Geographic populations of the medfly may be differentiated by mitochondrial DNA variation

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Abstract. Restriction enzyme cleavage sites of mitochondrial DNA (mtDNA) from the Mediterranean fruit fly were found to vary among introduced populations in the Neotropics. The survey included samples from 15 established natural populations and 5 laboratory cultures from Hawaii, Central America, South America and West Africa and samples from recent California infestations (1989, 1991). Based on restriction fragment length polymorphisms from 2 enzymes, Hawaii is an unlikely source for the 1989 and 1991 California infestations. Interpopulational variation in mtDNA demonstrates the potential for the technique to trace the process of colonization (geographic spread) by this insect.
Key words. Mediterranean fruit fly; *Ceratitis capitata*; mitochondrial DNA; colonization.

The Mediterranean fruit fly or medfly, *Ceratitis capitata* (Wiedemann), is recognized as one of the most serious pest of fruits and vegetables wherever it has become established in tropical and subtropical areas of the world. Although probably originating in equatorial Africa, it has expanded its geographical and host range over the course of the past 150 years, spreading to the Mediterranean (1842), southern Africa (1889), Australia (1887), South America (1901), Hawaii (1910), Central America (1955) and other areas[1-3]. Areas of permanent medfly establishment are indicated in figure 1.

Medfly continues to threaten important fruit production centers with subtropical or Mediterranean climates. The anathema of the medfly to agriculture is perhaps best illustrated by the efforts taken by uninfested countries to prevent colonization and, failing that, to eradicate outbreaks of the insect when they occur. Medfly first appeared in the continental United States in Florida in 1929 and $ 7.5 million was spent to eradicate it[4]. USDA Animal and Plant Health Inspection Service recently allocated $ 9.7 million annually for medfly detection and exclusion activities[5]. Preventative costs are small, however, compared to the cost of an infestation. In recent years, California in particular has been periodically plagued with medfly infestations[6]. Estimated expenditures by the State of California for medfly eradication efforts in 1989–1990 were over $ 60 million[5], not including loss of markets and quarantine costs to growers.

One uncertainty regarding medfly outbreaks in the United States is the geographic source(s) from which colonist flies originate. Based on circumstantial evidence, it is assumed that Hawaii could be a major source of medfly infestations in California[7]. Hard evidence concerning source areas would enhance quarantine interdiction ef-

Figure 1. Worldwide distribution of the medfly. Each dot represents a country or island where populations have become established.

forts and investigations of the dynamics of medfly colonist populations. Such information also might help determine whether an infestation is the result of a new introduction or the resurgence of an old one that had been reduced to a non-detectable level[6]. Attempts to address these uncertainties have been based largely on quarantine and interception data and medflies trapped as part of an eradication project. To date, available genetic markers have not been informative at the population level in medflies. Polymorphic proteins in medfly populations show significantly lower levels of variability in the New World compared to Africa and the Mediterranean region[8], which is consistent with expectations for an introduced species. This dearth of allozyme variability limits the ability of the technique to characterize New World populations. Genetic markers based on mitochondrial or nuclear DNA variation have been widely used in insect genetic studies, including studies on introduced insect populations[9] and *Anastrepha fraterculus* (Wiedemann)[10], another fruit fly species. We report here the existence of restriction fragment length polymorphism (RFLP) in mitochondrial DNA among introduced and endemic medfly populations.

Materials and methods

Adult medflies were obtained from 15 wild populations in Venezuela (2), Hawaii (7), Nigeria (1), Liberia (1), Guatemala (2) and California (2). In addition, samples from laboratory cultures initiated from local, feral populations were obtained from Argentina (3), Guatemala (1) and Hawaii (1). The California samples were taken from the 1989 and 1991 outbreaks in Los Angeles County. Samples were frozen and maintained at −80 °C before analysis (except for the 1991 California flies, which were received in 70% ethanol). A preliminary screening of 25 restriction enzymes was conducted on a subsample of the populations and, with the exception of California (1991) and Liberia, at least 10 individuals from each population were analyzed for the informative restriction enzymes.

Total nucleic acids were isolated from individual adults using a modification of methods developed for honey bees[11]. Flies were each gently homogenized in a 15-ml Corex tube with 1 ml of grinding buffer (10 mM Tris, 60 mM NaCl, 300 mM sucrose, 10 mM EDTA). One ml of a lysis buffer (300 mM Tris, 40 mM SDS, 20 mM EDTA, 0.7% freshly mixed DEPC) was added immediately and the solution was placed on ice for 15 min. The resulting lysate was centrifuged at 1400 × g for 8 min. One ml of buffer-saturated phenol was added to the supernatant and the mixture centrifuged at 1400 × g for 8 min. The aqueous phase was retained and extracted first with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), then with chloroform:isoamyl alcohol (24:1). The supernatant was twice precipitated with ethanol and centrifuged (1700 × g for 8 min) to pellet the DNA. The pellet was dried under vacuum, then resuspended in 90 µl of TE buffer (10 mM Tris, 0.1 mM EDTA). DNA thus isolated provided enough material for 3–4 digests. Restriction enzyme digestion conditions were those recommended by the suppliers. Digested DNA was separated on 1.0% agarose gels and then transferred to nitrocellulose filters using the Southern method described by Maniatis et al.[12].

Purified mtDNA from *Anastrepha suspensa* (Loew) or *C. capitata* was used to make a probe for the transferred mtDNA fragments. *A. suspensa* was obtained from a laboratory culture in Gainesville, Florida, and *C. capitata* was obtained from a laboratory culture in Honolulu, Hawaii. 8 g of adults or pupae were homogenized in a mitochondrial isolation medium, MIM (220 mM mannitol, 70 mM sucrose, 2 mM HEPES, 1 mM EDTA, 1 mM EGTA) on ice. Mitochondria were concentrated by differential centrifugation at 4 °C in a Sorvall SS34 rotor as follows: homogenate (5% w:v of tissue:MIM) was centrifuged at 2500 rpm for 10 min. The supernatant was collected and placed on ice. The pellet was again homogenized in MIM and centrifuged as above. Supernatants were combined and centrifuged at 10000 rpm for 10 min. The resulting pellet was homogenized in MIM solution and then centrifuged at 2500 rpm for 10 min. Supernatant was collected and centrifuged at 10000 rpm to form a mitochondrial pellet. Mitochondria were lysed with SDS, protein was precipitated with cesium chloride and pelleted by centrifugation at 10000 rpm. Closed circular mitochondrial DNA in the supernatant was separated from linear DNA and purified by two successive equilibrium centrifugations in CsCl and ethidium bromide[12]. Afterward, ethidium bromide was removed by butyl alcohol extraction, and CsCl was removed using a 30000 mol. wt Millipore filter and centrifugation or by drop-dialysis[13]. DNA isolated by the preceding procedure is suitable for production of mtDNA probe in other insects[11,14] and compares favorably with cloned mtDNA for such purposes in the honey bee (WSS, unpublished data). A radioactive probe was produced using nick-translation of intact purified mtDNA and hybridization was carried out under standard conditions[12] optimized for medfly (25% formamide, 50 °C). Visualization of mtDNA fragments was achieved with autoradiography.

Results and discussion

Restriction fragment length polymorphisms exist within (*Hae* III) and among (*Xba* I, *Eco*R V) medfly populations. *Eco*R V and *Xba* I pattern variation among populations from differing geographic locations could be used to define two distinctive haplotypes within New World medflies. Samples from Hawaii and Venezuela exhibited one haplotype, while samples from Argentina, California and Guatemala were fixed for the alternate haplotype (fig. 2). The Hawaiian haplotype was not present in samples taken from California medfly populations in 1989 or 1991, providing strong evidence that Hawaii was not the geographic source for the medflies introduced to Califor-

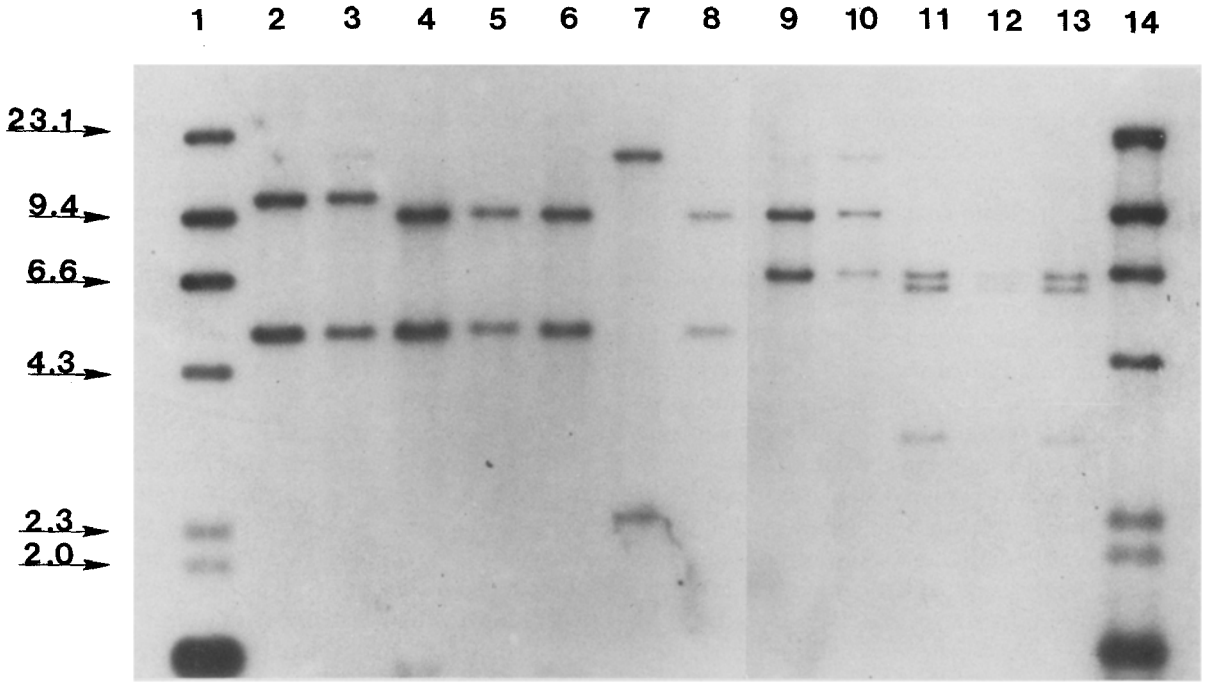

Figure 2. Autoradiograph of Southern blot showing medfly mtDNA variation in six populations digested with *Eco*R V (lanes 2–8) and *Xba* I (lanes 9–13). Lanes 2 and 9 = HI; lanes 3 and 10 = Venezuela; lanes 4 and 11 = Argentina; lanes 5 and 12 = Guatemala; lanes 6 and 13 = CA (1989 infestation). Lanes 7 and 8 show *Eco*R V variation found within a single Nigerian population. Lanes 1 and 14 contain a lambda/*Hind* III size standard shown in kilobase pairs.

nia in those years. Specimens from California suitably preserved for restriction enzyme analysis were very limited. Data from multiple infestation sites and other years of infestation remain unavailable. Thus, we do not imply that other recorded infestations could not have originated from Hawaii.

While the sampling of populations from some countries was limited, Hawaii was sampled relatively thoroughly. Seven different sites from the four major islands were sampled, with collections from two of these sites on two separate occasions. All of these samples exhibited a single mitochondrial haplotype. Presuming our collections are a representative sampling of medflies from Hawaii, our analysis comprises a sample from a binomial distribution; confidence limits are a function of sample size (approximately $1/2n$; $N = 70$)[15]. Hence, the frequency of other, undiscovered haplotypes can be estimated at less than 1%. This apparent lack of polymorphism is consistent with the hypothesis that introductions into various New World habitats were made from single sources by small populations or even single foundresses.

The laboratory cultures from Hawaii and Guatemala had haplotypes identical to wild populations taken from their respective geographic regions. These two distinct mtDNA haplotypes, already in culture, could be exploited as genetic markers to identify particular stocks of medfly for laboratory and field research. We expect that substantial additional mtDNA polymorphism exists in the medfly, especially in endemic areas of Africa, as medflies from a single population in Nigeria exhibited both *Xba* I and *Eco*R V polymorphisms described from the New World, as well as an additional *Eco*R V pattern (fig. 2). Another fruit fly, *A. fraterculus*, is known to be quite polymorphic in its endemic range[10].

These data clearly indicate the potential for mtDNA RFLP analysis in the study of medfly population genetics. The addition of samples from South American and African countries and Australia may permit diagnosis of the patterns of introduction and spread in recent colonization events in California and elsewhere. If colonization events in this insect are rare or arise from a single individual or group of siblings, then the technique may be exceptionally powerful to map the colonization pattern of this pest in past and future events.

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Juvenilizing effect of ecdysone mimic RH 5849 in *Galleria mellonella* larvae [1]

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Abstract. The response of the final instar larvae of *G. mellonella* to topical application of the non-steroidal ecdysone mimic, RH 5849, was age-related as well as dose-dependent. In young final instar larvae, moderate doses of RH 5849 induced perfect supernumerary larval moults, but doses equal to and higher than 8.5 µg per larva caused premature formation of larval cuticle and were lethal. Application of RH 5849 significantly increased allatotropic activity of the brain, and also activated synthesis of juvenile hormone (JH) by the corpora cardiaca/corpora allata complex. Simultaneous application of RH 5849 and FMev, a potent inhibitor of JH synthesis, to young final instar larvae lowered the incidence of perfect supernumerary larval moults. We conclude that the effect of RH 5849 on the developmental programme in *G. mellonella* is mediated by the corpora allata.
Key words. Galleria mellonella; ecdysone mimic RH 5849; juvenile hormone; allatotropic activity; corpora allata; supernumerary larval moult; juvenilizing effect.

It is generally accepted that in lepidopteran species a drop in juvenile hormone (JH) titre at the beginning of the final larval instar facilitates the metamorphic developmental programme, which is then controlled by two ecdysteroid peaks. A small peak precedes onset of wandering and a larger peak occurs just before pupation [2–6]. In *G. mellonella* the metamorphic programme is labile and the larva can revert to the larval programme under the influence of chilling stress [7], brain implantation [8] or JH application [9]. This shift is externally manifested by a supernumerary larval moult or a moult to the larval-pupal intermediate [9, 10]. The larval programme is characterized by a high JH titre throughout instar and a peak of ecdysteroids which reaches about 300 ng per g of body weight in *G. mellonella* [2, 11]. According to Sehnal et al. [2] the shape and size of the ecdysteroid peak during the larval cycle is regulated by levels of JH. Following bilateral allatectomy or application of FMev, an inhibitor of JH synthesis [12], larvae lose the ability to produce supernumerary larvae [8, 13, 14]. It is claimed that high levels of ecdysteroids mimic the action of JH, possibly by activating the insect's own corpora allata, or by acting synergistically with low levels of endogenous JH (for review see Willis [14]). However, the rapid degradation of ecdysteroids in insect tissues has been a serious obstacle in these studies. Recently, a highly potent, metabolically stable, non-steroidal ecdysone mimic, RH 5849, has been discovered [16]. This compound causes premature initiation of the larval moult at all stages of larval development of *Manduca sexta* [17] and in other insect species [18]. In larvae of *Plodia interpunctella*, RH 5849 stimulates supernumerary larval moults only when applied together with methoprene [19]. Application of RH 5849 performed very early in the final larval instar of diapausing and non-diapausing *Ostrinia nubilalis* larvae induced supernumerary larval ecdysis, and the effect has been correlated with a high systemic JH titre [20]. In this paper we describe the effects of RH 5849 on the development of final instar *G. mellonella* larvae. We have found that RH 5849 has a typical juvenilizing effect in this insect species, eliciting the formation of perfect supernumerary larvae. We are particulary interested in the effects of RH 5849 on the activity of the brain corpora cardiaca/corpora allata axis during transition from the metamorphic to larval developmental programme.

Materials and methods

Experiments were performed on the wax moth, *Galleria mellonella* (Lepidoptera, Pyralidae). The larvae were reared in constant darkness at 30 °C on a semi-artificial