DISCRIMINATION OF CARIBBEAN AND MEDITERRANEAN FRUIT FLY LARVAE (DIPTERA:TEPHRITIDAE) BY CUTICULAR HYDROCARBON ANALYSIS

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ABSTRACT

Larvae of the Caribbean fruit fly, *Anastrepha suspensa* (Loew) can be differentiated from those of the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) with nearly 100% accuracy by analysis of cuticular hydrocarbon (CHC) patterns. A discriminant model is presented based on samples of feral and laboratory Caribbean and Mediterranean fruit flies. The difference in the ratio of two components is sufficient to discriminate third instars of the species. Accuracy in discrimination, utilization of specimens that are damaged, dried or otherwise unusable for morphometric, isozyme or DNA analysis, low cost per sample and automation of the process, all make CHC analysis a particularly effective solution for identification of these two species.

Key Words: *Anastrepha suspensa*, *Ceratitis capitata*, gas chromatography, discriminant analysis.

RESUMEN

Las larvas de la mosca Caribeña de las frutas, *Anastrepha suspensa* (Loew) y las de la mosca del mediterraneo, *Ceratitis capitata* (Wiedemann) pueden ser diferenciadas entre sí con aproximadamente un 100% de precisión mediante análisis de los patrones de los hidrocarburos cuticulares. Se presenta un modelo para ambas moscas basado en muestras ferales y de laboratorio. La diferencia en la relación de dos componentes es suficiente para discriminar terceros estadíos de cada especie. La precisión en la discriminación, la posibilidad de trabajar con especímenes que se encuentran dañados, secos, o de alguna manera inutilizables para el análisis morfométrico, de isozimas o DNA, el bajo costo por muestra y la automatización del proceso, hacen el análisis de hidrocarburos una solución particularmente efectiva para la identificación de estas dos especies.

The state of Florida, as a major producer and exporter of fruits and vegetables, maintains a number of programs to deal with tephritid fruit fly pests. *Anastrepha suspensa* (Loew), the Caribbean fruit fly or caribfly, successfully invaded and rapidly colonized the southern half of the state during the 1960's (Clark & Weems 1989) necessitating costly treatment and population monitoring programs to protect commercial crops from damage, and to certify fruit free of this insect for exportation (Riherd 1993). Additionally, state and federal agencies maintain a vigilant quarantine and trapping program to minimize introductions and provide early detection of other exotic fruit flies [e.g. *Ceratitis capitata* (Wiedemann), Mediterranean fruit fly or medfly, or other *Anastrepha* species]. Once new fruit fly introductions are detected, usually by capture of adult fruit flies in specially baited traps, a well-defined action plan must go into effect. Captured flies are identified and evaluated for reproductive condition, trap density in the vicinity of the original trap site is intensified, and fruits, if present, are checked for the presence of larvae. Detection of an active infestation of

an exotic species and its focal point, i.e., fertile adults ovipositing into susceptible fruits and producing viable larvae, is critical before undertaking an effective action plan. Due to the widespread occurrence of *A. suspensa,* however, Florida faces a special problem in finding such focal points. In practical terms, we face a needle-in-a-haystack problem if the new detection occurs in an area where *A. suspensa* is already actively breeding. Accurate identification of fruit fly larvae is difficult to impossible given current knowledge (Steck et al. 1990). The problem in Florida is simplified to the extent that the identification is an either-or decision, i.e., *A. suspensa* or newly detected exotic. The third instar of *A. suspensa* has been described in useful detail by Heppner (1984) and is included in the key of Steck et al. (1990), but other immature stages of this species have not been adequately described. Immature stages of medfly also have been described (Phillips 1946, Hardy 1949). Usually it is possible to differentiate larvae of the two species using morphological criteria; however, the natural range of morphological variability includes overlap in character state distributions, and some specimens (especially earlier instars) cannot be reliably identified. About 10% of *A. suspensa* and *C. capitata* larvae fall into the indeterminate category [H.A. Denmark, Florida Department of Agriculture and Consumer Services (FDACS), pers. comm.]. Given the economic costs that may accrue from the mis-identification of fruit fly larvae, it is desirable that effective adjuncts to morphological identification of larvae be found and implemented.

In this paper we describe the use of cuticular hydrocarbon (CHC) analysis to differentiate *C. capitata* and *A. suspensa* immature stages. Carlson & Yocom (1986) provided the groundwork for this analysis by describing CHC chromatographic patterns of six economically important fruit fly taxa including *C. capitata* and *A. suspensa*. The third instar larvae of *A. suspensa* and *C. capitata* were found to exhibit quantitatively different hydrocarbon patterns. More recently, Sutton & Carlson (1993) investigated CHC variation in larvae of *A. fraterculus* (Wiedemann), *A. obliqua* (Macquart), *A. suspensa* and other *Anastrepha* taxa and constructed discriminant models. Unfortunately, it is not possible to reconstruct realistic classification error estimates from the sample statistics as published, given the limited number of samples and the potential bias to which it could lead. Thus, it is necessary to sample the range of variation before an effective discriminant model can be constructed that not only distinguishes between *A. suspensa* and *C. capitata* larvae based upon CHC patterns, but also specifies robust classification error rates. Consequently, we have extended the previous analyses to include representative samples of third instar *A. suspensa* larvae from a number of host fruits in Florida and the Caribbean, and third instar *C. capitata* larvae from various hosts in Central America and Hawaii. A small number of second instar larvae was also analyzed.

Materials and Methods

A total of 159 third instar larvae of *A. suspensa* was analyzed. The samples originated from eight feral collections in Dade County, Florida, taken from loquat (*Eriobotrya japonica* (Thumb.) Lindl.), calamondin (*Citrofortunella* x *mitis* Blanco), guava (*Psidium guajava* L.), cattley guava (*Psidium cattleianum* Sabine), Surinam cherry (*Eugenia uniflora* L.) and tropical almond (*Terminalia catappa* L.); one feral collection from Lake Co., Florida, from guava; and four collections from Puerto Rico from guava and tropical almond. Additional third instar larvae of *A. suspensa* were obtained from the USDA-ARS laboratory colony (Gainesville, FL) and the FDACS mass-rearing facility (Gainesville, FL), together with 12 second instar larvae.

C. capitata samples comprised 99 specimens of third instar larvae taken from six feral collections in Hawaii originating from coffee (*Coffea arabica* L.), persimmon (*Diospyros kaki* L. f.), and Jerusalem cherry (*Solanum pseudocapsicum* L.); samples from the USDA-APHIS mass-rearing facility at Waimanalo and the USDA-ARS col-

ony at Waimea; one feral collection from calamondin from Guatemala, and samples from laboratory colonies at USDA-APHIS and the Petapa mass-rearing facility. A total of 12 second instar larvae was obtained from the Waimanola facility and two feral second instars were collected from coffee in Hawaii.

All specimens are vouchered with the Florida State Collection of Arthropods, FDACS, Gainesville, Florida.

Individual whole larvae (fresh, freshly frozen, or dried) were extracted and prepared as previously described (Carlson & Yocom 1986, Sutton & Carlson 1993). GC analyses were made with a Tracor Model 540 Gas Chromatograph (Austin, TX) fitted with a nonpolar fused silica capillary column (BP-1, 50 m x 0.15 mm id, 0.25 µm film thickness, Scientific Glass Engineering, Austin, TX), cool on-column injector (OCI-3, Scientific Glass Engineering, Austin, TX) and Tracor flame-ionization detector. Purified hydrogen was used as the carrier gas at a pressure of 80 psi providing a linear flow-rate of 35-45 cm/s; the oven was temperature programmed from 60-225°C at 20° per minute, and 225-320°C at 1.5° per minute.

Areas were calculated for each of the selected peaks for each sample using the Nelson Model 2100 Chromatography Data System, Revision 5.0 (Nelson Analytical, Inc., Cupertina, CA). The SAS System, Version 6.01, (SAS Institute, Inc., Cary, NC) was utilized for the discriminant analysis and the density plot was constructed using Sygraph, Version 1.1 (SYSTAT, Inc., Evanston, IL).

Results and Discussion

The CHC composition of larvae of *A. suspensa* and *C. capitata* is dominated by homologous series of n-alkanes and 2-monomethylalkanes with lesser quantities of other mono- and dimethylalkanes (Carlson & Yocom 1986, Sutton & Carlson 1993). The unsaturated hydrocarbons of *A. suspensa* larvae include squalene together with small quantities of as yet unidentified components (Sutton & Carlson 1993). Chromatograms of third instar *A. suspensa* and *C. capitata* extracts (Figure 1) exhibit consistent quantitative differences in the relative proportions of 2-methyloctacosane at Kovats Index (KI) (Kovats 1965) 2865 and 2-methyltriacontane at KI 3065 (Table 1). A density plot of the ln transformed ratios of the peak areas at KI 2865 and KI 3065 [R_{65} = ln (KI 2865 peak area/KI 3065 peak area)] with an Epanechakov kernel density estimator (Silverman 1986) (Fig. 2) resulted in unimodal distributions for *A. suspensa* and *C. capitata* having well separated modes and a small amount of overlap in the distribution tails. No consistent quantitative differences in CHC pattern were evident between feral Puerto Rico and Florida *A. suspensa*, nor between Hawaii and Guatamala *C. capitata*. Likewise, the laboratory colonies of *C. capitata* exhibited R_{65} values within the range of variation seen in wild collections. The R_{65} values for feral Florida *A. suspensa* are consistent with those reported previously (Carlson & Yocom 1986); however, the *A. suspensa* colony maintained for many years by USDA-ARS, Gainesville, as well as the extant colony at FDACS, Gainesville, exhibited R_{65}

TABLE 1. Univariate Statistics for the ln of the ratio of the peak areas at KI 2865 and KI 3065 (R_{65}), for third instar larvae of *A. suspensa* and *C. capitata*.

	A. suspensa	*C. capitata*
N	159	99
Mean	3.45	-0.05
S. D.	2.80	0.15
Minimum	1.43	-3.50
Maximum	4.54	2.14

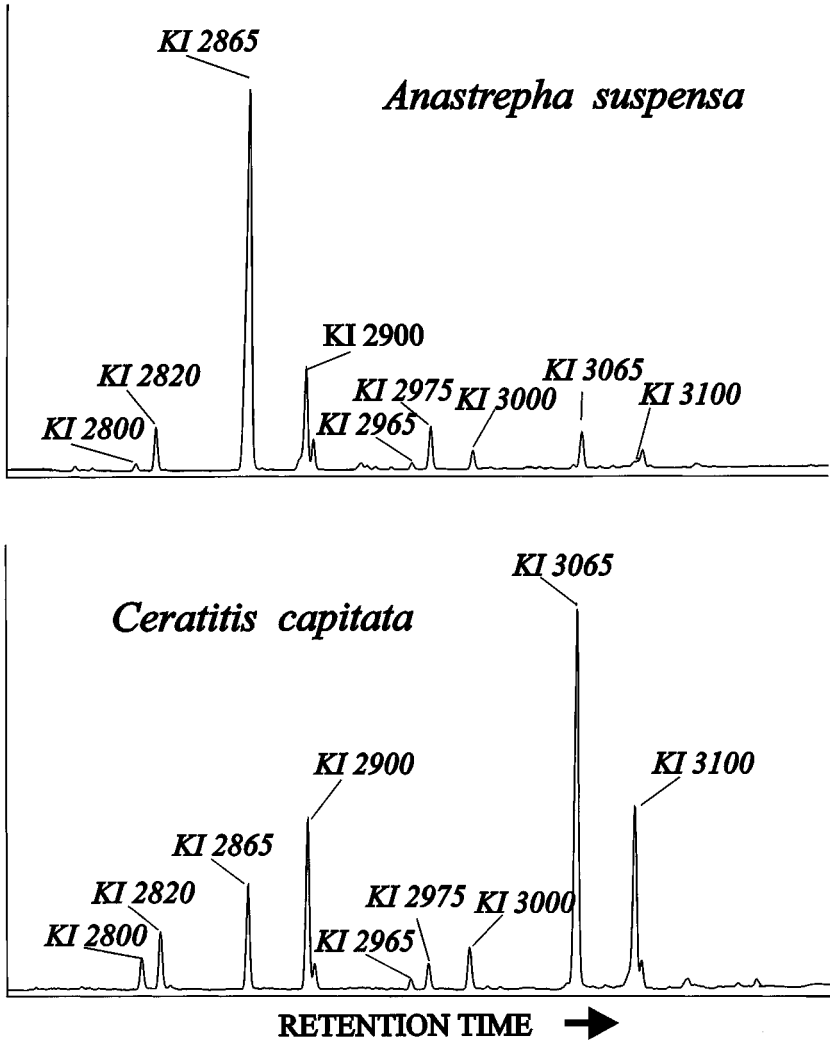

Fig. 1. Capillary gas chromatograms of hydrocarbons extracted from individual third instar larvae of tephritid fruit flies: a. *A. suspensa*, b. *C. capitata*.

values substantially lower than those seen in wild collections. This was confirmed by reexamination of CHC patterns from a later sampling (Sutton & Carlson 1993) of the USDA colony. The reduced R_{65} values in the USDA colony are the consequence of an as yet unidentified eluant of KI 3060 that co-elutes with the KI 3065 peak under the chromatographic conditions used in the previous studies. This peak is also present in wild *A. suspensa* from Florida and Puerto Rico but in much reduced quantities compared to those seen in individuals from the USDA colony. The origin of the FDACS colony is unclear, but it probably originated from the USDA colony. To avoid this bias, only wild *A. suspensa* or laboratory colonies recently established from wild individuals were used for comparison with *C. capitata*. There was no evidence that CHC patterns are correlated with host fruits for either species.

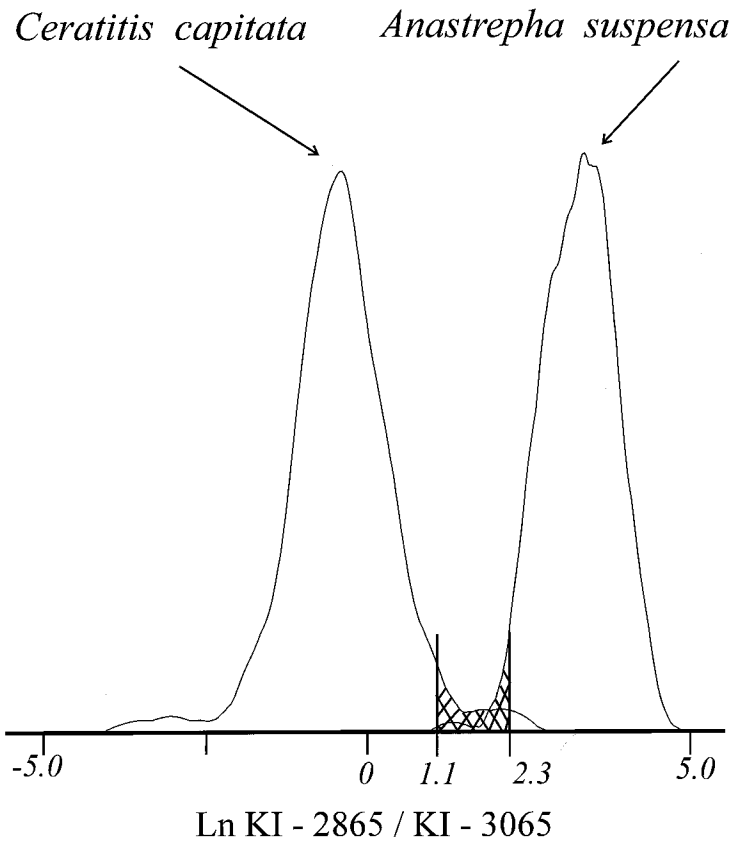

Fig. 2. Density plot using a kernel density estimator with Epanechakov kernel for the ln transformed ratio of the areas of the 28-and 30-carbon backbone 2-monomethylalkane peaks (R_{65}) for *A. suspensa* and *C. capitata*. Cross-hatching indicates the region of possible overlap.

The specification of a decision criterion and reliability estimates for classification errors based upon R_{65} values for third instar larvae of *A. suspensa* and *C. capitata* depends upon the accurate reconstruction of the distribution tails for each species and the degree of overlap between them. Our sample distributions exhibited a small degree of overlap in the region of $1.10 \leq R_{65} \leq 2.30$. Given the economic costs of misidentification, it is safer to assume that even this sampling may be insufficient to fully describe population distributions in the range of possible overlap. A linear discriminant model resulted in jack-knifed classification errors of 0.63% and 2.02% for *A. suspensa* and *C. capitata*, respectively. However, given that false negative errors are potentially more costly than false positives, a more conservative bias is indicated. The values for *A. suspensa* drop off sharply for R_{65} less than 2.30, with only 3% (5 of 159 individuals) below 2.30; a single wild individual had an R_{65} value less than 2.08. Three percent (3/99 individuals) of the third instar *C. capitata* larvae exhibited R_{65} values within the interval (1.10,2.30), with two individuals having R_{65} values greater than 1.39. Any specific value is somewhat arbitrary given the uncertainties involved, but a classification threshold of about $R_{65} = 2.30$ would seem appropriate.

Rare individuals of *A. suspensa* (3% or so) would be expected to exhibit R_{65} values below this threshold and would classify as *C. capitata*. Thus, we must accept the possibility of false positives for larvae having R_{65} values in the range of 1.10 to 2.30.

It should be noted that all third instars analyzed could be correctly identified visually using secondary aspects of the hydrocarbon patterns (Fig. 1). Low R_{65} values in *A. suspensa* were due more to variation in the KI 2865 peak than to variation in the KI 3065 peak, i.e., the latter peak remains small with respect to the overall pattern. Similarly, higher R_{65} values in *C. capitata* are a consequence of a relative increase in the KI 3065 peak rather than a significant reduction in the KI 2865 peak so that the KI 3065 peak remains large with respect to the overall pattern. Unfortunately, such aspects of chromatographic patterns are difficult to quantify, and interpretations based upon small peaks can be rendered difficult, if not impossible, by suboptimal chromatographic conditions.

Second instar larvae of *A. suspensa* and *C. capitata* from laboratory colonies exhibited significant quantitative shifts of the CHC pattern compared to third instars. In both species, second instars show a relative increase in the KI 2865 peak and a reduction in the KI 3065 in comparison to the third instar larvae. *A. suspensa* from the FDACS colony exhibited mean R_{65} values of 4.16 ± 2.09 (n=12) and 2.09 ± 0.88 (n=12) for second and third instars, respectively. No feral second instars of *A. suspensa* were available for analysis. The R_{65} values for second and third instar larvae of *C. capitata* from the lab colony at Waimea were 1.26 ± 0.60 (n=12) and -0.58 ± 0.03 (n=12) respectively. Two feral *C. capitata* second instars showed corresponding R_{65} values of 2.36 and 2.81. It is unclear whether there is a more or less continuous shift in the 2-monomethylalkane series with age, or if the CHC pattern remains relatively invariant over a larval stage. The sampling of second instars was insufficient to construct a realistic classification model; however, it is clear that use of the third instar model would result in a substantial increase in mis-classified *C. capitata* second instars.

Larvae utilized for analysis were either fresh, fresh-frozen or dried. Alcohol preserved specimens generally did not give clean chromatograms and are not recommended for use in CHC analysis. The CHC patterns were not appreciably altered by larval decomposition or physical damage; hence it is possible to utilize specimens that are not identifiable by other means. Very good extractions were also obtained from larval skins alone, e.g. following removal of internal contents for protein electrophoresis or other analysis. Thus, CHC analysis is compatible with simultaneous analysis of a single specimen by both morphological and biochemical or molecular means. Given the possible economic ramifications of misidentification and the ease with which the analysis of a critical individual may be botched, it is imperative that alternative identification systems be available for backup and/or verification. This is particularly important in the case of larvae having R_{65} values in the region of possible overlap between the two taxa.

We found use of 28-200 mesh activated silica gel to be a particularly effective method for drying larvae in the field. While this procedure precludes the use of the larvae for analyses such as protein electrophoresis, solvent extraction of specimens dried in silica gel was significantly enhanced in comparison to fresh or frozen material. Possibly there are highly polar or hydrophilic components in the larval cuticle that shield the hydrocarbons from the non-polar solvent used for extraction, but are removed by adsorption onto the silica gel.

Implementation of CHC analysis for the discrimination of larvae of *A. suspensa* and *C. capitata* will require further development. No attempt was made in this study to optimize either the larval extraction procedure or the gas chromatography parameters to minimize analysis time and cost. Further sampling will be required to construct a reliable discriminant model for second instar larvae. In addition, the potential exists to adapt and/or extend CHC analysis to the identification of other economically important species, especially *Bactrocera*, *Dacus* and other *Anastrepha* species.

ACKNOWLEDGMENT

We gratefully acknowledge the assistance of several colleagues in providing specimens. These include S. Bloem, H. Chang, M. J. Hayes, M. Hennessey P. J. Landolt, L. Stange, and R. Vargas. We also thank D.A. Carlson and S.R. Yocom for reviewing this manuscript.

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