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Biology, damage potential and molecular identification of *Conogethes punctiferalis* Guenee in cocoa (*Theobroma cacao* Linn.)

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Abstract

Conogethes punctiferalis is an important polyphagous pest attacking many economically important crops. Recently, *C. punctiferalis* has been found to be an emerging pest in cocoa and was found to feed and bore into cocoa pods. The larvae feed on the rind of cocoa cherelles/pods, later bore into pods, feed the internal contents of the pods, the granular faecal pellets are seen outside the pods. When pods/cherelles touch each other, it is easy for the larvae to damage more than one pod/cherelle. Pods damaged by *Conogethes* are exposed to secondary infection by pathogens that lead to pod rot. The larvae sometimes feed on flower buds and flowers cushions. The damaged flower cushions may dry and shed prematurely. The damage of *C. punctiferalis* on cocoa is observed from December and peak incidence is noticed during March to May. On an average 2 per cent damage was recorded in the Central Plantation Crops Research Institute, Regional Station, Vittal. In order to develop a DNA-based molecular identification system for this species, primers were designed based on two nuclear genes *viz*., ribosomal protein S5 (RPS5) gene and carbamoyl phosphate synthetase/aspartate transcarbamylase/dihydroorotase (CAD). PCR-amenable DNA was isolated from *C. puntiferalis* larva. The designed primers amplified single bands of expected sizes using genomic DNA as template. The amplicons were purified, cloned and sequenced and sequence analysis revealed close homology to the gene of interest from related moths.

Keywords: Conogethes punctiferalis, cocoa, emerging pest, molecular identification

Introduction

Cocoa (*Theobroma cacao* L.) is a small understorey tree endemic to the lowland rainforests of the Amazon basin (Wood and Lass, 1985). It is grown throughout the humid tropics, often in agro-forestry ecosystems with other fruit and commodity crops. Among the insect pests, a few could cause severe damage during the initial years and some sap sucking insects cause direct crop loss by feeding on young and maturing pods.

Conogethes punctiferalis Guenee (Crambidae: Lepidoptera) is an important polyphagous pest reported not only from South-East Asia and Australia (Pena *et al*., 2002), but also from Britain and Europe as introduced pest. In India, this pest has a host range of 36 crop plants belonging to 23 families. It is the most serious insect pest of papaya in Australia (Chay-Prove *et al*., 2000), *Durio zibethinus* in Thailand, fruits and maize crop in China (CPCI, 2005), more than 20 fruit crops including *Dimocarpus longan, Averrhoa carambola, Litchi chinensis* in Korea and *Helianthus annuus, Macadamia ternifolia* in New Zealand (CPCI, 2005).

The caterpillars of this pest caused damage of upto 80 per cent of the flower cushions in about 40 per cent of the trees. During 1975, a study of cocoa pests in Thrissur district of Kerala revealed

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that *C. punctiferalis* was found to damage cocoa flower cushions severely and occasionally on cocoa bark (ChandraMohanan and Harishu Kumar, 1975).

The use of DNA sequences represents a promising and effective tool for fast and accurate species identification (Hebert *et al*., 2003; Waugh, 2007; Pereira *et al*., 2008). Molecular identification possesses several advantages over conventional techniques of examination, some of them being requirement of only a very small amount of material for DNA extraction and its high accuracy. Molecular techniques have been successfully applied in various vertebrate and invertebrate taxa for species delimitation and identification (Smith *et al*., 2005; Clare *et al*., 2006; Hubert *et al*., 2008; Smith and Fisher, 2009; Zhou *et al*., 2009).

Presently, *C. puntiferalis* is emerging as a pest on cocoa pods in India. In view of importance of cocoa crop, the present study was undertaken to assess the damage potential of the pest in cocoa pods, the pest biology and its molecular identification.

Materials and methods

Biology of *C. punctiferalis*

The study was conducted at Central Plantation Crops Research Institute (CPCRI), Regional Station, Vittal, Karnataka, India and CPCRI, Kasaragod, Kerala, India during 2010 to 2013. Field collected *C. punctiferalis* larvae were reared on castor using plastic trays (Rajabaskar, 2003). Emerging adults were sexed and kept in large cages for oviposition. Cotton pads soaked with 10 per cent sugar solution were given as adult food. Castor inflorescence (panicle) with flowers and immature capsules were kept as ovipositional substrate with the cut end dipped in water in conical flask. Newly hatched first instar larvae were transferred to immature capsules of castor and reared in trays. New capsules were given as feed once in four days, *i.e*. when dried or eaten by the larvae.

Ten neonate larvae immediately after hatching were transferred to individual plastic boxes containing cocoa pods. The boxes were covered with muslin cloth for adequate aeration and rubber bands to prevent the escape of larvae. Each set up was replicated three times with ten larvae per replication. The larvae were provided with fresh food once in three days. Observations were recorded on the time interval between different larval instars, pupal duration and longevity of adults.

DNA extraction and PCR

DNA was extracted from fresh fifth instar larva of *C. punctiferalis* using DNeasy blood and tissue kit (QIAGEN) as per the manufacturer's instructions. The quality and quantity of the extracted DNA was assessed using spectrophotometer and agarose gel electrophoresis.

Primers were designed based on ribosomal protein S5 (RPS5) and carbamoyl phosphate synthetase/aspartate transcarbamylase/ dihydroorotase (CAD) genes from sequences of these genes of moths from subfamily Spilomeliane deposited in NCBI. PCR was carried out using the designed primers, in volumes of 20 µl containing 35 ng genomic DNA, 0.3 µM each of forward and reverse primers, 100 µM of each dNTPs (M/s Bangalore Genei Pvt. Ltd., India), 1X buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$) and 1.0 Unit of *Taq* DNA polymerase (M/s Bangalore Genei Pvt. Ltd., India). PCR amplifications were performed on a BIORAD thermal cycler with a PCR profile of 94 °C for 2 min followed by 30 cycles of 30 sec at 94 °C, 1 min at 52 °C and 1 min at 72 °C with a final extension for 10 min at 72 °C.

After amplification, a volume of 3 µl of loading buffer (98% formamide, 10 mM EDTA, 0.005% each of xylene cyanol and bromophenol blue as tracking dyes) was added to the amplified product. The amplified products were run on 1.2 per cent agarose gel, stained with ethidium bromide and were visualized in a gel documentation system. The approximate band length was scored by comparing with the molecular ladder.

Purification and cloning of the amplicons

PCR products of expected sizes were purified using Strataprep PCR purification kit. Cloning of the eluted fragments was carried out using the InsT/A clone™ PCR product cloning kit (M/s MBI Fermentas Inc., USA). Ligation of PCR amplified purified DNA fragment was performed using pTZ57R/T vector as described in supplier's manual. Vector (pTZ57R/T) and inserts were taken in 1:3 ratio. Ligation was conducted in volumes of 30 µl

containing 3 µl of plasmid vector pTZ57R/T DNA (0.165µg, 0.18 pmol ends), 4 µl of eluted PCR fragment (approx. 0.54 pmol ends), 3 µl of 10X ligation buffer and 1 µl of T4 DNA Ligase (5 Units). The ligation mixture was incubated at 22 °C overnight. Transformation was carried out using TransformAid™ Bacterial Transformation System. After transformation, the cells were finally plated on pre-warmed LB- agar plates [ampicillin (100 ppm)/IPTG (100 ppm)/X-gal (160 ppm)] and incubated overnight at 37 °C. The recombinant clones were identified by blue/white colony selection.

Clone analysis by PCR

Colony PCR was carried out for direct analysis of the positive transformants.

One colony was picked up and resuspended in 20 µl of the PCR mixture. The reaction mixture was incubated for 5 min at 94 °C to lyse the cells and inactivate the nucleases. PCR amplification was carried out as described earlier and the PCR products visualized by agarose gel electrophoresis.

Isolation of recombinant plasmid DNA

Clones selected after colony PCR were re-inoculated into LB broth with amplicillin overnight (100 ppm). Highly pure plasmid DNA was extracted from the overnight culture by nucleospin plasmid quick pure kit (M/s Macherey-Nagel) following the manufacturer's instructions.

Sequencing of cloned fragments and sequence analysis

The recombinant plasmids were sequenced commercially at M/s Scigenom, Kochi, India. The nucleotide sequences from the M13 forward primer was screened for the presence of vector contamination using VecScreen (Altschul *et al*., 1997). The contaminated region was cut using the software BIOEDIT (version 2.31).

The amino acids were deduced from the nucleotide sequences in all possible frames using the translate option at the ExPASy (expert protein analysis system) proteomics server at the Swiss Institute of Bioinformatics. The resulting amino acids were subjected to similarity search using a local alignment search algorithm, BLAST (Altschul *et al*., 1997). The scores of alignment were computed with reference to block substitution matrix (BLOSUM). The result of the homology search is detailed as maximum identity for increased length of subject sequences, higher positive values and decreasing E-values. Lower the E-value, the more similar the sequences in terms of homology to the previously reported similar sequences in the database.

DNA sequences were analyzed using MEGA 5.10. Nucleotide sequences were aligned using MUSCLE programme. A genetic matrix was calculated from the alignment data and analyzed with the neighbor-joining method to reconstruct phylogeny. Bootstrap consensus trees were constructed using MEGA.

Results and discussion

Biology of *C. punctiferalis* on cocoa

Medium sized adult moth with small black dots on pale yellowish wing, the moth has a wingspan of about 3 cm. Mating was observed only in the dark after 7.30 PM. Female moth lay pinkish oval flat eggs singly or in groups of 2 or 3 mostly in between wart or grooves of pods/cherelles/flower cushions of cocoa. Eggs were rarely observed on unopened flowers and new flush leaves. The incubation period of eggs was 2.9 days. The full grown larvae were very active, 3 to 3.5 cm long, reddish brown, having brown marks on each segment with pinkish tinge, fine hairs on the body with dark head and prothoracic shield (Fig. 1). The results of biology of *C. punctiferalis* revealed that the total larval period was 23.82 days. The larvae after hatching feed on flower cushions, flower buds, rind of cocoa cherelles/pods later bore and feed the

Fig. 1. Larva of *C. punctiferalis*

internal contents of the pods and granular faecal pellets were seen outside the pods (Fig. 2). Wherever, pod/cherelle touches each other it is easy for the larvae to damage more than one pods/ cherelles. Pods damaged by *C. punctiferalis* are sometimes exposed to secondary infection by pathogens that lead to pod rot (Fig. 3a and b). The attacked cushions dry off and shed prematurely.

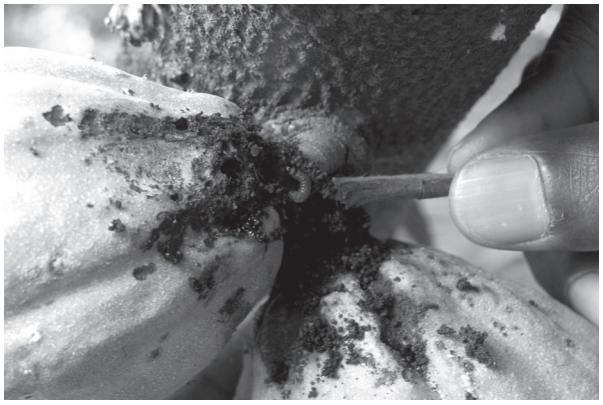
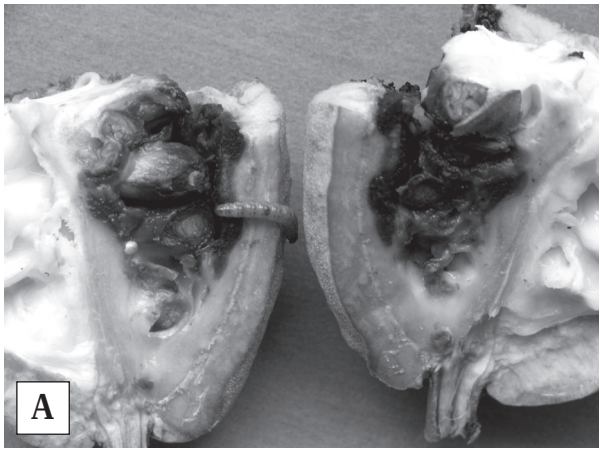


Fig. 2. Damaged cocoa pods with larva



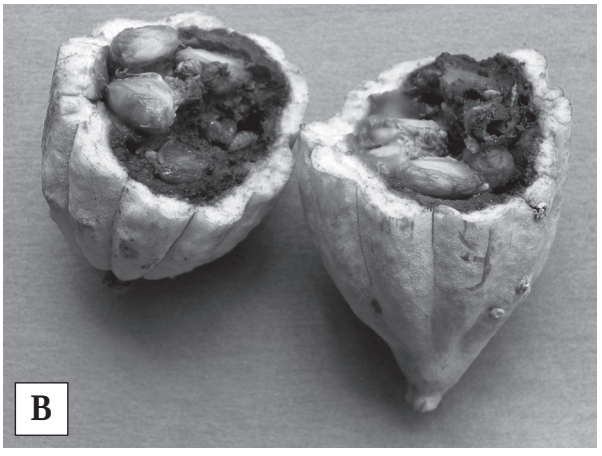


Fig. 3. A and B. Internal content of cocoa pods rotten due to *C. punctiferalis* damage

The total developmental period was 34.7 days. The longevity of male and female moth was 5.7 and 6.5 days respectively. The larvae throughout their life were seen under a cover of silk and frass or excreta which was on damaged pods and cherelles. The full grown larva was pupated inside the damaged pods or cherelles or in a thin silken cocoon outside the damaged pods/ cherelles. Adult emerged in 7 to 10 days. The life cycle at laboratory condition ranged from 25 to 33 days (Table 1).

Table 1. Biology of *C. punctiferalis* on cocoa

Stages	Period in days*
I Instar	3.10
II Instar	3.97
III Instar	4.30
IV Instar	5.75
V Instar	6.70
Pupae	7.95
Total	31.77
Adult male longivity	5.70
Adult female longivity	6.50

*Mean of three replications

Damage potential of *C. puntiferalis* on cocoa

The damage of *C. punctiferalis* was observed from December 2010 to May 2013. The infestation of *C. punctiferalis* started immediately after monsoon and the peak incidence was observed during March to May. On an average 2 per cent damage was observed in the research farm at CPCRI, Regional Station, Vittal (Table 2).

Table 2. Damage of *C. punctiferalis* on cocoa

Period	Mean per cent damage of *C. puntiferalis**		
	2010-2011	2011-2012	2012-2013
December	1.53	1.45	1.55
January	1.67	1.56	1.65
February	1.75	1.73	1.78
March	2.05	2.35	1.85
April	2.25	2.55	2.55
May	2.50	2.75	2.87
Mean damage	1.96	2.07	2.04

*Mean of 20 cocoa trees

Molecular identification

To confirm the species identity, an attempt was made with molecular tools. Intact DNA was obtained from fifth instar larva of *C. punctiferalis* using DNeasy as visualized using agarose gel electrophoresis followed by ethidium bromide staining in a gel documentation system. Primers designed in the present study, based on RPS5 and CAD genes are given in Table 3. The primers were used for amplification of genomic DNA and amplicons of expected sizes were obtained (Fig. 4). These were eluted, purified and cloned. Recombinant plasmids, extracted from clones confirmed by colony PCR, were sequenced. The sequences were deposited in Genbank (KC595364 and KC595365). Sequence analysis using BLASTn revealed close homology to related moths. The phylograms (Fig. 5a and 5b), constructed by BLASTn analysis, show the close association of *C. punctiferalis* from cocoa to *C. punctiferalis* from Hawaii and to other related moths. The nodes were supported by high bootstrap values.

In the present study *C. punctiferalis* was found to damage the pods by boring into the pods, feed the internal content of the pods, attract secondary infection and emerging as a pest in cocoa. Even though the recorded damage potential was less (2.07%), the main concern is, its feeding behaviour like cocoa pod borer *Conophomorpha gramerella* (Snellen) (Gracillariidae: Lepidoptera), which is a very serious pest in Malaysia, Indonesia, Java, East New Britain, Papua New Guinea (Ooi *et al*., 1987; Azhar, 1995; Azhar *et al*., 2001) causing 20-50 per cent yield loss (Mumford, 1984). Fortunately, this pest so far has not been reported in India. The biology of *C. punctiferalis* were in accordance with the reports of Jacob (1981) and Stanley *et al*. (2009).

Recently, the use of various nuclear genes in insect phylogenetic and taxonomic studies has been reported (Wahlberg and Wheat, 2008). Many advantages of the use of nuclear genes over mitochondrial genes in phylogenetic studies of insects have been proposed (Lin and Danforth, 2004). Some of these advantages include the slower rate of evolution of nuclear genes, their higher consistency index and higher symmetrical transformation rate matrices compared to mitochondrial genes (Lin and Danforth, 2004). In the present study, primers designed to amplify RPS5 and CAD genes, which have been previously demonstrated for their utility in deducing phylogenetic relationships in Hawaiian moths of Spilomelinae subfamily (Haines and Rubinoff, 2012). Nuclear genes, because of their occurrence in low copy number, have difficulty in amplifying through PCR (Lin and Danforth, 2004). Further, amplification of two or more paralogous loci of a nuclear gene may prove complicated to interpret (Lin and Danforth, 2004). The results demonstrate that both RPS5 and CAD genes behave as single copy in PCR reactions for the species examined and a combination of these two genes could be useful in

Table 3. Details of the primers designed, their sequences and expected product sizes

Sl. No.	Gene		Primers	Length	TM	GC	Product size
1.	CAD	Forward primer	GCTTCAAGAACCAACAGAC	19	56.0	47.4	473
		Reverse primer	AACAACCTACGGCACACCA	19	58.0	52.6	
2.	RPS5	Forward primer	GCATGGTTGTCGACTCCACG	20	64.0	60.0	528
		Reverse primer	CATCAGCTACACACTCAGCG	20	62.0	55.0	

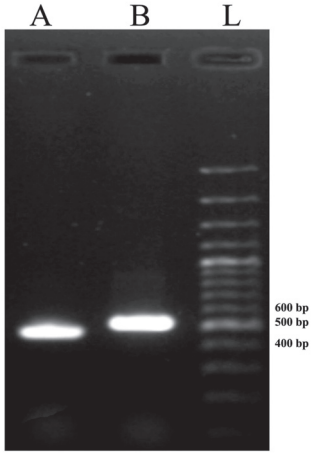


Fig. 4. Amplification of *C. punctiferalis* from cocoa using gene specific primers

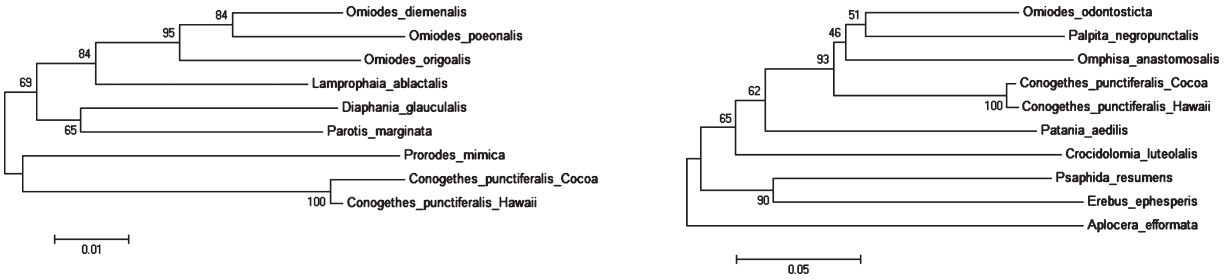


Fig. 5. Neighbour-joining phylograms of *C. punctiferalis* from cocoa using (A) RPS5 and (B) CAD genes

molecular systematics for specific amplification of *C. punctiferalis* DNA.

In the present study, two sets of primers were designed and validated for identification of *C. punctiferalis*. Bioecology of the pest is highly variable depending upon the host plant and habitat and therefore, any generalization on management strategy of the pest under different cultivated ecosystems is difficult. Hence, situation/location-specific studies on *Conogethes* are necessary for evolving rational pest management strategies.

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References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**: 3389-3402.

Azhar, I. 1995. An overview on the management of key insect pests of cocoa with major emphasis on the cocoa pod borer *Conopomorpha cramerella*. *The Planters* **71**: 469-480.

Azhar, I., Alias, A. and Meriam, M. 2001. Research on the cocoa pod borer in Malaysia. In: *Proceedings of Incoped 3rd International Seminar*, 16th-17th October 2000, Kota Kinabalu Sabah, Malaysia. pp. 105-113.

ChandraMohanan, R. and Harishu Kumar, P. 1975. The castor capsule borer, *Dichocrosis punctiferalis* Guen. as a pest of cocoa in India. *Agricultural Research Journal of Kerala* **14**: 79-80.

Chay-Prove, P., Ross, P., O'Hare, P., Macleod, N., Kernot, I., Evans, D., Grice, K., Vawdrey, L., Richards, N., Blair, A. and Astridge, D. 2000. *Agrilink Series: Your Growing Guide to Better Farming*. Queensland Horticulture Institute and Department of Primary Industries, Nambour, Queensland.

Clare, E.L., Lim, B.K., Engstrom, M.D., Eger, J.L. and Hebert, P.D.N. 2006. DNA bar-coding of neotropical bats: species identification and discovery within Guyana. *Molecular Ecology Notes* **7**: 184-190.

CPCI. 2005. Crop Protection Compendium on Internet, accessed in 2005. Wallingford, UK: CAB International.

Haines, W.P. and Rubinoff, D. 2012. Molecular phylogenetics of the moth genus *Omiodes Guenée* (Crambidae: Spilomelinae), and the origins of the Hawaiian lineage. *Molecular Phylogenetics and Evolution* **65**: 305-316.

Hebert, P.D.N., Cywinska, A., Ball, S.L. and de Waard, J.R. 2003. Biological identifications through DNA barcodes. *Proceedings of the Royal Society B: Biological Sciences* **270**: 313-321.

Hubert, N., Hanner, R., Holm, E., Mandrak, N.E., Taylor, E., Burridge, M., Watkinson, D., Dumont P., Curry, A., Bentzen, P. and Zhang, J. 2008. Identifying Canadian freshwater fishes through DNA barcodes. *PLoS ONE* **3**: e2490.

Jacob, S.A.1981. Biology of *Dichocrosis punctiferalis* Guen. on turmeric. *Journal of Plantation Crops* **9**: 119-123.

Lin, C.P. and Danforth, B.N. 2004. How do insect nuclear and mitochondrial gene substitution patterns differ? Insights from Bayesian analyses of combined datasets. *Molecular Phylogenetics and Evolution* **30**: 686-702.

Mumford, J.D. 1984. Control of the cocoa pod borer (*Acrocercops cramerella*): A critical review. In: *International Conference on Cocoa and Coconut*, Kuala Lumpur, Malaysia, 15th -17th October, 1984.

Ooi, P.A.C., Chan, L.G., Khoo, K.C., Teoh, C.H., Jusoh, M.M., Ho, C.T. and Lim, G.S. 1987. *Management of the Cocoa Pod Borer*. Malaysian Plant Protection Society, Kuala Lumpur. 192p.

Pena, J.E., Nadel, H., Barbosa-Pereira, M. and Smith, .D. 2002. Pollinators and pests of *Annona* species. In: *Tropical Fruit Pests and Pollinators: Biology, Economic Importance, Natural Enemies and Control.* (Eds.) Pena, J., Sharp, J. and Wysok, M., CABI Publication, UK. pp. 197-222.

Pereira, F., Carneiro, J. and Amorim, A. 2008. Identification of species with DNA-based technology: current progress

and challenges. *Recent Patent on DNA and Gene and Sequences* **2**: 187-200.

Rajabaskar, D. 2003. Studies on the evaluation of IPM modules against *Conogethes punctiferalis* Guenee and *Sciothrips cardamomi* Ramk. on cardamom. Ph.D. Thesis, Tamil Nadu Agricultural University, Coimbatore, India.

Smith, M.A. and Fisher, B.L. 2009. Invasion, DNA barcodes, and rapid biodiversity assessment using the ants of Mauritius. *Frontiers in Zoology* **6**: 31.

Smith, M.A., Fisher, B.L. and Hebert, P.D.N. 2005. DNA barcoding for effective biodiversity assessment of a hyper diverse arthropod group: The ants of Madagascar. *Philosophical Transactions of Royal Society B: Biological Sciences* **360**: 1825-1834.

Stanley, J., Chandrasekaran, S. and Preetha, G. 2009. *Conogethes punctiferalis* (Lepidoptera: Pyralidae) its biology and field parasitization. *Indian Journal of Agricultural Science* **79**: 906-909.

Wahlberg, N. and Wheat, C.W. 2008. Genomic outposts serve the phylogenomic pioneers: designing novel nuclear markers for genomic DNA extractions of lepidoptera. *Systematic Biology* **57**: 231-242.

Waugh, J. 2007. DNA bar-coding in animal species: progress, potential and pitfalls. *Bioessays* **29**: 188-197.

Wood, G.A.R. and Lass, R.A. 1985. *Cocoa*. 4th Edition, Tropical Agricultural Series, Longman Publications, New York, 620 pp.

Zhou, X., Adamowicz, S.J., Jacobus, L.M., DeWalt, R.E and Hebert, P.D.N. 2009. Towards a comprehensive barcode library for arctic life - Ephemeroptera, Plecoptera, and Trichoptera of Churchill, Manitoba, Canada. *Frontiers in Zoology* **6**: 30.