

Elucidating food plants of the aggregative, synchronously flashing Southeast Asian firefly, *Pteroptyx tener* Olivier (Coleoptera, Lampyridae)

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Abstract

The aggregative, synchronously flashing firefly, *Pteroptyx tener* congregates on a nightly basis on Berembang trees (*Sonneratia caseolaris*) growing along the lower reaches of the Selangor River (West Malaysia). Every night, the males and females of this species engage one another in a pre-mating ritual of flash communication. Little is known of the dietary requirements of the adults of *P. tener* apart from suggestions that these beetles feed on the nectar and sap of mangrove trees. The drastic reduction in their numbers in recent years has sparked an urgency to understand their dietary needs. Here, we report on a series of probing experiments where we sequenced and analysed DNA fragments obtained from the gut contents of adult *P. tener* specimens. We detected coding and non-coding chloroplast DNA (cpDNA) gene sequences in the gut DNA extracts of *P. tener*. One DNA sequence was in reasonably good condition to allow us to match it to the cpDNA sequence of a Malvacean, that is, *Heritiera littoralis*, a common inhabitant of estuarine habitats. We also detected the DNA sequences of plants (cultivated and natural) that may have come from hamlets or isolated freshwater swamps located further inland. The findings reported here provide early indication that *P. tener* may be able to travel further inland to search for their hosts. Future research should focus on visually confirming if *P. tener* feeds on *H. littoralis* and obtaining a more complete reference DNA database of plants in the firefly habitat.

Keywords

Pteroptyx tener, host, firefly, plants, chloroplast DNA

Introduction

The synchronous firefly, *Pteroptyx tener* Olivier (Coleoptera: Lampyridae), congregates in the thousands in estuaries in several locales throughout Peninsular Malaysia. Here, they perform their synchronous flashing behaviour throughout the year (Figure 1). During their nightly aggregation in estuarine habitats, the males and females of this species engage one other in a pre-mating ritual of flash communication before deciding upon their partners. Females which have mated then fly inland to lay their eggs on the ground (Nada and Kirton 2004). Lampyrid larvae require land snails for food with the type of hosts utilised highly dependent upon the snail species found locally (Madruga and Hernandez 2010). In comparison, little is known of the dietary requirements of *P. tener* with some speculating that the beetle feeds on the nectar of *Sonneratia caseolaris* Lythraceae (in Buck 1988; Nallakumar 2003) or that it required no food at all in the adult stage. In North America, a similar hypothesis was suggested for *Photinus carolinus* Green (Lampyridae), which was rejected after laboratory studies showed that firefly individuals reared on fruit had an increased lifespan (Faust 2008).

In temperate regions, fireflies have been reported to feed on a diverse range of host plants such as ginger lilies, honey-dew, pomegranate and floral segments of the milkweed, *Asclepias syriaca* (L.) (Lloyd 1998; Wedincamp Jr. et al. 1996; Ballantyne et al. 2013; Faust and Faust 2014). The males of *Photinus* and *Luciola* fireflies also provide nutrition to conspecific females during copulation (Rooney and Lewis 1999; Cratsley et al. 2003; South et al. 2008a; South et al. 2008b; South and Lewis 2012). These nuptial gifts contain nutrients essential for egg production in *Photinus* females (Rooney and Lewis 2002; Cratsley et al. 2003; Lewis et al. 2004). *Photuris* fireflies however have a slightly different way of obtaining nutrition. Considered the femme fatales of the insect world, the *Photuris* mimic the flash display of *Photinus* females in order to ensnare and devour male *Photinus*. This is done to obtain nutrients and defensive steroids missing in the *Photuris*, which would otherwise leave them defenceless against their natural enemies (Eisner et al. 1997).

Unfortunately, fireflies are not afforded the same attention, funding or structured research programmes given to insects of economic importance such as agricultural, livestock or stored product pests. The situation is also dire for *P. tener* in Malaysia, as the country's best-known insect species is threatened by waste pollution and destruction of its breeding habitat for the purpose of commercial planting of oil palms (Chiew 2009). The limited nature of funding for firefly conservation work in Malaysia has led us to conduct some probing experiments to solve the mystery of the nutritional requirements of *P. tener*. We set out first and foremost to determine if adult fireflies require plants or insects for food by extracting the whole gut content of adult *P. tener* beetles and screening them with plant chloroplast and invertebrate mitochondrial DNA (mtDNA) markers. We then compared these sequences with the DNA barcodes of plants from the firefly habitat. When a match to these reference plant DNA sequences was not available,



Figure 1. Aggregative, synchronously flashing firefly, *Pteroptyx tener* (Coleoptera: Lampyridae) performing their nightly mating ritual along the Selangor River.

we compared our DNA sequences with sequences on the GenBank database. We also compared mtDNA gene sequences obtained from the gut DNA extracts of *P. tener* with mtDNA gene sequences obtained from the tissue material of *P. tener*.

Materials and methods

Insect and plant DNA extraction

Adult *P. tener* fireflies were collected from the wild from Selangor, Sepetang (Perak) and Rembau Rivers (Negeri Sembilan) along the west coast of Peninsular Malaysia from their display trees, namely, *S. caseolaris* and *Hibiscus tiliaceus* (Malvaceae). Prior to DNA extraction, specimens were sterilised to remove contaminants such as pollen or plant residues from their bodies. Fireflies were sterilised by immersing them in a solution containing 0.5% sodium hypochlorite and 0.01 µl/ml Triton X-100 (Fisher BioReagents™, USA) and agitating them for 1 minute (in Matheson et. al. 2008). Specimens were then rinsed in double distilled water (ddH₂O) for 1 minute and air-dried on filter paper. Fireflies were then dissected and their alimentary canal removed with

a pair of dissecting forceps and an insect pin attached to a vice before using them in DNA extraction experiments. DNA from the gut of adult *P. tener* were extracted with Wizard Genomic DNA Purification Kit (Promega Corporation, Maddison, USA), while plant DNA was extracted from leaf samples using the CTAB extraction method (Murray and Thompson 1980). Laboratory bench tops and pipettes were sterilised with DNA Remover (Minerva Biolabs GmbH) before the start of our experiments. We also used filter tips to reduce the risk of contamination in our experiments.

Botanical surveys to search for members of the Malvaceae, Thymelaeaceae and Lythraceae, were carried out along parts of the riverbank inhabited by *P. tener*. Target plant families were ascertained earlier from preliminary laboratory screening of the gut contents of *P. tener* (unpublished). Voucher collection of twigs and stems with flowers and fruits, were made of the flora from the firefly habitat. DNA samples of the voucher materials, in the form of leaves of each targeted plant species were preserved in zip-lock bags containing silica gel. Plant voucher specimens were identified to the species-level by comparing them with identified materials in the Kepong Herbarium in FRIM.

Amplification of plant and insect barcoding genes

PCR was performed on a GeneAmp 9700 Thermal Cycler (Applied Biosystem, Foster City, CA) in a reaction containing 1 µl of template DNA (10ng/µl), 0.6 µl of each primer, 5.0 µl of 2× Transtaq Hifi Supermix (TransGene Biotech, Beijing) and 3.4 µl of Ultrapure ddH₂O (Invitrogen, USA). The following parameters were used to perform the PCR: initial denaturation at 95°C for 3 minutes, denaturation at 95°C for 1 minute, annealing for 1 minute at 47°C for matK, trnH and rbcL, extension at 72°C for 1 minute and 30 seconds, final extension at 72°C for 10 minutes. Amplification of insect barcoding genes used the following conditions: initial denaturation at 95°C for 3 minutes, denaturation at 95°C for one minute, annealing for 1 minute at 48°C for the 16S rRNA and cytochrome oxidase subunit I genes, extension at 72°C for 1 minute and 30 seconds, final extension at 72°C for 10 minutes. The primers used to amplify the three different genic regions (rbcL, trnH-psbA and matK) from the gut contents extracted from *P. tener* were rbcL-aF (5'-ATGTCACCACAAACAGAGAC-TAAAGC-3') (Levin et al., 2003) and rbcLa-724r (5'-TCGCATGTACCTGCAG-TAGC-3') (Fay et al., 1997); trnH-f (5'-CGCGCATGGTGGATT CACAATCC-3') (Tate and Simpson, 2003) and psbA-r (5'-GTTATGCATGAACGTAAT GCTC-3') (Sang et al., 1997); and the maturase K gene using the primer pair matK-f (5'-GTA-CAGTAGTTTTGTGTTTACGAG-3') and matK-r (5'-ACCCAGTCCATCTG-GAAAT CTTGGTTC). The cytochrome oxidase subunit I or *cox1* gene was amplified with the primer pair HCO2198 (5'-TAACTTCAGGGTGACCAAAAAAT-CA-3') and LCO1490 (5'-GGTC AACAAATCATAAAGATATTGG-3') while the 16S ribosomal RNA gene was amplified with the primer pair 16S-ar-JJ (5'-CGC-CTGTTTATTAAAAACAT-3') and 16S-1472-JJ (5'-GGTCCTTTCGTAATAA-3') (Folmer et al. 1994).

DNA and PCR products were electrophoresed on 0.85% agarose and 2.0% agarose gels, respectively, and visualised under ultraviolet light after staining with GelRed (Biotium, USA). Unincorporated primers and dNTPs were removed from the PCR products with shrimp alkaline phosphatase (USB ExoSAP-IT PCR Product Cleanup, Affymetrix, USA) following the manufacturer's protocol. The PCR products were then used in Big-Dye[®] Terminator ver. 3.1 cycle sequencing reactions after which they were injected into a 3130xl Genetic Analyzer (Applied Biosystems). Sequencing was performed in both the forward and reverse direction after which the sequences were assembled in Sequencher ver. 4.9 (Gene Codes Corp., Ann Arbor, MI). The following GenBank accession numbers KX909577-99 and KX80349-50 were generated from this study. The identity of the DNA sequences were determined by comparing the query sequences against reference DNA sequences on the National Centre for Biotechnology Information (NCBI) database (Madden 2003) using the Basic Local Alignment Search Tool (BLAST) and the reference DNA barcodes of riverine plants in the vicinity of the firefly habitat.

Results

Amplification of mtDNA and chloroplast genes from *Pteroptyx tener* gut extracts

Amplification of insect mtDNA barcoding genes from gut DNA extracts with 16S rRNA and *cox 1* markers showed the absence of non-*P. tener* DNA among our samples (Suppl. materials 1 and 2). MtDNA gene sequences obtained from the gut DNA extracts of *P. tener* were found to be identical to the DNA sequences obtained from the legs, thorax, and head of the firefly. We detected no overlying or underlying peaks in all electropherograms which would indicate the presence of mixed or multiple DNA samples in the material we analysed. Separately, the gel image in Figure 2 shows the amplification of plant genes in 12 firefly individuals obtained from the Selangor River. We detected the presence of PCR bands of the following size ranges: 200 to 300 base pairs (bp) amplified with the ribulose-biphosphate carboxylase (*rbcL*) marker; 100 to 200 bp, from the *trnH-psbA* intergenic spacer region (*trnH-psbA*) marker; and 300 to 500 base pairs, from the maturase K (*matK*) markers (Figure 2).

Several samples showed distinct PCR bands, for example, in lanes 5 through 8, lanes 16 through 23 (*rbcL* genes), lanes 25 through 28 (*trnH-psbA* genes) and lanes 33 through 38 (*matK* genes) (Figure 2). Similarly, Figure 3 showed that attempts to amplify the *trnH-psbA* gene resulted in smears in many of the samples although in some samples, single distinct PCR bands that were approximately 1,000 bp in size were obtained. *RbcL* genes between 700 and 800 bp in size were amplified in 4 samples from Sepetang River while a smear was obtained from an individual collected from Kuala Selangor (Figure 4). Amplification with *matK* markers resulted in smears in almost all the firefly samples (Figure 4). We were unable to amplify any plant DNA genes from the gut DNA extracts of firefly samples from the Rembau River.

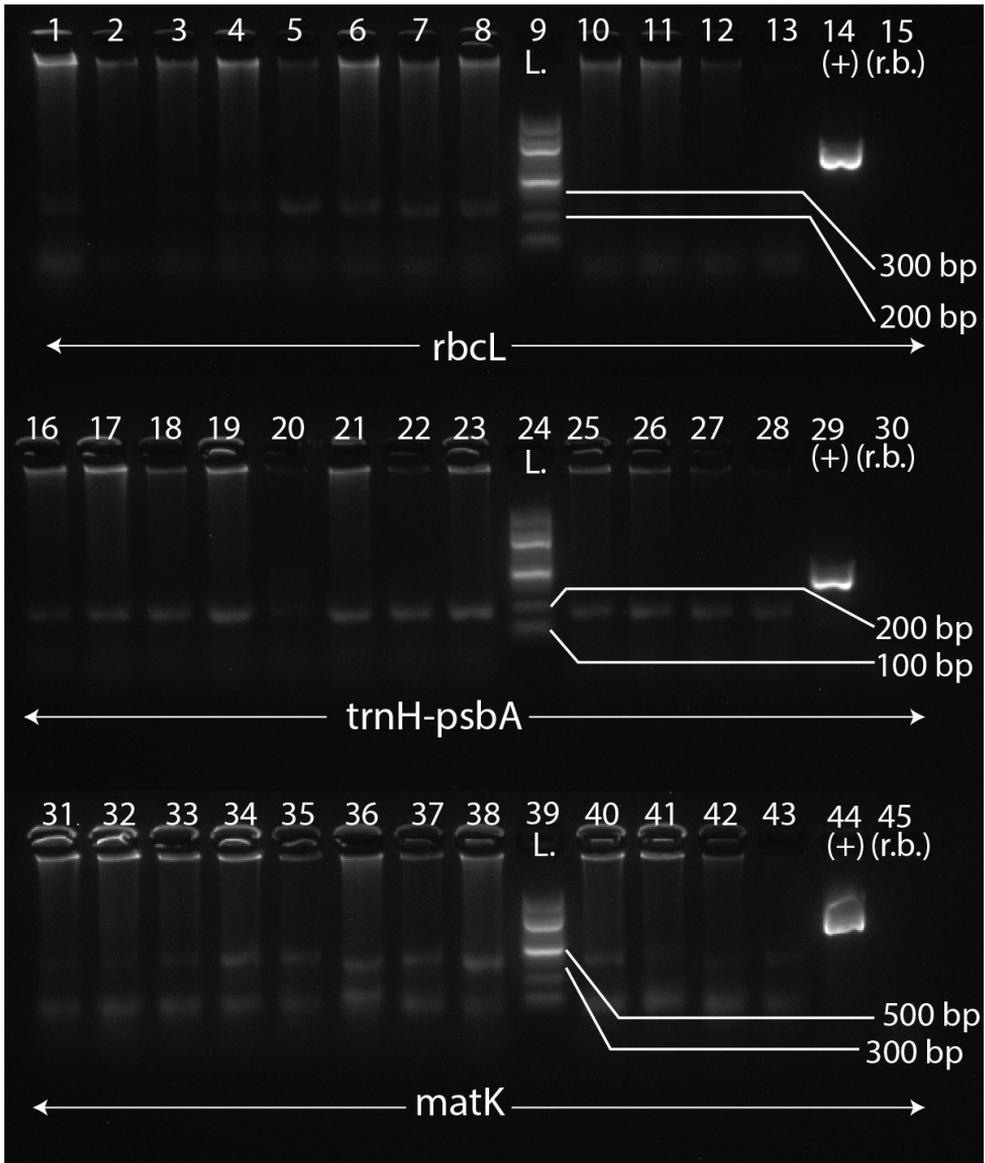


Figure 2. PCR amplification of *rbcL*, *trnH-psbA* and *matK* genes from *P. tener*. Note: “+” for positive control; “r.b.” for reagent blank; “L.” for 100 bp DNA ladder.

DNA sequences from *Pteroptyx tener* and plants from the firefly habitat

Approximately 26 plant genes between 124 and 744 bp in size were amplified and sequenced from the gut DNA extracts of male and female *P. tener* (Figure 5). DNA fragments amplified with *trnH-psbA*, *rbcL* and *matK* markers were found to be coding chloroplast and non-coding chloroplast-like genes. Only coding chloroplast DNA sequences

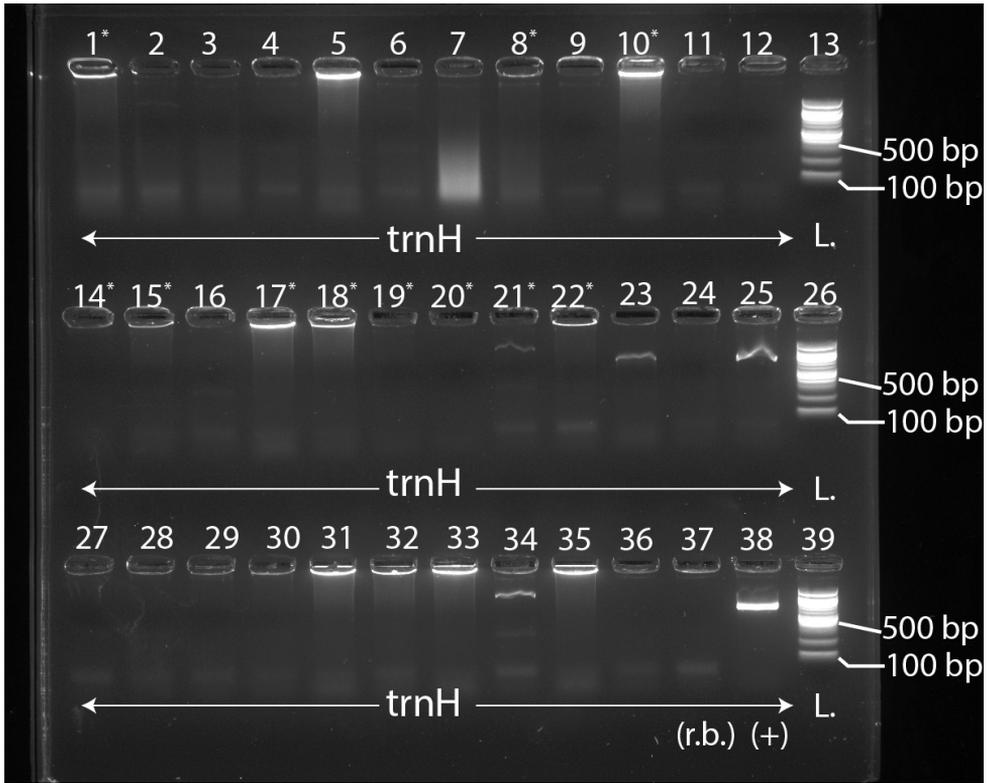


Figure 3. Amplification of *trnH-psbA* gene from *P. tener* from Selangor and Perak*. Note: “+” for positive control; “r.b.” for reagent blank; “L.” for 100 bp DNA ladder.

that were longer than 200 bp were deposited into the GenBank database as per the requirements of the National Centre for Biotechnology Information. One query sequence (Query 1) was identical to the *rbcL* sequence of *Heritiera littoralis* (KX909587), a Malvacean found along riverine areas (Figure 5). Prior to this, the sequence (Query 1) was found to be 99% similar to a sequence of *Tilia paucustata* from GenBank. Several plant meal sequences, that is, KX909584, KX950349, KF950350 and Query 5, returned a 97-99% (similarity) match to GenBank sequences of *Lawsonia inermis* (Lythraceae). We were however unable to locate the plant along the riverbanks of the Selangor River where the specimens were obtained.

We found no traces of *S. caseolaris* or *Hibiscus tiliaceus* in firefly gut DNA extracts (the display trees utilised by *P. tener* adults). A majority of our query DNA sequences (Queries 2-10) obtained from the gut DNA extracts of *P. tener* also did not match reference plant sequences from the riverine habitat of *P. tener*. The comparison of our other query sequences, that is, Query Sequences 6 to 10, showed similarity to the sequences of *Gonystylus bancanus* and *Aquilaria* sp. from GenBank. *Gonystylus bancanus* typically inhabits peat and freshwater swamps while *Aquilaria* is gaining popularity as a cultivated plant species in Malaysia. The remaining DNA or query sequences were non-coding, chloroplast-like

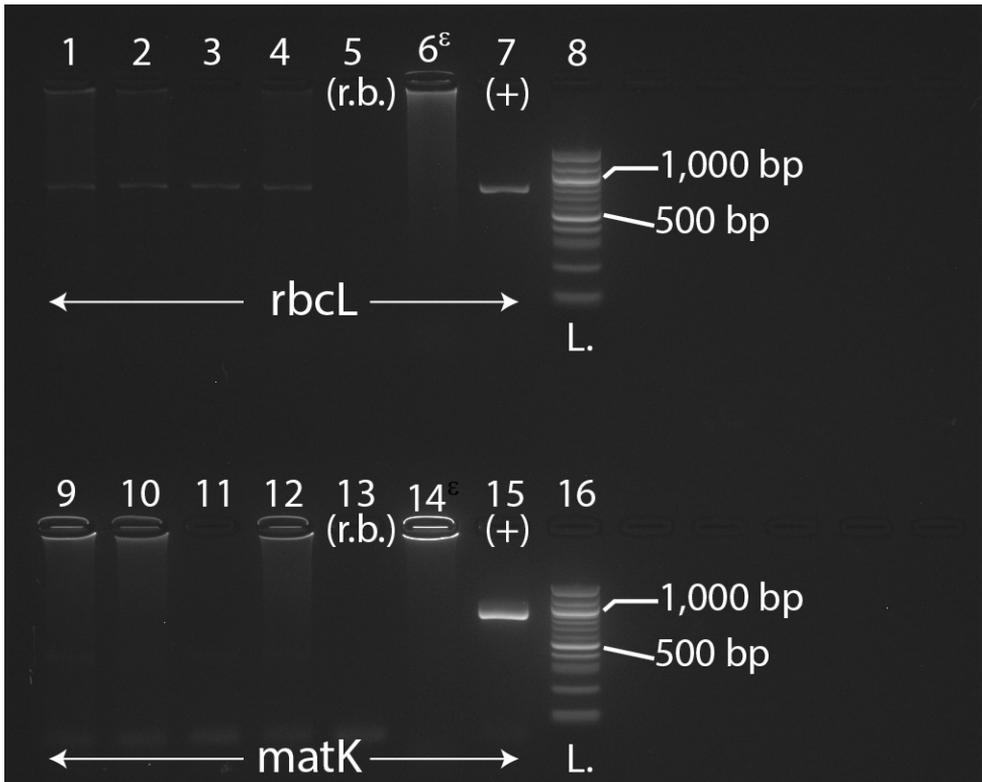


Figure 4. Amplification of *rbcL* and *matK* genes from *P. tener* from Perak and Selangor⁶. Note: + = positive control; “r.b.” = reagent blank; “L.” = DNA ladder.

DNA sequences. The results of the BLAST analysis to reference sequences on the GenBank database are shown in Figure 5. Most sequences had extremely low query coverage of between 6% and 24% of their gene length, although their similarity index was high.

Discussion

Plant DNA genes detected in gut DNA extracts of adult *Pteroptyx tener*

Only plant DNA could be detected in gut DNA extracts from *P. tener*, apart from its own DNA. Although *P. tener* spends a large portion of its adult life on *S. caseolaris* and *H. tiliaceus*, the plants were not found in their gut DNA extracts. However, this does not preclude the plants from being utilised for food by fireflies especially if the beetles fed on nectaries, fruit or sap of these plants. In our initial investigation (unpublished), plant sequences detected in the gut of the fireflies returned a match to the DNA sequences of Thymelaeaceae, Malvaceae and Lythraceae. The results of this experiment however showed that a single plant DNA sequence obtained from *P. tener* was identi-

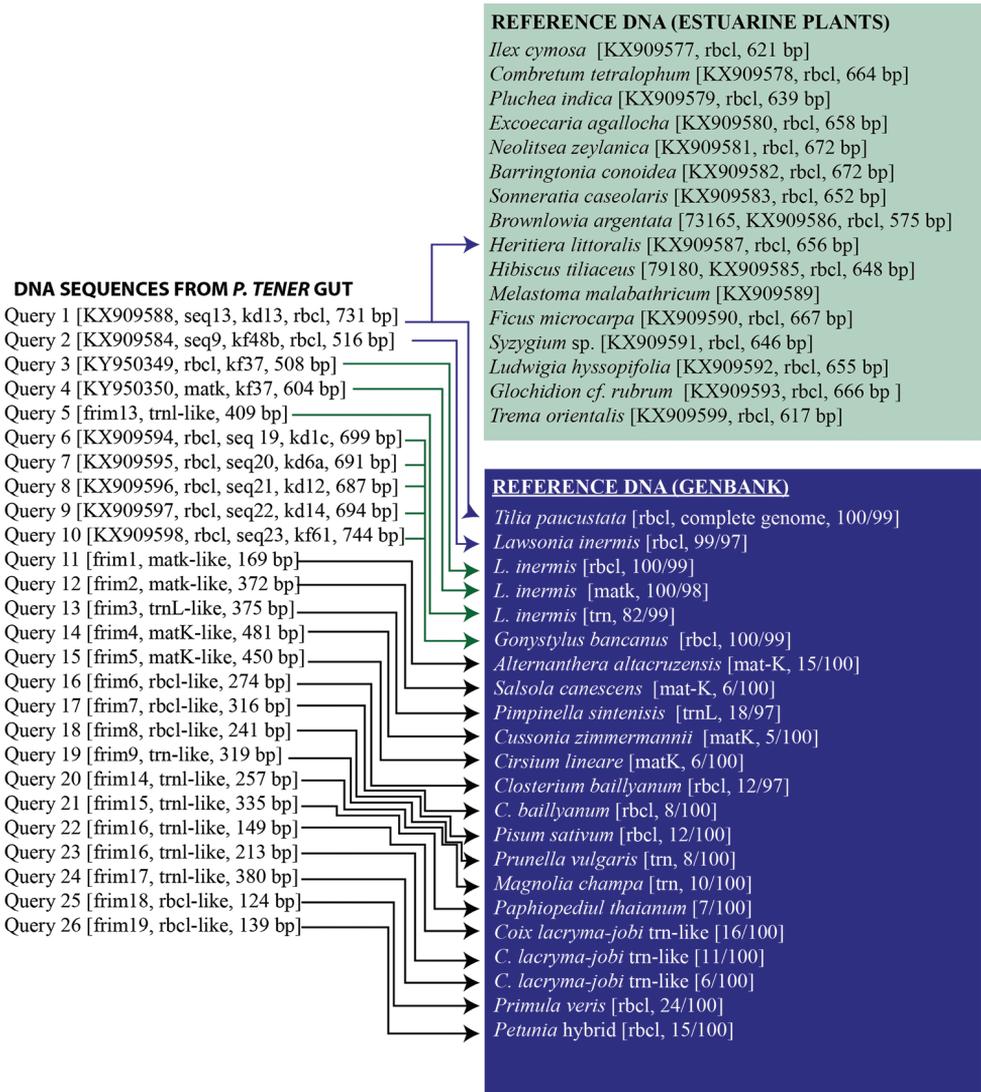


Figure 5. Table of relationships between plant DNA sequences detected in the gut contents of *P. tener*, reference DNA plants from the firefly habitat, and DNA sequences from GenBank. BLAST analysis results include the identity of the matching plant species, gene, query cover and similarity index (after forward slash sign).

cal to the rbcl sequence of *H. littoralis* (Malvaceae). The discovery of several DNA sequences that were highly similar to the DNA sequence of *L. inermis* (Lythraceae), a non-native, but relatively common garden plant in Malaysia, *G. bancanus* (Thymelaeaceae), an inhabitant of peat and freshwater swamps, and *Aquilaria* sp., a popular, cultivated plant species in Malaysia, has led us to speculate that *P. tener* travels further inland to obtain these host plants. Alternately, the plants may be in difficult to access or isolated forest patches.

Plant herbivory and determining food plants of insects

The larvae and adults of some beetles have mouthparts that have been adapted for feeding on fluids. The fluids are absorbed via canals or internal ducts located at the tip of their mandibles (Waldbauer 1998). The larvae of *P. tener* paralyse its snail host, *C. carinata*, with a toxin before liquidifying their tissue with digestive enzymes before the fluids are absorbed via these canals (Labandeira 1997; Fu and Ballantyne 2008). The mandibles of the larvae of *Pteroptyx valida* (Coleoptera: Lampyridae), similarly, contain canals. However, it is unclear if these canals are retained in the mandibles of their adults (Ballantyne and Menayah 2002) as there have been no attempts to describe the mouthparts of adults of *P. tener* and *P. valida* in detail.

Some firefly species remain in the larval stage for a longer period but are short-lived as adults. Others however spend a considerable amount of time in the adult stage after having only spent a brief period in the larval stage. Lewis and Cratsley (2008) found that short-lived adult *Photinus ignitus* Fall (approximately 2 weeks) did not feed in the adult stage, while long-lived adult *Ellychnia corrusca* Linnaeus (approximately 10 months) fed in adulthood. Preliminary studies in Malaysia however indicate that the adults of *P. tener* live for 3 to 4 weeks (Nada et al. 2012).

Plants play an important role in the life-cycle of insects. Plants are utilised as a stage for their mating, as food or for egg-laying (Kaiser et al. 2016). The manner in which insects feed is also dependent upon their environment and interaction with their host plant(s). Plant-insect interactions have evolved so intricately and have enabled insects to adopt a generalist or specialist feeding behaviour (Mello and Silva-Filho 2002). This interaction is also greatly influenced by the defence mechanisms developed by their host plants (Petschenka and Agrawal 2016). Specialists have developed highly efficient mechanisms to counteract the effects of chemical toxins while generalists compromise certain aspects of their lifecycle to overcome the defence mechanisms of plants (Mello and Silva-Filho 2002; Price et al. 1980).

It is probably unlikely that *P. tener* feeds on the leaves and seeds of *H. littoralis* which contain chemicals that interfere with the growth and reproduction of insects (Yu et al. 2011; Wangenstein et al. 2013). However, the bell-shaped flowers of *Heritiera* spp., which offer pollen as a reward and are thought to be beetle-pollinated, may possibly be their food source (Bernhardt 2000). Pollen has been found to encourage egg maturation in some beetle species (Romeis et al. 2005; Rana and Charlet 1997). In addition, the presence of *H. littoralis* in the vicinity of firefly display trees provides further support that the tree is a possible food source for *P. tener*.

Three approaches are generally available to determine the hosts or food plants of fireflies. These include conducting a DNA analysis of their gut contents; observational studies in the field and/or laboratory; or the use of biochemical methods to detect sugars and/or cellulose in their gut. Observational studies may help in the identification of the host plants of the firefly, however, some clues as to where to look would be helpful. Clear choices of host plants would be plants or flowers that are constantly frequented by the insect. Sugar and cellulose assays such as the anthrone test or calcofluor fluo-

rescent staining (Matheson et al. 2008) can help determine if nutrients are being obtained from plants. Junnila et al. (2010) demonstrated how sugar and cellulose assays developed by Schlein and Jacobson (1994) and Schlein and Muller (1995) were able to detect the presence of sugar in the gut of *Anopheles sergentii*'s (Diptera: Culicidae) although the tests were not able to help identify the plants utilised by the mosquitoes.

More recent studies however have employed the use of DNA barcodes amplified from the gut content of insects to determine their plant hosts (e.g., Matheson et al. 2008; Hoogendoorn and Heimpel 2001; Lee et al. 2002; Junnila et al. 2010; Kishimoto-Yamada et al. 2013). The method is however limited by the difficulty in amplifying DNA barcoding genes from the insect gut because plant tissues usually begin to break down post-ingestion (Matheson et al. 2008; Hoogendoorn and Heimpel 2001). Hoogendoorn and Heimpel (2002) shed some light on what to expect from these studies when DNA analysis is possible. They found that in general, shorter DNA sequences were more resilient and easier to detect compared to longer DNA sequences. Detectability was also unaffected by sex, weight or meal-size. Other studies however show that degradation of plant materials in the insect gut varies from species to species, and may prevent accurate molecular analysis (Matheson et al. 2008; Jurado-Rivera et al. 2009).

Plant DNA barcoding limitations

There were limits as to what we could barcode from the firefly habitat due to the significant amount of cost required to collect, barcode and identify plants from the area, which was beyond our project funding ability. In addition, most tropical plants have still not been barcoded. Accurate species identification of land plants also requires multiple DNA regions or loci to be sequenced. Plastid genes such as the *rbcL* and *matK* are important DNA barcoding genes for land plants (CBOL Plant Working Group, 2009). However, genes such as the plastid intergenic spacer *trnH-psbA* and nuclear ribosomal internal transcribed spacers (ITS) are sometimes required to increase species resolution (Fazekas et al. 2012). The possibility of sequencing the genomic content of the firefly gut has not been explored to date. However, sequencing their guts is only half the problem solved. There will still be a need to match these DNA sequences with plant species found in their habitat, a procedure which would require a relatively complete DNA database of plants in the firefly habitat or area to be obtained.

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Supplementary material 1

Supporting Information S1. 16S ribosomal RNA sequence alignment

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Data type: molecular data

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Supplementary material 2

Supporting Information S2. Cytochrome oxidase subunit 1 sequence alignment

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Data type: molecular data

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