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Endocrine and reproductive differences and genetic divergence in two populations of the cockroach *Diploptera punctata*

L.E. Lenkic [a], J.M. Wolfe [a], B.S.W. Chang [b], S.S. Tobe [a,*]

[a] Department of Cell and Systems Biology, University of Toronto, Toronto, Ont., Canada M5S 3G5
[b] Departments of Ecology & Evolutionary Biology, Cell & Systems Biology, Centre for the Analysis of Genome Evolution & Function, University of Toronto, Toronto, Ont., Canada M5S 3G5

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ABSTRACT

The viviparous cockroach, *Diploptera punctata*, has been a valuable model organism for studies of the regulation of reproduction by juvenile hormone (JH) in insects. As a result of its truly viviparous mode of reproduction, precise regulation of JH biosynthesis and reproduction is required for production of offspring, providing a model system for the study of the relationship between JH production and oocyte growth and maturation. Most studies to date have focused on individuals isolated from a Hawaiian population of this species. A new population of this cockroach was found in Nakorn Pathom, Thailand, which demonstrated striking differences in cuticle pigmentation and mating behaviours, suggesting possible physiological differences between the two populations. To better characterize these differences, rates of JH release and oocyte growth were measured during the first gonadotrophic cycle. The Thai population was found to show significantly earlier increases in the rate of JH release, and oocyte development as compared with the Hawaiian population. Breeding experiments to determine the degree of interfertility between the two populations demonstrated greatly reduced fertility in crosses between the two populations. Additionally, levels of genetic divergence between the two populations estimated by sequencing a fragment of the mitochondrial 16S rRNA gene were surprisingly high. The significant differences in physiology and mating behaviours, combined with the reduced interfertility and high levels of sequence divergence, suggest that these two populations of *D. punctata* are quite distinct, and may even be in the process of speciation. Moreover, these studies have important implications for the study of JH function in the reproductive cycle of insects, as differences in timing of rates of JH biosynthesis may suggest a process of heterochrony in reproduction between the two populations.

1. Introduction

Diploptera punctata (Dictyoptera: Blattaria: Blaberidae) is the only truly viviparous cockroach known, and only one of a few truly viviparous insects (Roth, 1970, 1999). They are a burrowing species, living in leaf litter, which serves as a moist substrate for breeding, and a protection from predators (Schal et al., 1984). They often eat the bark of selected tree species and are considered pests in the tropics, particularly Hawaii, where they were introduced (Stay, 1999).

Hawaiian *D. punctata* has been a model organism for physiological studies of the regulation of juvenile hormone (JH) biosynthesis, the key hormone in reproduction in insects. As a consequence of its truly viviparous mode of reproduction, JH biosynthesis and oocyte development must be tightly regulated in *D. punctata* (Stay and Tobe, 1978; Stay et al., 1983; Tobe, 1980), since the inappropriate production of JH during the period of gestation (pregnancy) results in abortion of the developing embryos. JH production must therefore remain at low levels to permit a viable pregnancy (Stay and Coop, 1973). In addition, precise control of the timing of JH biosynthesis is not limited to pregnancy, but is required at all stages of reproduction. The mechanical stimulation of mating and spermatophore insertion releases the inhibition on the corpora allata (CA) (possibly mediated by allatostatins), resulting in a rapid increase in JH biosynthesis (Engelmann, 1959; Rankin and Stay, 1987). JH biosynthesis occurs exclusively in the paired CA, endocrine glands associated with the retrocerebral complex; these glands increase in size during the reproductive cycle as a result of cell growth and division. As a consequence of the ease of manipulation of the CA and the high rates of JH biosynthesis in this species, *D. punctata* has proven to be a useful model for the study of development and

* Corresponding author. Fax: +1 416 978 3522.
E-mail address: stephen.tobe@utoronto.ca (S.S. Tobe).

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reproduction. As JH is released into the haemolymph at rates equal to biosynthesis, production of vitellogenin is induced, as is its uptake by the basal oocytes in the bilateral ovaries (Stay and Tobe, 1978). Thus, JH biosynthesis in adult female *D. punctata* is closely correlated with the gonadotrophic cycle (Stay and Tobe, 1978; Stay et al., 1983; Tobe, 1980).

A population of *D. punctata* was collected from Nakorn Pathom, Thailand in 2004. Comparison of the laboratory Hawaiian and the Thai populations revealed readily apparent morphological differences as well as behavioural differences in mating. Rates of JH release were therefore measured during the gonadotrophic cycle in the two populations, as was oocyte development, to determine if the Hawaiian and Thai populations differ in these respects. Differences between the two populations were also investigated by performing mating experiments to determine if interpopulation crosses are fertile. Finally, a fragment of the mitochondrial 16S rDNA gene was sequenced in order to assess genetic divergence.

2. Materials and methods

2.1. Animal maintenance

Both Hawaiian and Thai populations of *D. punctata* were reared in an incubator at 27 °C in 12:12 photoperiod. Animals were fed water and Lab Chow (Purina, St. Louis, MO) *ad libitum*. Newly molted females were picked in the morning and maintained in glass containers until dissected. A 1:1 ratio of males and females were placed in each container to ensure all females were mated.

2.2. Radiochemical assay (RCA) for juvenile hormone

The RCA was performed *in vitro* as previously described by Tobe and Pratt (1974) and Pratt and Tobe (1974), and modified by Tobe and Stay (1977) and Tobe and Clarke (1985). Basal oocyte length was measured to ascertain the stage of physiological development of each animal.

2.3. Mating experiments

Reciprocal crosses were performed between Hawaiian and Thai *D. punctata*. Parameters for each cross were assembled by placing males and females of either population together in cages when males were all 7 days old (from adult emergence) and females were fourth instars (approximately 43 days since birth) so that their adult emergence would occur in the presence of the males (Stay, 1999). Parental crosses were performed in large groups of 70–162 individuals (25–72 females), housed in clear acrylic "cages" (of size $13.5 \times 31.5 \times 52.0$ cm). They were checked regularly for nymphs throughout the gestation period which is known to be about 70–71 days in Hawaiian *D. punctata* (Stay, 1999). Any nymphs found were then raised in separate glass jars until adults, if male, or fourth instars, if female. Then they were paired with 2–4 members of the opposite sex from either the pure Hawaiian or Thai populations to make the F_1 crosses. The jars were each checked for nymphs during the gestation period.

2.4. DNA extraction, PCR, and DNA sequencing

One head (~25 mg) of a pregnant female of each population of *D. punctata* was removed, snap frozen in a mixture of dry ice and ethanol, and ground into a powder using a small mortar and pestle. Total genomic DNA isolation was adapted from the DNeasy protocol for purification of total DNA from animal tissues (Qiagen). Lysis time using Proteinase K was 3 h at 56 °C. To further purify the genomic DNA, a phenol/chloroform extraction, followed by an ethanol precipitation, was performed (Sambrook and Russell, 2001).

PCR amplification of a 415-bp fragment of the mitochondrial 16S rRNA gene was performed using forward (5′-TTA CGC TGT TAT CCC TTA-3′) and reverse (5′-CGC CTG TTT ATC AAA AAC AT-3′) primers adapted from Kambhampati and Smith (1995). The resulting amplification product contained about 1/3 of the gene. PCR conditions were a modification of Kambhampati (1995), with an annealing temperature of 50 °C. The amplified product was gel-purified (QIAquick, Qiagen), and then cloned into the TOPO TA cloning vector (Invitrogen). For each individual, at least three clones were sequenced and compared in order to eliminate sequencing and other polymerase artifacts. Sequences were deposited in Genbank under the accession numbers EU580104, EU580105. Voucher specimens of each *Diploptera* population were deposited at the Royal Ontario Museum (Toronto), Ent Spec. No. 102957-102960.

2.5. Sequence analysis

Additional 16S insect sequences were downloaded from the NCBI databases, and aligned with sequences obtained from the two *Diploptera* populations using the program ClustalW (Higgins et al., 1992). This alignment was subsequently modified by hand in order to incorporate information concerning rRNA secondary structure (Buckley et al., 2000). Phylogenetic analyses, including maximum parsimony, neighbour-joining/minimum evolution distance methods, and maximum likelihood methods, were performed on the aligned nucleotide sequences using PAUP* v.4.0b10 (Swofford, 2002). Bootstrap methods were used to assess the degree of confidence of nodes in the phylogeny (Felsenstein, 1985). Corrected pairwise distances were also calculated in PAUP* (Swofford, 2002). The species included in the phylogenetic analyses were as follows: Blaberidae: *Angustonicus amieuensis* (GenBank accession no. AJ870994) *Archimandrita tessellata* (U17761), *Blaberus atropos* (U17763), *Blaberus craniifer* (U17765), *Blaberus discoidalis* (U17767), *Blaberus giganteus* (U17771), *Byrsotria fumigata* (U17769), *Epilampra azteca* (U17783), *Eublaberus posticus* (U17785), *Geoscapheus woodwardi* (AB036178), *Gromphadorhina portentosa* (U17787), *Macropanesthia rhinoceros* (AB036177), *Nauphoeta cinerea* (U17797), *Panchlora nivea* (U17814), *Panesthia angustipennis* (AB036179), *Phoeotalia pallida* (U17816), *Phortioeca phoraspoides* (U17819), *Pycnoscelus surinamensis* (U17821), *Rhyparobia maderae* (U17825), *Salganea esakii* (AB036180), *Salganea taiwanensis* (AB036181), *Schultesia lampyridiformis* (U17827), *Trichoblatta pygmaea* (AB036182); Blattellidae: *Parcoblatta pensylvanica* (U17818); Blattidae: *Blatta orientalis* (U17774); Cryptocercidae: *Cryptocercus darwini* (AF126779), *Cryptocercus garciai* (AF126774), *Cryptocercus kyebangensis* (AF310220), *Cryptocercus matilei* (AJ519677), *Cryptocercus primarius* (AY631406), *Cryptocercus punctulatus* (AF126773), *Cryptocercus relictus* (AY631412), *Cryptocercus wrighti* (AF126772); Acrididae: *Locusta migratoria* (AY856117).

2.6. Statistical analysis

One-way ANOVA was performed to determine if rates of JH release were different between the two populations at oocyte lengths of 0.6–0.72 mm on day 1, and 1.63–1.78 mm on days 6 and 7. For this test, the JH release data were square-root transformed. Two-way ANOVA was performed to determine if the difference between populations in overall rate of development during the gonadotrophic cycle was significant. Both Mann–Whitney and