.488
ww26761	CPBNG061-19	658	*Conopomorpha cramerella*	East Sepik	Wewak	-3.49211	143.488
ww26762	CPBNG062-19	658	*Conopomorpha cramerella*	East Sepik	Wewak	-3.49211	143.488
ww26763	CPBNG063-19	658	*Conopomorpha cramerella*	East Sepik	Wewak	-3.49211	143.488
ww26764	CPBNG064-19	658	*Conopomorpha cramerella*	East Sepik	Wewak	-3.49211	143.488
ww26765	CPBNG065-19	658	*Conopomorpha cramerella*	East Sepik	Wewak	-3.49211	143.488
ww26766	CPBNG066-19	658	*Conopomorpha cramerella*	East Sepik	Wewak	-3.49211	143.488
ww26767	CPBNG067-19	658	*Conopomorpha cramerella*	East Sepik	Wewak	-3.49211	143.488
ww26768	CPBNG068-19	658	*Conopomorpha cramerella*	East Sepik	Wewak	-3.49211	143.488
ww26769	CPBNG069-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.2399	155.564
ww26770	CPBNG070-19	658	*Conopomorpha cramerella*	Bougainville	Panguna	-6.40683	155.45
ww26771	CPBNG071-19	658	*Conopomorpha cramerella*	Bougainville	Panguna	-6.40683	155.45
ww26772	CPBNG072-19	658	*Conopomorpha cramerella*	Bougainville	Panguna	-6.40683	155.45
ww26773	CPBNG073-19	658	*Conopomorpha cramerella*	Bougainville	Panguna	-6.40683	155.45
ww26774	CPBNG074-19	658	*Conopomorpha cramerella*	East Sepik	Wewak	-3.49211	143.488
ww26775	CPBNG075-19	0	Insecta*	East Sepik	Wewak	-3.49211	143.488
ww26776	CPBNG076-19	0	Insecta*	East Sepik	Wewak	-3.49203	143.487
ww26777	CPBNG077-19	0	Insecta*	Bougainville	Arawa	-6.23968	155.565
ww26778	CPBNG078-19	0	Insecta*	Bougainville	Arawa	-6.23968	155.565
ww26779	CPBNG079-19	0	Insecta*	Bougainville	Arawa	-6.23968	155.565
ww26780	CPBNG080-19	658	Lepidoptera	Bougainville	Arawa	-6.23108	155.557
ww26781	CPBNG081-19	0	Insecta*	Bougainville	Arawa	-6.2399	155.564
ww26782	CPBNG082-19	0	Insecta*	Bougainville	Arawa	-6.2399	155.564
ww26783	CPBNG083-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.2399	155.564
ww26784	CPBNG084-19	0	Insecta*	Bougainville	Arawa	-6.2399	155.564
ww26787	CPBNG085-19	0	Insecta*	Bougainville	Singh vl.	-5.27812	154.694
ww26788	CPBNG086-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.23093	155.556
ww26789	CPBNG087-19	658	*Conopomorpha cramerella*	East Sepik	Wewak	-3.49211	143.488
ww26790	CPBNG088-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.23108	155.557
ww26791	CPBNG089-19	658	Lepidoptera	Bougainville	Tinpuz	-5.59942	154.997
ww26792	CPBNG090-19	658	*Conopomorpha cramerella*	Bougainville	Arawa	-6.2399	155.564
ww26793	CPBNG091-19	455	Crambidae				
ww27701	CPBNG092-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27702	CPBNG093-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27703	CPBNG094-19	658	*Conopomorpha cramerella*	East New Britain	Raulavat	-4.29111	152.018
ww27704	CPBNG095-19	658	*Conopomorpha cramerella*	East New Britain	Raulavat	-4.29111	152.018
ww27705	CPBNG096-19	658	*Conopomorpha cramerella*	East New Britain	Raulavat	-4.29111	152.018
ww27706	CPBNG097-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27707	CPBNG098-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27708	CPBNG099-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27709	CPBNG100-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27710	CPBNG101-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27711	CPBNG102-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27712	CPBNG103-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27713	CPBNG104-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27714	CPBNG105-19	658	*Conopomorpha cramerella*	East New Britain	Raulavat	-4.28778	152.019
ww27715	CPBNG106-19	658	*Conopomorpha cramerella*	East New Britain	Raulavat	-4.28778	152.019

ww27716	CPBNG107-19	658	*Conopomorpha cramerella*	East New Britain	Raulavat	-4.28778	152.019
ww27717	CPBNG108-19	658	*Conopomorpha cramerella*	East New Britain	Raulavat	-4.28778	152.019
ww27718	CPBNG109-19	658	*Conopomorpha cramerella*	East New Britain	Raulavat	-4.28778	152.019
ww27719	CPBNG110-19	658	*Conopomorpha cramerella*	East New Britain	Raulavat		
ww27720	CPBNG111-19	658	*Conopomorpha cramerella*	East New Britain	Raulavat		
ww27721	CPBNG112-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27722	CPBNG113-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27723	CPBNG114-19	658	*Conopomorpha cramerella*	East New Britain	Raulavat		
ww27724	CPBNG115-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27725	CPBNG116-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27726	CPBNG117-19	658	*Conopomorpha cramerella*	East New Britain	Raulavat		
ww27727	CPBNG118-19	658	*Conopomorpha cramerella*	East New Britain	Raulavat		
ww27728	CPBNG119-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27729	CPBNG120-19	658	*Conopomorpha cramerella*	East New Britain	Raulavat		
ww27730	CPBNG121-19	658	*Conopomorpha cramerella*	East New Britain	Raulavat		
ww27731	CPBNG122-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27732	CPBNG123-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27733	CPBNG124-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27734	CPBNG125-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27735	CPBNG126-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27736	CPBNG127-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27737	CPBNG128-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27738	CPBNG129-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27739	CPBNG130-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27740	CPBNG131-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27741	CPBNG132-19	658	Noctuidae	East New Britain	Raulavat		
ww27742	CPBNG133-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27743	CPBNG134-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27744	CPBNG135-19	0	Insecta*	East New Britain	Raulavat		
ww27745	CPBNG136-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27746	CPBNG137-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27747	CPBNG138-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27748	CPBNG139-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27749	CPBNG140-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27750	CPBNG141-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27751	CPBNG142-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27752	CPBNG143-19	658	*Thaumatotibia zophophanes*	East New Britain	Tavilo		
ww27753	CPBNG144-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27754	CPBNG145-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27755	CPBNG146-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27756	CPBNG147-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27757	CPBNG148-19	658	*Thaumatotibia zophophanes*	East New Britain	Tavilo		
ww27758	CPBNG149-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27759	CPBNG150-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27760	CPBNG151-19	658	Gracillariidae	East New Britain	Kerevat		
ww27761	CPBNG152-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27762	CPBNG153-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27763	CPBNG154-19	658	*Conopomorpha cramerella*	East New Britain	Kerevat	-4.32986	152.131
ww27764	CPBNG155-19	658	*Conopomorpha cramerella*	East New Britain	Raulavat		
ww27765	CPBNG156-19	658	*Conopomorpha cramerella*	East New Britain	Raulavat		
ww27766	CPBNG157-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27767	CPBNG158-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27768	CPBNG159-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27769	CPBNG160-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27770	CPBNG161-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27771	CPBNG162-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27772	CPBNG163-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27773	CPBNG164-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27774	CPBNG165-19	658	*Conopomorpha cramerella*	East New Britain	Kerevat	-4.32986	152.031
ww27775	CPBNG166-19	658	*Conopomorpha cramerella*	East New Britain	Raulavat		
ww27776	CPBNG167-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		
ww27777	CPBNG168-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo		

ww27778	CPBNG169-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo
ww27779	CPBNG170-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo
ww27780	CPBNG171-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo
ww27781	CPBNG172-19	658	Trigonidiidae	East New Britain	Raulavat
ww27782	CPBNG173-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo
ww27783	CPBNG174-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo
ww27784	CPBNG175-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo
ww27785	CPBNG176-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo
ww27786	CPBNG177-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo
ww27787	CPBNG178-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo
ww27788	CPBNG179-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo
ww27789	CPBNG180-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo
ww27790	CPBNG181-19	599	*Conopomorpha*	East New Britain	Raulavat
ww27791	CPBNG182-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo
ww27792	CPBNG183-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo
ww27793	CPBNG184-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo
ww27794	CPBNG185-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo
ww27795	CPBNG186-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo
ww27796	CPBNG187-19	658	*Conopomorpha cramerella*	East New Britain	Tavilo
ww27797	CPBNG188-19	0	Lepidoptera*	East New Britain	Tavilo
ww27798	CPBNG189-19	0	Lepidoptera*	East New Britain	Tavilo
ww27799	CPBNG190-19	0	Lepidoptera*	East New Britain	Tavilo

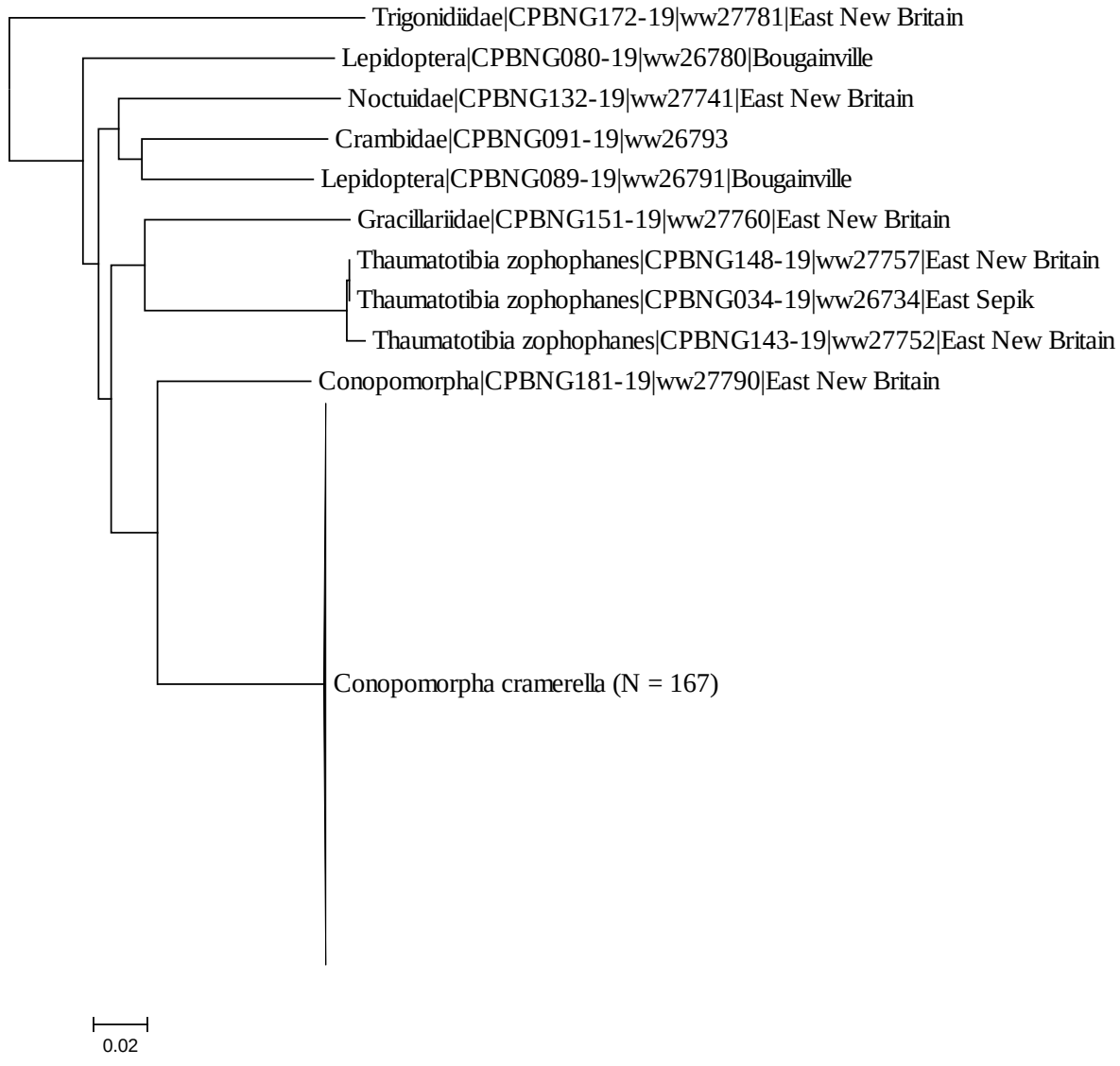
Supplementary Table S2 Closest matched taxonomic identities for DNA barcodes of ten non *Conopomorpha cramerella* specimens sampled among CPB from PNG. Closest matched genetic identity (sequence similarity %) of specimen DNA barcodes determined using the identification engine at BOLD (http://v4.boldsystems.org/index.php/IDS_OpenIdEngine). Identification to species indicated where best matched sequence similarity is > 98%. Taxonomic description below family level available (N/A) for some specimens. * non lepidoptera specimen, possible contamination.

	BOLD best matched identification		
Sample ID	Family	Genus	sequence similarity %
ww26791	Cosmopterigidae	*Limnaecia*	93.4
ww26793	Crambidae	N/A	92.1
ww27760	Gracillariidae	*Stomphastis*	96.9
ww27790	Gracillariidae	*Conopomorpha* sp. (BIN: AAI5875)	99.2
ww27741	Noctuidae	N/A	91.9
ww26780	Riodinidae	N/A	89.6
ww27757	Tortricidae	*Thaumatotibia zophophanes*	100
ww27752	Tortricidae	*Thaumatotibia zophophanes*	99.2
ww26734	Tortricidae	*Thaumatotibia zophophanes*	100

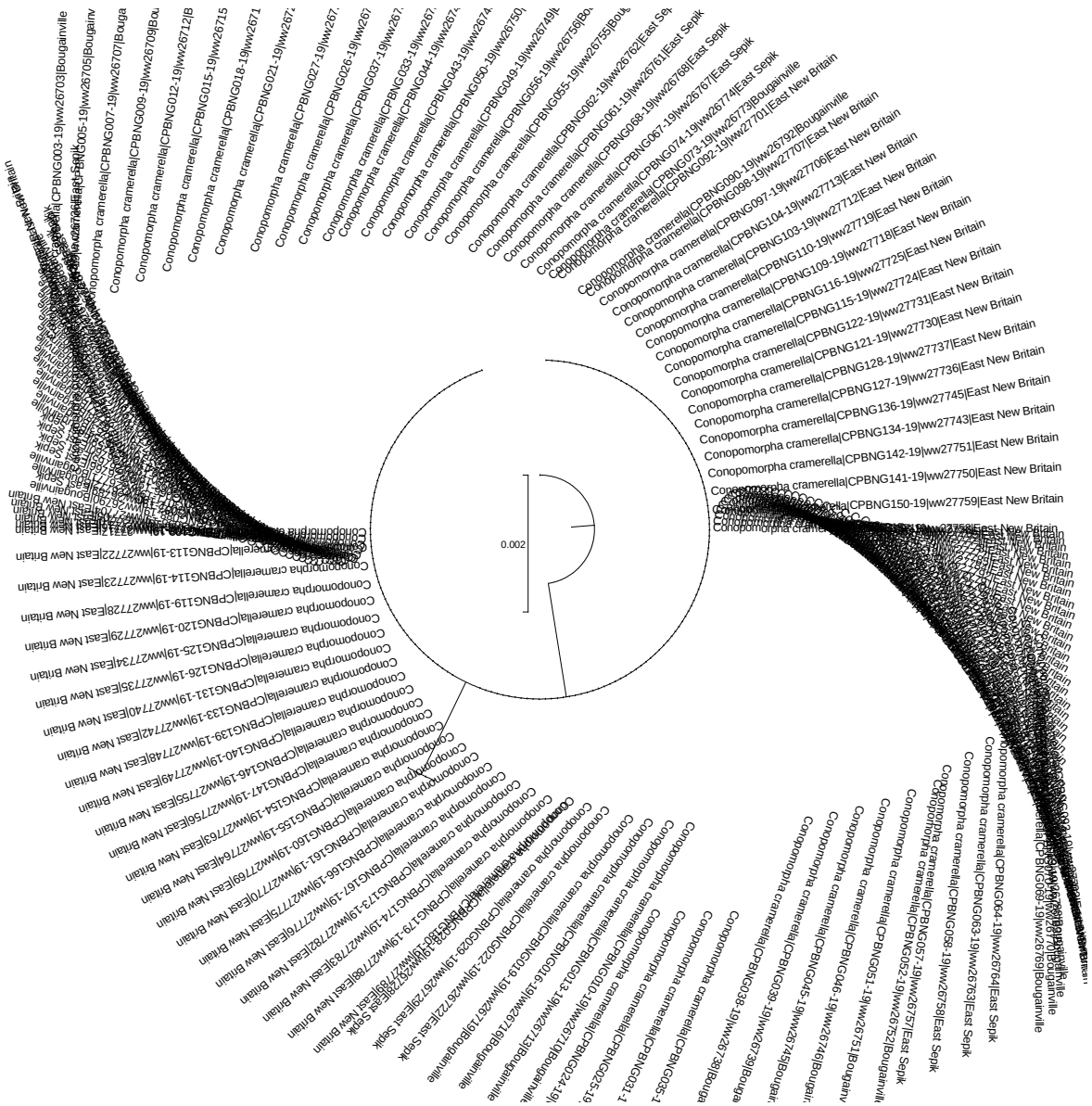
ww27781	Gryllidae*	N/A	86.7

Supplementary Figure S3 A & B. Neighbour joining distance tree of specimen DNA barcodes (N=177) deposited at BOLD (http://v4.boldsystems.org) under project "Cocoa pod borer in Papua New Guinea" (project code: CPBNG). Tip labels indicate specimen taxonomic identification of barcode sequence, BOLD process ID linked to NSW DPI specimen ID, and PNG province location, as listed in Supplementary Table S1. Best matched specimen taxonomic identifications using BOLD ID genetic search engine. Distance tree generated at BOLD using online Taxon ID Tree tool incorporating BOLD aligner option and Kimura 2 Parameter distance model. Tree in figure S2A/ modified with all sequences of *Conopomorpha cramerella* (N=167) collapsed as a clade, with *C. cramerella* clade expanded as a circularised clade in Figure B. Scale bar in S2A/ = 2%, bar in S2B/ = 0.2% K2P adjusted sequence distance.

S3A/



S3B/



Appendix 4.

Sustainable diets for Microlepidoptera: a novel approach to dietary development.

To be submitted to: Journal of Economic Entomology.

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Abstract

The comparative slaughter technique (or carcass milling technique) involves the slaughter of an animal for which a diet is to be developed. The carcass of which is then analysed for dry matter, protein, fat, and energy using proximate and amino acid analyses to determine its nutritional needs. Additionally, the animal's natural food is analysed in the same way to determine how this provides these nutritional needs. A diet is then developed to mimic the natural food to provide the nutritional needs of the animal. Although common for mammals and birds, this technique has only recently been utilised for developing diets for Insects, but not for Microlepidoptera. Here we demonstrate the application of this method to developing an artificial diet for *Conopomorpha cramerella* (Snellen) (Lepidoptera: Gracillaridae) a significant pest of cocoa pods across most of its range in South East Asia and the Pacific region. The ideal amino acid profile, and proximate chemical composition of *C. cramerella* and its host plant, cocoa, are reported here. These provided the nutrient and dietary information on which to base ingredients that could be formulated to rear this pest on an artificial diet. Six larval diets for *C. cramerella* were formulated using this methodology. We also suggest two control diets for comparison; one a modified version of the diet developed for the coffee berry borer, *Hypothenemus hampei* Ferrari (Coleoptera: Scolytidae), which included readily obtainable ingredients and ground up host plant material, and the other formulation based on the diet used to rear the bean pod borer, *Muruca vitrata* (Fabricius, 1787) (Lepidoptera: Crambidae)).

Introduction

For hundreds of years large numbers of insects have been successfully reared on their plant hosts and artificial diets (Wheeler & Zahniser, 2001). Artificial diets reduce time, space, labour and costs compared to rearing insects on their actual host plants (Conlong, 1992; Hervet *et al.,* 2016). In recent times, artificial diet development, and insect rearing in general, has become more refined. For example, over the past 25 years, a series of studies have established the basic nutritional requirements of most insect groups at different life stages, and the behavioural and physiological mechanisms by which the insects respond to changes in diet quality (Ojeda-Avila *et al.,* 2003). In addition, books have been written outlining specific diets for different insect species (e.g. Singh, 1977), on how to develop insect diets (e.g. Cohen, 2015) and outlining principles and procedures for rearing high quality insects (e.g. Schneider, 2009).

According to Davis (2007), the first known plant-feeding insect to be reared from egg to adult on an artificial diet was the European corn borer *Ostrinia nubilalis* Hübner (Lepidoptera: Noctuidae) in 1949. This diet has since then formed the basis for many phytophagous insect diets. Adkisson *et al.* (1960) were the first to use wheat germ as an ingredient in artificial diet to rear the pink bollworm *Pectinophora gossypiella* Saunders (Lepidoptera: Gelechiidae). The recipe was later modified by Vanderzant *et al.* (1962) to rear the corn earworm *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae). Berger (1963) further modified this diet to rear several Noctuid insect species. McMorran (1965) modified this diet even more to rear species of the Tortricidae. Grisdale (1973) consequently added linseed oil to the recipe as an ingredient to reduce wing deformities in some of the Lepidopteran species. Based on Grisdale's (1973) recipe, Atkinson (1978) successfully developed the first artificial diet for mass production of *Eldana saccharina* Walker (Lepidoptera: Pyralidae) in South Africa. Rutherford and Van Staden (1991), identified essential nutritional requirements for *E. saccharina*, and the nutrients on the synthetic diet developed for *E. saccharina* were similar to those found in sugarcane stalks. They found that the balance of sugars, fatty acids, phenolics, tannins and amino acids had an effect on survival and growth of *E. saccharina* larvae. They also state that most synthetic diets used to rear large numbers of insects essentially consists of crude natural products such as wheat germ or some other bulk plant material, such as chickpea flour. Brewer's yeast, casein and glucose were the main nutritional factors in developed diets.

Diet development is a continuous process, as demonstrated in the *E. saccharina* sugarcane IPM program. Following the work of Atkinson (1978), Graham and Conlong (1988), Graham

(1990), Gillespie (1993) and Walton and Conlong (2016) developed diets that improved quality and production of the insects needed for the various IPM programs. A new and improved artificial diet based on one developed for *O. nubilalis* (Nagy 1970), with lucerne meal supplied in rabbit pellets as the main ingredient, recently replaced the previous conventional sugarcane based diet used to routinely rear *E. saccharina*, as insects were produced faster, were of higher quality, and less expensive than those reared on the previously used diets (Ngomane *et al.*, 2017).

In contrast, published literature on artificial diet development for microlepidoptera, a subjective grouping of lepidopteran families, commonly known as the 'smaller moths', such as *Conopomorpha cramerella* (Snellen), a devastating lepidopteran pest of cocoa pods in South East Asian countries and Papua New Guinea, and other species in its Family Gracilliaridae, is limited. These 'smaller moths' are notoriously difficult to rear successfully. The most efficient diet for this pest was developed by Malaysian Cocoa Board researchers in the late 1990's, but was not very successful at rearing successive generations of this insect (Furtek *et al.*, 2000). Santoso *et al.* (2004) had similar results when they attempted *C. cramerella* rearing in Indonesia. Due, in part to contamination, and other issues, artificial diet development for this insect in general has not been successful. In 2006, Azwang *et al.* composed 50 different meridic artificial diets for *C. cramerella*. The best formulation enabled them to get a colony going from fertile eggs, through to moths (but with only a 1% success rate), which could not be sustained. They acknowledged that, like in the preceding research, their diet quality and contamination issues were of concern, and needed further research.

Determining the quality of the diet plays a major role in insect growth and development. According to Karowe and Martins (1992), major determinants of the diet's quality include its toughness, texture and pH, all of which may consequently influence microbial growth thereby compromising the diet's nutritional value and in turn affecting its palatability and the resultant consumption of the diet by the insect. For *C. cramerella* in particular, this may mean the development of a more liquid, or gel-based diet, as done by Chang *et al.* (2009) for tephritid fruit flies, compared to the more solid diets generally developed for Lepidoptera.

In addition, the balance of nutrients in artificial diets for insects is very important because it directly influences insect growth, tissue maintenance, reproduction and energy allocation (Genç, 2006). In order to obtain a balance of nutrients, a fair amount of carbohydrates, proteins,

lipids, vitamins and amino acids are required (Genç, 2006). Generally the majority of animal (i.e. livestock) feeds (and insect artificial diets) are composed of carbohydrates or energy producing nutrients (i.e. high grain diet) (De Goey, 1973; Genç, 2006; Sahtout, 2012). The animals consume approximately 70 to 75% of energy from the feed or diet for maintenance (De Goey, 1973; Sahtout, 2012). Since the feed or diet expense is relatively higher than animal or insect production costs, it is essential to avoid over-supply of nutrients, because, once the animal or insect's nutrient requirements are supplied, any excess nutrients are wasted by excretion or removal of unwanted fat (De Goey, 1973; Sahtout, 2012). Thus, in order to minimise this cost, a number of techniques have been developed to evaluate nutritional requirements to help formulate animal and/or insect diets (De Goey, 1973; Sahtout, 2012).

The use of the comparative slaughter technique (or carcass milling technique), defined as the method for determining energy retention in animals, plays an important role in the development of animal feeds or insect diets (Babinszky and Bársony, 2013). This comparative slaughter technique requires that representative animals are slaughtered and their carcasses analysed for dry matter, protein, fat, and energy using proximate and amino acid analyses. Also analysed for the same constituents are the animals natural host plants. The comparative slaughter technique is quite expensive when applied to large animals but cheaper when applied to insects (Babinszky and Bársony, 2013). Using the information collected from the comparative slaughter technique and proximate and amino acid analyses (i.e. partitioning of compounds in a feed into categories based on the chemical properties of the compounds), relatively inexpensive artificial diets for both animals and insects can be formulated using feed formulation programmes such as WinFeed (Windows-based feed formulation program developed by EFG Software).

This approach has been used to successfully improve diets for larger Lepidopteran species, known to be more readily cultured and/or mass-reared, including the African sugarcane borer, *Eldana saccharina* Walker (Lepidoptera: Pyralidae) (Woods *et al.,* 2019a), and the False codling moth, *Thaumatotibia leucotreta* Meyrick (Lepidoptera: Tortricidae) (Woods, 2019b). It has also been used to improve the diet of the black soldier fly,(*Hermetia illucens* Linnaeus (Diptera: Stratiomyidae) (Woods *et al.,* 2019c), However, it has never been used to develop diets for microlepidoptera.

This paper describes the first use of the comparative slaughter technique and proximate and amino acid analyses to develop an artificial diet to rear the microlepidopteran *C. cramerella*. In addition, two control diets are proposed, given there are no published effective diets available for this species, against which the newly formulated diets may be compared in future studies.

Materials and Methods

Harvest of infested cocoa pods

To ensure the supply of larval stages needed for analyses, *C. cramerella* infested cocoa pods were harvested from two plantations, Block 4 (S04.29094°, E152.01794°) and Block 12 (S04.30192°, E152.02551°) at the Cocoa Board, Tavilo, East New Britain Province, Papua New Guinea. Approximately 3000 pods were collected. Susceptible cocoa varieties were targeted, but infested tolerant varieties were also sourced. Cocoa pods were a minimum of three months old and at least 10cm in length.

Sampling of insects

To collect the *C. cramerella* larvae, green banana leaves were placed on a tarpaulin and the harvested infested cocoa pods placed in a single layer on top of the leaves. The cocoa pods were then covered with another layer of green banana leaves and a tarpaulin was placed over the top to emanate the humid, warm conditions under which *C. cramerella* typically exit the fruit in the field to pupate. Late larval instars that were ready to pupate, and some smaller instars exited the fruit and made their way onto the underside of the banana leaves, or in between the furrows of the cocoa pod. To capture the larvae before they pupated and their nutrients were partitioned (Niogret pers. comm. 2018), leaves and cocoa pods were checked every three hours, 24h/day for seven consecutive days to obtain the minimum required mass of larvae for the proximate and amino acid analyses (see below).

Sampled larvae were placed inside a 70ml screw-capped plastic vial (Thermo Fisher Scientific Inc. Scoresby, Victoria Australia 3179), together with several cocoa seeds with their mucilage intact. These were placed in a fridge at 4°C until they were ready to be processed. All samples were processed within 24h. Only live larvae were utilised; dead larvae were discarded.

Laboratory processing of insect material

C. cramerella larvae for processing were removed from the fridge and separated from the cocoa seeds and mucilage. Live larvae were placed in a baking sieve in batches of 1-50 individuals. The sieve containing larvae was immersed in boiling water for exactly 1min 10s. The blanched larvae were then placed on paper towel to dry for 1-2 mins. Larvae were weighed (g) in batches on a 4 decimal place balance (VWR Analytical balance LA124i, VWR International GmbH. Graumanngasse 7 1150 Wien) and the wet weight recorded. A total of 6559 larvae were sampled.

After their wet weight was recorded, the larvae were placed in an oven at 60°C and weighed at regular intervals (after 3h 30mins and then every 10 minutes afterwards) until a constant mass (i.e. dry weight) was achieved. The larvae were allowed to cool down to room temperature for a few minutes, prior to weighing.

Once the final dry mass was achieved, the larvae from each 'batch' was placed in 0.5ml BIOplastics screw capped tube (Austral Scientific Pty Ltd Gymea NSW), sealed tightly and placed in the freezer at -20°C until ready to transport to the University of Stellenbosch, South Africa for analyses.

Collection of plant material

Cocoa

Mature, uninfested *C. cramerella* susceptible cocoa pods (varieties: KA2-106-47, K82XKEE-42, K82XKEE-12, KA-106-43, K82XKW5, K82-43, K82-42, KA2-106-12, K82-47, KA2-106-43, KA2-106-5, KA2-106-23) were harvested from Block 2 (S04.30323°, E152.01350°) at the Cocoa Board, Tavilo, East New Britain Province, Papua New Guinea and taken back to the Cocoa Board Entomology Laboratory for processing. The uninfested cocoa pods were split and the mucilage cut off a total of 898 individual seeds.

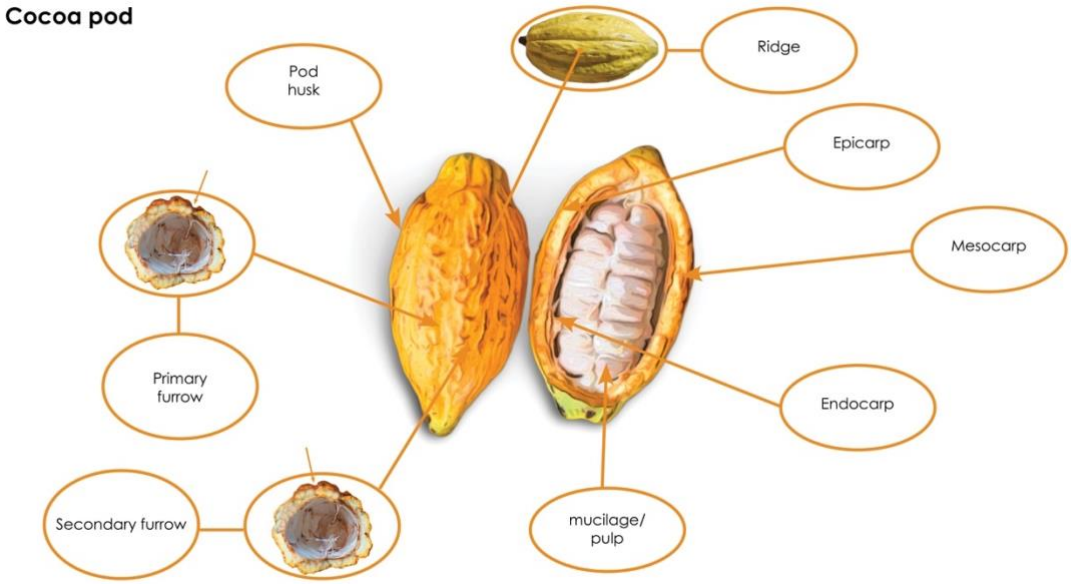

Figure 1. Cocoa (*Theobroma cacao*) pod illustrating the various structures. Image credit: Leo McGrane, **cesar** pty ltd.

Laboratory processing of plant material

The cocoa mucilage was placed on a 9cm diameter plastic petri dish (MicroAnalytix Pty Ltd., Taren Point NSW 2229) in batches and weighed on a 4 decimal place balance as described above and the wet weight recorded. The mucilage was then placed in an oven at 60°C for 24h and then weighed every 1h until an even mass (i.e. dry weight) was achieved. The mucilage was allowed to cool down to room temperature for several minutes before weighing.

Once the final dry weight was achieved, the mucilage was placed in a 70mL yellow PE screw cap vial 70 mL (Thermo Fisher Scientific Inc. Scoresby, Victoria Australia 3179), sealed tightly with a layer of parafilm around the top, and placed in a freezer at -20°C until ready to transport.

Transport of insect and plant material

A layer of techniice (Techniice, 3 Finch St, Frankston VIC 3199, Australia) was placed on the base of a 4L polystyrene container and covered in bubble wrap. The vials containing insect and plant samples were placed on top of the bubble wrap, ensuring they would not move during transport. Another layer of bubble wrap was placed on top, covered with techniice and then sealed. All appropriate documentation was acquired and the samples were sent by courier from Papua New Guinea to Stellenbosch University, Stellenbosch, South Africa for proximate and amino acid analyses analyses.

Dietary analyses of insect and plant material

Proximate analyses

Proximate chemical analyses were completed on the processed *C. cramerella* and host plant material. Total percentages of moisture, protein, fibre and ash were determined according to AOAC methods (AOAC, 1997; Table 1). The protein content was determined by the Dumas combustion method and ashing was done at 500 °C for 5 h. The moisture content was determined by drying at 100 °C for 24 h. The lipid content of the larvae was determined by means of acid hydrolysis and the plant material by means of ether extract. The fibre content was determined by solvent extraction using the ANKOM method.

Amino acid analyses

To determine the amino acid profiles of the processed *C. cramerella* and host plant material, a sample weighing 0.1 g was placed in a specialized hydrolysis tube. Six mL hydrochloric acid (HCl) solution and 15% phenol solution was then added to the sample. The tubes were then vacuated and nitrogen (N) added under pressure. The tubes were subsequently sealed off with a blue flame and the samples left to hydrolyse at 110˚C for 24 hours. After hydrolysis, the samples were transferred to Eppendorf tubes and refrigerated until sent to the Central Analytical Facility of Stellenbosch University. Amino acid composition was determined by means of the Waters AccQ Tag Ultra Derivatization method. The following amino acids were analysed: histidine, serine, arginine, glycine, asparagine, glutamine, throenine, alanine, proline, lysine, tyrosine, methionine, valine, isoleucine, leucine and phenylalanine. From the analysed amino acids the ideal amino acid profiles were determined (IAAP). Protein that has a perfect amino acid balance is often referred to as an ideal protein. The IAAP supplies the optimum balance of essential amino acids together with sufficient nitrogen for the synthesis of non-essential amino acids. In theory it matches an animals requirements exactly. To determine the IAAP the proportion of amino acids are expressed relative to the amount of lysine. Lysine is the first limiting amino acid for animal growth in most organisms, and has also been studied the most extensively (Boisen et al., 2000).

Treatment diets

For each diet, 250 g of dry ingredients were measured, and to this 750 mL of boiling water was added and stirred. The moisture content was corrected to 72±1%. Using a 650 W microwave, the diet was heated in a glass beaker for approximately 1 min until it boiled and

rose slightly. The glass beaker containing the dietary contents was then removed from the microwave, stirred and placed in the microwave again for approximately 1 minute until boiling and risen. The beaker was removed, stirred and using 60 mL syringes, approximately 3-4 mL diet was dispensed into 25 mL plastic screw top vials (Lasec SA, Container Faeces S/Cap Spoonst Pp 25x80mm 25ml; Stock Code PLPS109048; www.lasec.co.za). The diet was allowed to completely cool before screwing on the lid. The diet containing vials were placed in a fridge at 4 °C overnight and inoculated the next day. Vials containing diet were allowed to reach room temperature to allow them to be rid of the condensate, and to not shock *C. cramerella* upon inoculation. The pH of each developed diet was determined using a Sper Scientific Benchtop pH Meter (Instrument Choice, 22-24 Cavan Road, Dry Creek, South Australia 5094).

C. cramerella egg collection

C. cramerella infested cocoa pods were collected from the entomology trial block in Raulavat (S04.29094°, E152.01794°). Susceptible cocoa cultivars (37 -13/1, CCI B2, and 38 –10/3) were targeted, however more tolerant cultivars (16 -2/3, 36 -3/1 and the 73 -2/2) were also sampled. Cocoa pods were placed in either 20x30x40 cm plastic tubs with a mesh top or 30 x 30 x 30 cm mesh Bugdorm cages (Bugdorm, Taiwan) under ambient conditions in a laboratory at the Cocoa Board, Tavilo, PNG. Eclosed adults were collected daily and grouped together in equal sex ratios in 30x30x30 cm mesh Bugdorm cages (Bugdorm, Taiwan) containing no more than ten adults per cage. Detached uninfested mature cocoa pods (4-6 months old) were wrapped in a single ply of paper tissue (Thick and Soft Kitchen Towel 2 ply/Softex 2 ply, Tropicana Limited, Kokopo, PNG). Two wrapped pods were placed at the base of each of the cages/tubs. Two honey patches were placed in each of the cages/tubs as food.

Female *C. cramerella* laid their eggs in the grooves of the paper tissue. Thirty replicates of each diet treatment were inoculated on the 5 June 2019 with eggs laid from the 1-4 June 2019, and the remaining replicates were inoculated on the 6 June 2019 with eggs laid from the 5-6 June 2019. The viability of eggs were examined under a microscope and ony those that were fertile, i.e. orange in colour, were used. The tissue paper containing eggs, was cut such that each piece of paper contained only a single egg.

Diet inoculation

Vials containing the treatment diets were retrieved from the fridge and allowed to come to room temperature (1-2 h) prior to inoculation. The top of each diet was scarified gently using forceps in a cross hatched manner, ensuring that the gel top diets were not mixed in with the diet beneath. Using forceps, the eggs on tissue paper were added singly to the top of each diet, ensuring that the egg was facing upwards and the paper was flat on the diet. Forceps were rinsed in ethanol between each diet. Each diet treatment was replicated 100 times in 25 ml plastic screw top vials (Lasec, South Africa). Diets were inoculated over two consecutive days. Treatment diets were placed in a growth room under ambient conditions (Temperature range: 28-35°C; Relative Humidity 56-77%) and were observed daily for contamination. Fourteen days after egg inoculation, cocoa pod leaves were sampled from the surrounding cocoa plantations. These were brought back to the lab and autoclaved (Autoclave model HL42E, Hirayma MFG. Corp., Japan). To autoclave the leaves, four litres of distilled water were added into the base of the autoclave up to the designated water level. Forty-five healthy fresh cocoa leaves were shared equally into three autoclave plastic bags i.e. 15 leaves per bag, and then laid into the autoclave just above the water level on top of petri dishes placed on the base tray. The plastic bags were not sealed but were folded at the opening. The autoclave was closed, switched on and the pressure maintained at 1 kilopascal (kPa) for 15 minutes before it was switched off and left to cool. The autoclaved leaves were removed the following day. Scissors cleaned with 70% ethanol were used to cut the autoclaved leaves into approximately 4 cm^2 (2 cm x 2 cm). Subsequently, each treatment vial was randomly and horizontally placed on a tray, and a 4? cm^2 of autoclaved leaf was placed in each treatment vial, to provide a medium on which the larvae could pupate. From fifteen days after egg inoculation, the vials were checked daily for pupation until 25 days after egg inoculation. As pupation was not observed, the diet in all vials was carefully searched for emerged larvae, and egg hatch was recorded.

Results

Insect material

A total of 6,559 larvae were sampled to obtain enough dried material for analyses. The total wet weight of insect material obtained was 43.72g. The total dry weight of larvae was 14.96g.

Plant material

The sampled cocoa mucilage had a wet weight of 556.85g, and a dry weight of 103.76g.

Dietary analyses of insect and plant material

Proximate chemical composition

The proximate chemical composition revealed that the moisture content of the mucilage is high (Table 1). This can make it difficult to formulate an artificial diet as it is challenging to reach such a high moisture content and bind that amount of water over an extended period. The protein content of the mucilage is very low compared with food sources of other Lepidoptera (Woods et al., 2019a) and with conventional raw materials it is difficult to formulate for such a low protein value. Excess protein cannot be stored and must be excreted in the form of uric acid which places a lot of metabolic stress on the animal due to the high cost of deamination.

Table 1. Proximate chemical composition of *Conopomorpha cramerella* larvae and cocoa mucilage used to formulate treatment diets.

Sample	Original moisture (%)	Moisture (%)	Ash (%)	Crude fat (%)	Crude fibre (%)	Nitrogen (%)	Protein (%)
C. cramerella larvae	65.78	5.92	3.54	35.93	n/a	7.18	44.88
Cocoa mucilage	81.37	19.3	8.57	1.57	2.94	0.64	4.00

Amino Acid Analyses

Comparing the ideal amino acid profiles of *C. cramerella* larvae and the cocoa pod mucilage, revealed that the mucilage has significantly higher methionine and histidine levels whereas the rest of the essential amino acids i.e. arginine, threonine, valine, isoleucine, leucine and phenylalanine are comparable (Table 2). This suggests that the protein and more specifically amino acids provided by the cocoa mucilage closely represents the demand of *C. cramerella* larvae with the slight exception of methionine and histidine.

Table 2. Amino acid compositions (% m/m) and ratio (%) of amino acids (in relation to lysine) of *Conopomorpha cramerella* larvae and cocoa pod mucilage.

Amino Acid	Amino acid composition (% m/m)		Amino ratio (%) to lysine	
	C. cramerella larvae	Cocoa mucilage	*C. cramerella* larvae	Cocoa mucilage
His	1.38	0.18	29	75
Ser	3	0.03	63	13
Arg	2.94	0.21	61	88
Gly	2.04	0.18	43	75
Asp	5.51	0.54	115	225
Glu	6.54	1.07	136	446

Thr	2.23	0.12	46	50
Ala	2.54	0.17	53	71
Pro	1.7	0.16	35	67
Lys	4.8	0.24	100	100
Tyr	3.09	0.2	64	83
Met	1.54	0.21	32	88
Val	2.5	0.17	52	71
Ile	1.94	0.12	40	50
Leu	3.55	0.26	74	108
Phe	2.18	0.19	45	79

Treatment diets

A total of six diets (i.e. treatments) were developed and tested (Table 3). The control diet was adapted from the diet developed by Wang *et al.,* (2013) to rear the bean pod borer, *Maruca vitrata* (Fabricius) (Lepidoptera: Crambidae). The Chang diet, which was modified from a diet developed to rear the coffee berry borer, *Hypothenemus hampei* (Ferrari) (Coleoptera: Scolytidae), and included readily obtainable ingredients and incorporated ground up host plant material (Brun *et al.,* 1993). The carcass milling technique was used to determine the amino acid composition of the larvae and expressed as a percentage of lysine (Table 2); this represents the ideal amino acid profile (IAAP). The supply of amino acids in this ratio results in an amino acid profile most closely representing the requirements of the insect (Gous, 1986). The IAAP of the larvae (Table 2) was used to formulate the IAAP treatment diet (Table 3). The natural (NAT) diet was formulated to resemble the nutrient composition of the natural diet of the larvae i.e. cocoa mucilage (Table 2&3). Diets were formulated according to the novel nutrient specifications using similar ingredients to the control diet (Table 3).The IAAP and the NAT diets were tested with and without a gel top. The pH of each developed diet was determined using a Sper Scientific Benchtop pH Meter (Instrument Choice, 22-24 Cavan Road, Dry Creek, South Australia 5094).

Table 3. Ingredients and calculated nutritional value of *Conopomorpha cramerella* larval diets.

	CONT	**IAAP**	**NAT**	**CHANG**	**Gel top**
Ingredients (%)					
Agar	8.91			12.33	
Arbocel		12.96	11.40		
Sodium benzoate		1.61	1.61	1.08	1.61

Ascorbic acid	1.49	1.38	1.29		
Brewers yeast	3.71	3.00	3.00		
Carrageenan gel		3.00	3.00		3.00
Casein				3.85	
Citric acid		0.52	0.52		
Cocoa pods (1:3 husk:mucilage)		10.00	10.00	57.81	91.68
DL methionine		0.02			
Limestone		27.46	1.05		
Monocalcium phosphate			4.16		
Sodium propionate		2.06	2.06	0.46	
Nipagen	0.52	1.29	1.29	0.67	1.29
Streptomycin		0.40	0.40	0.27	0.40
Salt			10.00		
Sorbic acid	0.52				
Soybean 46		11.82	47.52		
Soybean full fat	59.43				
Sugar	5.94			7.71	
Trisodium citrate		0.52	0.52		0.52
Vanderzants vitamins				0.39	
†Vitamin and mineral premix	0.16	0.15	0.15		
Wheat germ meal	19.31	22.32	0.54	15.42	
Whole egg powder		1.50	1.50		1.50
Calculated nutritional value (%)					
Dry matter	91.41	93.26	92.78		
Moisture	8.59	6.74	7.22		
Crude protein	27.49	13.19	23.90		
Crude fiber	4.14	10.00	10.0		
Crude fat	12.00	2.90	1.57		
Calcium	1.47	10.68	1.82		
Phosphorous	2.60	2.68	1.58		
Sodium	0.13	0.13	3.97		
Potassium	3.56	3.21	1.64		
‡DE (Pig) (MJ kg-1)	13.67	5.45	7.52		
Lysine	1.71	0.77	1.50		
Methionine	0.40	0.22	0.35		
*TSSA	0.79	0.39	0.67		
Tryptophan	0.32	0.13	0.31		
Isoleucine	1.24	0.53	1.17		

Leucine	1.99	0.87	1.82
Threonine	1.07	0.49	0.95

where, CONT = Control, IAAP = Ideal amino acid profile, NAT = Natural, CHANG = modified coffee berry borer diet. The IAAP and NAT diets were made both with and without a gel top. †=Broiler starter vitamin and mineral premix, ‡=Digestible energy as determined for pigs, *Total Sulphur containing amino acids. **Note:** Oxytetracycline was omitted from the final diets due to concerns over this compound's safety and was replaced with streptomycin. Acetic acid was not deemed necessary in the final diets due to other compounds, such as nipagen, that play a similar role. The 'mucilage' also contained some of the endocarp.

pH of diets

Variations in the pH between the internal and external layers of the cocoa pod were evident (Table 4). The developed diets were closer in pH to the husk of the cocoa pod.

Table 4. pH of the developed diets and the cocoa mucilage and husk.

	Cocoa mucilage	**Cocoa husk**	**CONT**	**CHANG**	**IAAP**	**IAAPG**	**NAT**	**NATG**
pH	3.29	5.48	5.15	4.71	4.96	4.90	5.20	5.04

Diet inoculation

A large number of larvae did not eclose (Table 5) and was deemed due to both lacking sufficient stimulation and/or adequate conditions. Those larvae that did eclose, were unable, or were not enticed to bore through the paper towel. No contamination of any of the diets were observed.

Table 5. The proportion of eclosed cocoa pod borer larvae on each of the six treatment diets.

Treatment diet	**Uneclosed larvae (%)**	**Partially eclosed larvae (%)**	**Eclosed (dead) larvae that left egg patch (%)**	**Total egg hatch (%)**	**Missing eggs (%)**
Control	85	5	3	8	7
Chang	92	7	0	7	1
IAAP	80	0	1	1	19
IAAPG	86	0	0	0	14
NAT	96	2	0	2	2
NATG	95	0	1	1	4

Discussion and Conclusions

Research groups in the south east Asian and Pacific regions, where this pest is a serious problem to cocoa production, have spent over a decade in attempts to develop an artificial diet for *C. cramerella*, so that effective control measures for it can be researched. These have met with very limited success (Furtek *et al.*, 2000, Santoso *et al.* 2004). Our attempts using the proximate and amino analysis approach, that has been very successful at developing cost effective artificial diets for mammal and bird species, and more recently insect species such as BSF and FCM, has not worked for rearing CPB. However, as stated, this is the first attempt, and we have demonstrated a method largely used to develop large animal diets, but is now also being used to successfully develop diets for insects (Woods *et al.,* 2009a,b), that could be utilised to develop an artificial diet for microlepidoptera such as *C. cramerella*, that once perfected, can be used to mass rear the insect for IPM bioassays, end even for mass rearing to provide individuals for SIT programs.

Much research is still needed in developing this approach, and aspects that have to be considered include dietary through to environmental considerations for all life stages.

A crucial component for larval development, and successful moult of the insect is the nutritional content of the diet. Using the approach described in this study, this optimises the chance of developing a diet that will meet the nutritional needs of the insect. Ensuring the diet has the correct toughness and texture will also be an important aspect to encourage the larvae to successfully feed through the instars.

It is preferable to develop a diet that has a similar pH to that of the host plant material (Cohen, 2015). In our study, the pH of the different developed diets demonstrate that it will be necessary to adjust the pH of the developed artificial diets. As the husk of the cocoa pod, which the *C. cramerella* larvae must enter to reach the mucilage upon which it feeds, has a higher pH than the mucilage, then we could mimic this using the gel top with a pH similar to that of the husk. Similarly, we could aim to lower the pH of the diets to more closely reflect the pH of the host plant mucilage.

The importance of water for survival and performance of insects is apparent (Slansky & Scriber, 1985). Indeed, it has been shown that an artificial diet moisture content, similar to that of the host material is preferable for successful larval development (Wang *et al.*, 2014).

Achieving a high water content of the artificial diet similar to that of the host plant mucilage of *C. cramerella* is an area that will require future research attention.

Understanding requirements by invertebrates, such as feeding stimulants, may also be an important component if larvae are not incited to enter the artificial diet. Some insects require a feeding stimulant to commence feeding (Vanderzant, 1969; Cohen, 2015). This is where the gel top may play an important role, mimicking the stimulants found in the plant host rind, which encourage the larvae to bore into the fruit. In the current study, a gel top was added to the top of the NAT and IAAP diets, incorporating a larger proportion of the cocoa pod than the diet beneath, as it is possible that the husk contains some sort of feeding stimulant.

Contamination of artificial diets by pathogens and other insects is a significant issue in insect mass-rearing (Sampson et al., 2016; Ni & Streett, 2005). A range of antimicrobials are used in insect diets to prevent microbial contamination and propagation. However, these same agents can also be toxic to the insect, preventing the normal development of larvae and are thus important considerations in an insect-rearing program (Alverson & Cohen 2002; Mizuno & Hisashi 2009). Other factors such as pH and moisture content can also impact microbial contamination (Jay 2000; Marin et al. 2003). Therefore, careful consideration of these factors will be an important component in the development and success of an artificial diet for *C. cramerella*.

In most insect rearing enterprises, much of the diet is not used by the growing insect and goes to waste, thus increasing costs. Lessons can be learnt from animal scientists, in the development of animal feeds to reduce this waste, and produce insect feed that is more suited to the individual needs of the insect being reared, thus maintaining quality and reducing wastage and thus costs.

This study reports a novel method, i.e. the carcass milling technique, not previously used to develop a diet for members of the microlepidoptera. This technique has been applied to a pest species, *C. cramerella*, to not only demonstrate this approach, but provides the foundational underpinnings, including the nutritional and chemical composition, and IAAP to develop trial diets for *C. cramerella*.

Acknowledgements

Thank you to the field team who collected the cocoa pods and sampled the larvae including Tovue Moses, Nox Jason, Simion Mark, Andrew Jimmy, George Andrew, Theodore Tigero, Jason Mirox, Michael Kalauna, Ludwick Lelehite, Ken Mabu, Junior Jerenah, Darius Bakol and Sebastian Muku. Terry Osborne, Dorcas Lotu, Matiran Tutmulai and Olisha Wesley are thanked for their technical assistance. The Cocoa Board staff and others who assisted with and arranged the sending of insect and plant samples internationally are gratefully acknowledged. Thank you to Annie Johnson for formatting the manuscript.

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Prepared by Dr Jess Lye, cesar

14 February 2019

Media Release

The cocoa pod borer has been a major problem for cocoa growers across Papua New Guinea, and the Australian Centre for International Agriculture Research has commenced new research to help improve management of the pest.

Cocoa production represents nearly 20% of agricultural revenue per annum in PNG and is largely produced by small, family-owned farms. Following a 2006 outbreak in East New Britain, the cocoa pod borer has also been identified in several other provinces in PNG.

Project leader Dr Olivia Reynolds, **cesar** says one of the challenges of addressing the cocoa pod borer has been lack of knowledge about the pest. A small team of PNG and Australian experts are currently in PNG visiting East New Britain, East Sepik and the Autonomous Region of Bougainville to sample the cocoa pod borer to help answer this question.

The team will collect samples and resolve identification of this species, which will help scientists develop the best way to manage the pest in PNG.

"It will allow us to determine the species/race/biotype directly affecting crops in PNG," Dr Reynolds said. "This will aid the development of targeted management practices."

The NSW Department of Primary Industries project is funded by Australia through ACIAR in collaboration with the Papua New Guinea Cocoa Board, **cesar**, Stellenbosch University, South African Sugarcane Research Institute and the University of Sydney.

Ends

Facebook

The cocoa pod borer has been a major problem for cocoa growers across Papua New Guinea, and the @ACIARAustralia has commenced a research project to help improve management of the pest.

Project leader Dr Olivia Reynolds, **cesar** says one of the challenges of addressing the cocoa pod borer has been lack of knowledge about the pest. A small team of PNG and Australian experts are currently in East New Britain, East Sepik and the Autonomous Region of Bougainville to collect samples of the cocoa pod borer and identify the species, which will help scientists develop the best way to manage the pest in PNG.

The NSW Department of Primary Industries project is funded by Australia through ACIAR in collaboration with the Papua New Guinea Cocoa Board, **cesar**, Stellenbosch University, South African Sugarcane Research Institute and the University of Sydney.

Tweet

Scientists from @ACIARAustralia, Victoria, New South Wales and #PNG have commenced a project on cocoa pod borer species to help manage the pest in #PNG #PNGAusPartnership

Prepared by Dr Jess Lye, cesar
5th June 2019

chocoholic plant pest placed under the microscope

cesar *research scientist and team lead, Dr Olivia Reynolds, has been leading an intrepid new project investigating the biology of a priority pest in the cocoa production industry of Papua New Guinea. With two research field trips to affected cocoa production areas complete, the project has begun to yield some promising results.*

When I overheard that **cesar** Research Team Lead (and entomologist), Dr Olivia Reynolds, was on the hunt for livestock feed I was initially confused. Then realisation set in – it all comes down to a particular plant pest and biosecurity threat, the cocoa pod borer (*Conopomorpha cramerella*), and creating just the right mix of nutrients to sustain a colony outside of its common host plant.

As the name suggests, cocoa pod borer is a pest of cocoa. However, it also feed on tropical fruits, such as rambutan and longan. As a lepidopteran insect the cocoa pod borer develops from egg to caterpillar to pupa, before finally emerging as a small (approximately 7mm) adult moth. Notable production damage can be attributed to feeding of the caterpillar within the pod, which will cause premature or uneven ripening.[1] The interruption of cocoa bean development causes the beans to clump together, making them difficult to be harvested and processed and reducing yield.

The cocoa pod borer caterpillar feeds on connective tissue between beans within the pod. (Image credit: Dr Olivia Reynolds)

The cocoa pod borer is a pest throughout parts of South East Asia and the Western Pacific. As far back as the 1840's the borer was attributed with the translocation of the North Sulawesi cocoa industry to the Philippines and Java (the North Sulawesi cocoa industry has since recovered).[2] More recently, a cocoa pod borer outbreak in Papua New Guinea has placed farmers under pressure. Where recommended management steps have proved impractical, farmers have changed crops or opted to send cocoa pods to local markets.

"Cocoa plantations in Papua New Guinea tend to be largely small-holder family farms, and effectively managing this destructive pest has thrown up some

[1] Cocoa pod borer data sheet, CABI.

[2] Toxopeus H; Giesberger G, 1983. History of cocoa and cocoa research in Indonesia. Archives on Cocoa Research, 2:7-34

challenges. Any change, or improvement to current pest management practices to combat the cocoa pod borer needs to be achievable and cost-effective" stresses Dr Reynolds.

Pinning down cocoa pod borer biology, ecology and behaviour is an important part of the research, which is funded through the Australian Centre for International Agricultural Research (ACIAR) and is a collaboration between **cesar**, New South Wales Department of Primary Industries, the University of Sydney, the Cocoa Board of Papua New Guinea, the South African Sugarcane Research Institute, Stellenbosch University and the United States Department of Agriculture.

The project has three major objectives:

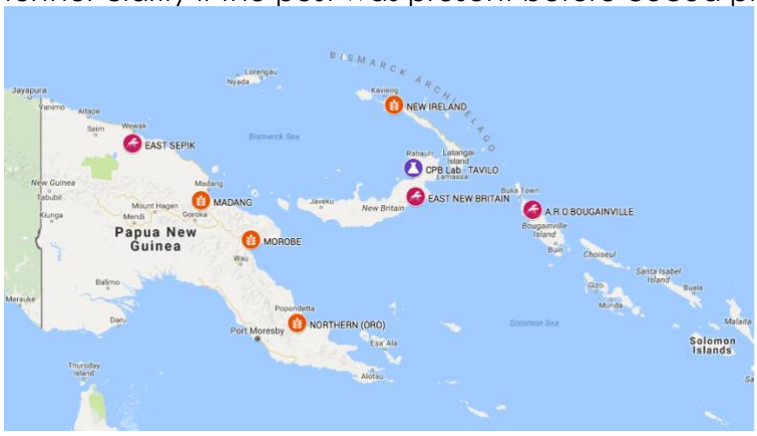
1. To review what research has been conducted to date on the pest, and identify the gaps in our knowledge on identification and management
2. To resolve the identification of the cocoa pod borer (it is not known if the borers affecting cocoa production regions overseas are different species, or genetic variants of the same species)
3. To determine the best method of culturing the pest in a laboratory environment (this explains the hunt for livestock feed)

With the first two field trips to Papua New Guinea completed during October 2018 and February 2019, the project is well underway. So, what has been found to date?

let the DNA do the talking

After collecting over 150 samples of cocoa pod borer from three cocoa producing provinces of Papua New Guinea (as well as receiving international specimens), caterpillars, pupae and adults were received by project collaborators in New South Wales for taxonomic and molecular identification. Preliminary molecular analysis of a subset of data has detected two of the five known cocoa pod borer haplotypes, but also an additional, novel haplotype. Further, molecular results have identified another lepidopteran species infesting cocoa that was previously not known to be a pest of the crop!

As the research progresses, further molecular analysis will support development of a diagnostic that will enable accurate identification and will assist with painting a picture of how the cocoa pod borer has moved through Papua New Guinea (and further clarify if the pest was present before cocoa plantations entered PNG).



Main cocoa growing provinces of Papua New Guinea (plant image; orange) with the three CPB sampling locations (fly image; pink) and CPB laboratory location (Tavilo). (Image credit: Deane Woruba, NSW DPI)

finding the Goldilocks recipe
The ability to raise, or 'culture' a pest is extremely useful. Once a colony can be kept in a controlled laboratory environment more sophisticated research into the biology, behaviour and ecology of the pest can be carried out. Such further research is a crucial step in employing additional methods to combat the pest, such as the Sterile Insect Technique – a methodology that has been used in several Australian states to aid eradication of Queensland fruit fly in fruit fly free areas for decades.

The method in use to determine the best diet for cocoa pod borer is termed 'the comparative slaughter technique', which is also colourfully known as 'the carcass milling technique'. This involves collecting caterpillars (after observing the hosts on which they have been feeding) and assessing their body compositions for dry matter, protein, fat, and key nutrients. Similarly, it also involves analysing the host material. These analyses can give scientists a template for developing the ideal diet.

contributing to a community of interest
Importantly, the trip also allowed the research team to build knowledge about cocoa pod borer in affected areas as many farmers recognise the damage, but not what the pest looks like.

"Correct identification, of both the pest and damage, is the first essential step to good pest management. A community driven approach to learning more about this pest and its biology and ecology will be extremely important in effectively controlling the cocoa pod borer in Papua New Guinea," says Dr Reynolds.

In turn, locals shared their observations about the pest damage they had observed.

The recent research excursion to Papua New Guinea cocoa producing regions also supported sharing of knowledge about the pest. (Image credit: Dr Olivia Reynolds)

where to from here?
In countries that remain unaffected by the pest, surveillance remains a priority as the borer can travel long distance via infested fruit. Thus, this research has benefits for both our neighbours in cocoa pod borer affected nations including Papua New Guinea and for tropical fruit farmers in Australia through the development of a diagnostic protocol. We are the only agricultural nation in the world to have eradicated cocoa pod borer after it was detected in Queensland in 2011. Further, the development of an artificial diet will not only enable a greater understanding of the biology, behaviour and ecology of the pest under controlled conditions, but will

allow the development of more sophisticated management techniques such as the sterile insect technique.

This research will continue with another visit to the PNG Cocoa Board entomology laboratory in June, where further activities will include testing the fitness and performance of the cocoa pod borer on eight diets developed using the comparative slaughter technique.

With all this talk of cocoa, you must ready for a treat or two… try sourcing single origin chocolate from PNG to try its distinctive fine flavour. After the completion of this third research mission we will have further pieces of the cocoa pod borer puzzle to share. Until then, good-bye,
Lukim yu, choco-*later*!

Have you seen something unusual? Suspect exotic plant pest detections should be made to the Plant Pest Hotline 1800 084 881

This research is funded by the Australian Centre for International Agricultural Research (ACIAR). Project partners are the New South Wales Department of Primary Industries, the University of Sydney, the University of Stellenbosch, the South African Sugarcane Research Institute, the United States Department of Agriculture (USDA) and the Papua New Guinea Cocoa Board.

MEDIA RELEASE
For immediate release

Cocoa pod borer research underway to aid farmers in Papua New Guinea

Cocoa borer is a serious threat to Papua New Guinea's emerging cocoa industry. A team of researchers is spending time in PNG, with support of the Papua New Guinea Australia Partnership, to find out as much as they can about the destructive pest and how best to manage its spread.

The team consists of researchers from the PNG Cocoa Board, agriculture and conservation research group **cesar,** New South Wales Department of Primary Industries, University of Sydney and South Africa's Stellenbosch University and Sugarcane Research Institute.

This is the third visit by the project team, after successfully collecting 150 samples of cocoa pod borer from East New Britain, East Sepik and the Autonomous Region of Bougainville on previous visits.

"Cocoa plantations in Papua New Guinea tend to be largely small-holder family farms, and effectively managing this destructive pest has thrown up some challenges. Any change, or improvement to current pest management practices to combat the cocoa pod borer needs to be achievable and cost-effective" says **cesar**'s Dr Olivia Reynolds.

"Correct identification of both the pest and damage, is the first essential step to good management. A community driven approach to learning more about this pest and its biology and ecology will be extremely important in effectively controlling the cocoa pod borer in Papua New Guinea," she says.

The project is also reviewing what research has already been done and where there are knowledge gaps in identification and management. The research will provide methods to accurately identify the cocoa pod borer and culturing it in a laboratory environment so it can be better studied and understood.

Cocoa pod borer (*Conopomorpha cramerella*) is a pest throughout parts of South East Asia and the Western Pacific. Feeding of the caterpillar within the pod causes premature or uneven ripening of cocoa plants, affecting cocoa harvest and yield.

This research is funded by the Australian Government, through the Australian Centre for International Agricultural Research (ACIAR).

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