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Resolution of three cryptic agricultural pests (*Ceratitis fasciventris, C. anonae, C. rosa*, Diptera: Tephritidae) using cuticular hydrocarbon profiling

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Abstract

Discrimination of particular species within the species complexes of tephritid fruit flies is a very challenging task. In this fruit-fly family, several complexes of cryptic species have been reported, including the African cryptic species complex (FAR complex). Cuticular hydrocarbons (CHCs) appear to be an excellent tool for chemotaxonomical discrimination of these cryptic species. In the present study, CHC profiles have been used to discriminate among three important agricultural pests from the FAR complex, *Ceratitis fasciventris*, *Ceratitis anonae* and *Ceratitis rosa*. Hexane body surface extracts of mature males and females were analyzed by two-dimensional gas chromatography with mass spectrometric detection and differences in CHC profiles between species and sexes tested through multivariate statistics and compared with species identification by means of microsatellite markers. Quantitative as well as qualitative CHC profile differences between sexes and species are reported. The CHC profiles consisted of a mixture of linear, internally methyl-branched and mono-, di- and tri-unsaturated alkanes. Twelve compounds were pinpointed as potential chemotaxonomical markers. The present study shows that presence or absence of particular CHCs might be used in the chemical diagnosis of the FAR complex. Moreover, our results represent an important first step in the development of a useful chemotaxonomic tool for cryptic species identification of these important agricultural pests.

Keywords: cryptic species complex, genus *Ceratitis*, cuticular hydrocarbons, polymorphic microsatellite loci, chemotaxonomy

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Introduction

The genus *Ceratitis* (Diptera: Tephritidae) consists of approximately 100 species, some of them being important agricultural pests. In addition, due to the polyphagous diet of many fruit flies, some species may potentially become cosmopolitan pests in the near future (Yuval & Hendrichs, 2001). The Afro-tropical fruit flies *Ceratitis fasciventris*, *Ceratitis anonae* and *Ceratitis rosa* constitute the so-called FAR species complex (Virgilio *et al.*, 2007*a*, *b*). Historically, *C. fasciventris* was considered as a variety of *C. rosa* (Bezzi, 1920), yet recently it has been recognized as a separate species (De Meyer, 2001). Unlike *Ceratitis capitata*, which has spread from its home range in East Africa and attained an almost worldwide distribution over the past century, *C. fasciventris*, *C. anonae* and *C. rosa* have so far not been reported outside the African continent (except for *C. rosa* on La Réunion and Mauritius islands). Nevertheless, they are potentially invasive (Fletcher, 1989; White & Elson-Harris, 1992; De Meyer *et al.*, 2008). As it is difficult to distinguish some members of the FAR complex morphologically (De Meyer & Freidberg, 2006), especially females, a number of molecular approaches for species recognition was developed in the past (Baliraine *et al.*, 2004; Barr & McPheron, 2006; Virgilio *et al.*, 2008; Steinke *et al.*, 2012; Virgilio *et al.*, 2012). Virgilio *et al.* (2013) have specified five genotypic groups within the FAR complex using comparison of allelic variations at 16 microsatellite loci. These genotypic groups were labeled as R1 and R2 (representing *C. rosa*), F1 and F2 (representing *C. fasciventris*) and A (representing *C. anonae*). However, the use of microsatellite variability for cryptic species discrimination is rather laborious and expensive. The uncertain taxonomic status of particular populations has important practical implications on the effective development and use of the sterile insect techniques (SIT), with respect to rearing the sterile males of the correct species, and in consequence affects the international movement of fruits and vegetables due to trade barriers to important agricultural commodities which are hosts to pest tephritids (Dyck *et al.*, 2005). Moreover, the identification of cryptic species is crucial for an accurate assessment of biodiversity estimates, for facilitating disease and crop–plant–pathogen control and for directing conservation efforts toward vulnerable endemic species (Paterson, 1991; Besansky, 1999; Copren *et al.*, 2005; Garros *et al.*, 2006; Bickford *et al.*, 2007). All these reasons prompt us to search for alternative tools for fruit-fly cryptic species identification.

In this study, we consider cuticular hydrocarbons (CHCs) as a potential tool for cryptic species discrimination. CHCs are the main constituents of insect epicuticle and play an important role in waterproofing of the cuticle (Gibbs, 2011) and inter-individual recognition of insects (Howard & Blomquist, 2005; Blomquist & Bagnères, 2010; Curtis *et al.*, 2013; Jennings *et al.*, 2014). They are most widely analyzed in numerous insect lineages since they are often distinct and stable over large geographical areas (Martin *et al.*, 2008; Martin & Drijfhout, 2009). Biochemical investigations have shown that insects synthesize *de novo* most of their hydrocarbons themselves (Wakayama *et al.*, 1985; Gozansky *et al.*, 1997; Martin *et al.*, 2008). The CHC composition is genetically determined and has a taxonomic potential (Lockey, 1991; Blomquist & Bagnères, 2010; Guillem *et al.*, 2012; Kather & Martin, 2012). Recently, CHC profiles have been used to resolve insect species complexes of the fruit-fly *Drosophila buzzatii* (Oliveira *et al.*, 2011), the ants *Pachycondyla villosa* (Lucas *et al.*, 2002) and *Tetramorium caespitum*/*impurum* (Schlick-Steiner *et al.*, 2006), termites (Haverty *et al.*, 2000), orchid bees (Pokorny *et al.*, 2014) or mirids *Macrolophus* (Gemeno *et al.*, 2012). Studies of CHCs of tephritid fruit flies (*C. rosa*, *C. capitata*, *Anastrepha ludens*, *Anastrepha suspensa*, *Anastrepha fraterculus*, *Dacus cucurbitae* and *Dacus dorsalis*) have shown the presence of *n*-alkanes, methyl-branched alkanes, alkenes and alkadienes (Carlson & Yocom, 1986; Lavine *et al.*, 1992; Sutton & Steck, 1994; Vaníčková, 2012; Vaníčková *et al.*, 2012). However, according to our present knowledge, there are no reports on the characterization of the CHC profiles of *C. anonae* and *C. fasciventris* concerning the sex- and species-specific differences.

Our objectives are: (a) to characterize the CHC profiles of *C. fasciventris*, *C. anonae* and *C. rosa*, (b) to quantify the differences between species and sexes and (c) to explore the possibility of using CHC profiling as a diagnostic tool.

Materials and methods

Insects

The experiments were performed with male and female laboratory-reared specimens of *C. fasciventris*, *C. anonae* and *C. rosa*. In order to evaluate the CHC differences within the FAR complex and between the FAR complex and a congeneric outgroup, laboratory-reared males and females of *C. capitata* were also included. The pupae of *C. fasciventris*, *C. anonae* and *C. rosa* were obtained from the International Centre of Insect Physiology and Ecology (ICIPE, Nairobi, Kenya), whereas pupae of *C. capitata* came from the entomological laboratory of the Food and Agriculture Organization/International Atomic Energy Agency (FAO/IAEA, Seibersdorf, Austria, originally from Argentina). The pupae were kept under identical laboratory conditions at the Institute of Organic Chemistry and Biochemistry (IOCB, Prague, Czech Republic). Eclosed adult flies were fed on artificial diet consisting of sugarcane: yeast (3:1) and mineral water and were kept at a relative humidity of 60%, at 25°C and a 12L:12D photoperiod.

Chemical analyses

Prior to chemical analyses, 20-day-old adult flies were frozen at −18°C and placed for 15 min into a desiccator to remove the surface moisture. In order to extract CHCs from insect body surface individual fly was placed in small glass vials, which contained 0.5 ml of hexane (Fluka, Germany) and gently agitate for 5 min. Bromdecane was used as an internal standard for quantification (50 ng per 1 ml of the extract). Each extract was concentrated to approximately 100 µl by a constant flow of nitrogen and stored in a freezer until analysis.

Two-dimensional (2D) gas chromatography with time-of-flight mass spectrometric detection (GC × GC/TOFMS) was used for the quantification and identification of CHC profiles. The analyses were performed on a LECO Pegasus 4D instrument (LECO Corp., St Joseph, MI, USA) equipped with a non-moving quad-jet cryomodulator. A DB-5 column (J&W Scientific, Folsom, CA, USA; 30 m × 250 µm i.d. × 0.25 µm film) was used for GC in the first dimension. The second-dimension analysis was performed on a polar BPX-50 column (SGE Inc., Austin, TX, USA; 2 m × 100 µm i.d. × 0.1 µm film). Helium was used as a carrier gas at a constant flow of 1 ml min^{-1}. The temperature program for the primary GC oven was as follows: 150°C for 2 min, then 150–300°C at 5°C min^{-1}

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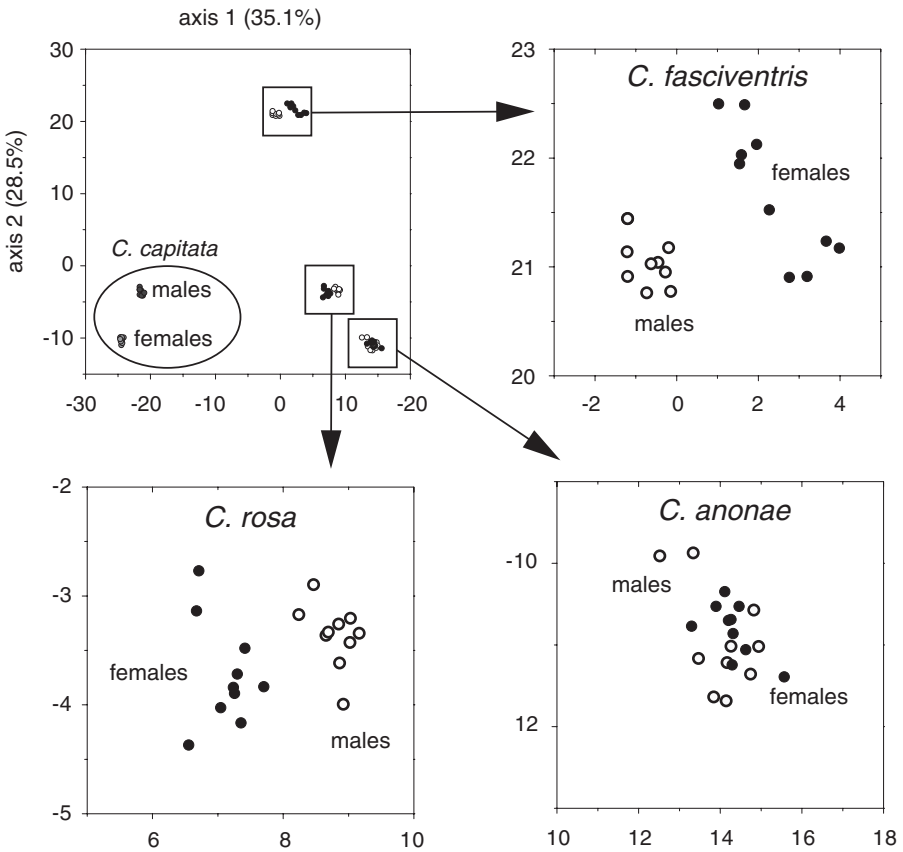

Fig. 1. PCAs of Euclidean distances between males and females *C. capitata*, *C. fasciventris*, *C. anonae* and *C. rosa* (as calculated from peak areas of 59 CHCs).

and finally a 10 min hold at 320°C. The program in the secondary oven was 10°C higher than in the primary one and was operated in an iso-ramping mode. The modulation period, the hot-pulse duration and the cool time between the stages were set to 3.0, 0.4 and 1.1 s, respectively. The transfer line to the TOFMS was operated at 260°C. The source temperature was 250°C with a filament bias voltage of −70 eV. The data-acquisition rate was 100 Hz (scans sec^{-1}) for the mass range of 29–400 amu. The detector voltage was 1750 V. For each sample, 1 µl was injected in the splitless mode. The inlet temperature was 200°C. The purge time was 60 s at a flow of 60 ml min^{-1}. The data were processed and consecutively visualized on 2D and three-dimensional chromatograms using LECO ChromaTOF™ software. The *n*-alkane standard (C_8–C_{38}; Sigma-Aldrich) was co-injected with authentic samples to determine the retention indices (*RI*s) of the analytes. The hydrocarbons were identified by a comparison of their MS fragmentation patterns and *RI* (Van Den Dool & Kratz, 1963; Pomonis *et al.*, 1978; Carlson & Yocom, 1986; Carlson *et al.*, 1989; Lavine *et al.*, 1992; Goh *et al.*, 1993; Sutton & Carlson, 1993; Sutton & Steck, 1994; Carlson *et al.*, 1998; Geiselhardt *et al.*, 2009; Vaníčková, 2012; Vaníčková *et al.*, 2012).

Table 1. PERMANOVA and *a posteriori* comparison (*t*-statistic) testing differences in multivariate patterns of 59 CHCs in relation to species (*C. fasciventris*, *C. anonae*, *C. rosa*, *C. capitata*) and sex (male × female) of 80 tephritid fruit flies.

Source of variability	*P*	df	MS	*F*
Species	***	3	12,650.38	594.54
Sex	n.s.	1	1595.88	1.10
Species × sex	***	3	1445.72	67.94
Residual		72	21.27	
C. capitata × *C. fasciventris*	***			
C. capitata × *C. anonae*	***			
C. capitata × *C. rosa*	***			
C. fasciventris × *C. anonae*	***			
C. fasciventris × *C. rosa*	***			
C. rosa × *C. anonae*	***			
C. capitata male × female	***			
C. fasciventris male × female	***			
C. anonae male × female	*			
C. rosa male × female	***			

P-values were obtained using 10^5 unrestricted permutations of raw data. Probability of Monte Carlo simulations: n.s.: not significant $P > 0.05$; ***: $P < 0.001$, **: $P < 0.01$; *: $P < 0.05$. df: degrees of freedom; MS: mean-square estimates; *F*: pseudo-*F*.

Statistics

The relative peak areas of 59 CHC compounds (as identified by the GC × GC/TOFMS in the deconvoluted total-ion chromatogram mode) were calculated in ten replicate specimens for each sex of the four species ($N = 80$). Principal component analysis (PCA) was used for the unconstrained

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Table 2. Compounds identified by chemical analyses (GC × GC/TOFMS) and statistical analyses (SIMPER and PERMANOVA) of body surface extracts of females and males of *C. fasciventris*, *C. anonae*, *C. rosa* and *C. capitata*.

No.	Compound	*RI*	Diagnostic ions (*m/z*)	*C. fasciventris*		*C. anonae*		*C. rosa*		*C. capitata*	
				Male	Female	Male	Female	Male	Female	Male	Female
1	4-MeC_{28}	2859	364/365, 408	*	*	*	*	–	*	*	*
2	7-MeC_{29}	2940	112/113, 336/337, 422	*	*	–	–	–	–	*	*
3	4-MeC_{29}	2958	378/379, 422	–	–	*	*	*	*	*	*
4	3-MeC_{29}	2978	392/393, 422	–	–	*	*	*	*	–	–
5	3-MeC_{30}	3077	406/407, 436	–	*	*	*	–	–	–	–
6	11,13-DiMeC_{31}	3269	168/169, 210/211, 280/281, 322/323, 464	*	*	–	–	*	*	–	–
7	13,17-DiMeC_{31}	3269	196/197, 224/225, 266/267, 294/295, 464	*	*	–	–	*	*	–	–
8	12,14-DiMeC_{31}	3269	182/183, 224/225, 266/267, 308/309, 464	*	*	–	–	*	*	–	–
9	Branched C_{35}	3488	492	*	*	–	–	*	*	*	–
10	$C_{36:1}$	3529	504	–	–	*	*	*	*	*	*
11	Branched $C_{37:1}$	3630	518	*	*	*	*	*	*	*	–
12	$C_{37:3}$	3635	514	–	–	–	–	*	*	*	*

$N = 10$ for each combination of gender and species. *RI*: retention index; *: present, –: absent, $C_{36:1}$ hexatriacontene, $C_{37:1}$ heptatriacontene, $C_{37:3}$ heptatriacontatriene.

ordination of multivariate data (Anderson, 2003) and the percentage contribution of each CHC to the average dissimilarity between (a) species and (b) sexes within each species was calculated with similarity percentage analysis (SIMPER) (Clarke, 1993). Permutation multivariate analysis of variance (PERMANOVA: Anderson, 2001) was used to verify the differences in the CHC distribution patterns across species and sexes. PERMANOVA considered species (four levels: *C. fasciventris*, *C. anonae*, *C. rosa*, *C. capitata*) as a random factor and sex (two levels: male, female) as a fixed factor orthogonal to species. The tests were based on 10^5 unrestricted permutations of raw data. Pair-wise *a posteriori* comparisons of factor levels were then implemented using the PERMANOVA *t-statistic* (Anderson, 2001). Following Clarke (1993), we log-transformed the multivariate data in order to reduce the differences in scale among the variables while preserving information on the relative abundance of CHCs across specimens.

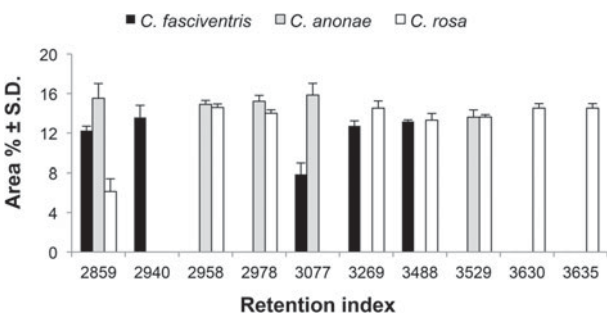

Fig. 2. Average log-transformed CHCs peak areas (standard deviations as error bars), represented by *RI*s that contributed the most to the differences (>2% contribution to species dissimilarity) between *C. fasciventris*, *C. anonae* and *C. rosa* (see Supplementary Material, table 2).

Molecular genetic analyses

All chemically characterized FAR specimens were then genotyped at 16 polymorphic microsatellite loci using the primers and laboratory protocols described in Anderson *et al.* (2010). The genetic data were combined with those of Virgilio *et al.* (2010) and a PCA was performed to assign the FAR specimens of this study to one of the five genotypic clusters previously described (Virgilio *et al.*, 2013) (*C. fasciventris* F1, F2, *C. anonae* A, *C. rosa* R1, R2). The PCA was based on Euclidean distances among a total of 669 multilocus genotypes and computed using the R-package adegenet 1.3–4 (Jombart, 2008).

Results

The CHC profiles of the four African fruit-fly species of the genus *Ceratitis* are complex mixtures of straight-chained and methyl-branched alkanes, alkenes, alkadienes and alkatrienes with a wide range of carbon backbones (C_{23}–C_{38}). Altogether, the GC × GC/TOFMS analysis identified 59 CHC peaks, whose log-transformed areas were used for the statistical analyses. The subsequent PCA of the quantified data is depicted in fig. 1. The first two axes of the unconstrained PCA explained 63.6% of the variability (35.1 and 28.5%, respectively). The PCA clearly separated the three species of the FAR complex and the out-group *C. capitata*, as well as males from females of *C. capitata*, *C. fasciventris* and *C. rosa*. The PERMANOVA analyses and *a posteriori* pairwise permutational tests (table 1) showed highly significant differences both across species and across species and sexes and indicated that (a) all species have significantly different CHC profiles and (b) within each species, males and females have significantly different CHC profiles.

For each pairwise comparison, SIMPER (tables S1 and S2, Supplementary Materials) allowed the identification of the first ten CHCs that contributed the most to the differentiation between the three species of the FAR complex. Bray–Curtis dissimilarity between the FAR species ranged from 6.54 (between *C. anonae* and *C. rosa*) to 9.24 (between *C. anonae* and *C. fasciventris*) and it was comparably higher between the out-group *C. capitata* and the three FAR species (range

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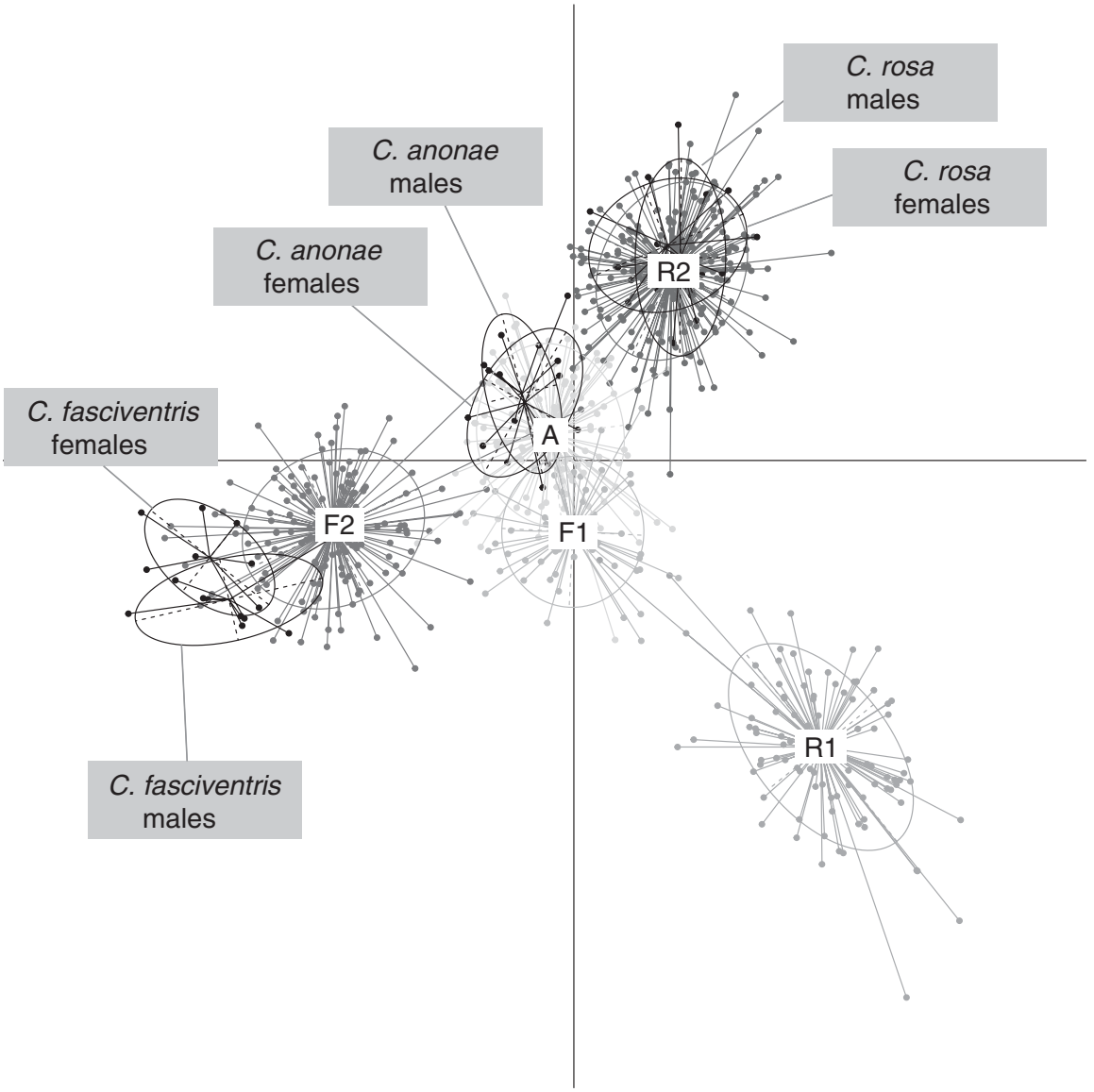

Fig. 3. PCAs of Euclidean distances among the individual genotypes of specimens sampled in this study (grouped according to the species and the sex, represented in black) and 621 genotypes of *C. fasciventris*, *C. anonae* and *C. rosa* assigned to five genotypic clusters (*C. fasciventris* F1 and F2, *C. anonae* A, *C. rosa* R1 and R2) like in Virgilio *et al.* (2013). The genotype groups are labeled inside their 95% inertia ellipses and connected to the corresponding group centroids.

10.0–11.06, table S1 in Supplementary Material). The CHCs that contributed the most to the differences between the FAR species were the ones occurring only in one or two species (fig. 2, table 2, tables S1 and S2, Supplementary Materials). The Bray–Curtis dissimilarity between sexes within each of the FAR species was comparably lower and ranged from 2.58 to 3.99 (in *C. rosa* and *C. fasciventris*, respectively, table S2 in Supplementary Material). The 12 CHC compounds that contributed (>2% contribution to species dissimilarity) the most to interspecific differentiation (fig. 2, table 2, table S1 in Supplementary Material) comprised nine methyl-branched alkanes, two alkenes and one alkatriene. The *RI*s of all 12 CHCs were calculated. Among them, *RI* 3077 (3-MeC_{30}) was detected in the females but not in the males of *C. fasciventris* and *RI* 2859 (4-MeC_{28}) was detected in the females but not in the males of *C. rosa*. The differences between the peak areas of *C. anonae* were comparably lower (table S2 in Supplementary Material). In the case of dimethyl alkanes (*RI* 3269) the given *RI*s represented co-eluting mixture of three compounds. In *C. capitata*, the compounds specific for the males were *RI* 3488 (branched pentatriacontane) and *RI* 3630 (branched heptatriacontene $C_{37:1}$) (table 2).

The PCA of microsatellite genotypes showed that the *C. fasciventris* and *C. rosa* specimens sampled in this study (males and females) belong to the genotypic clusters *C. fasciventris* F2 and *C. rosa* R2 (Virgilio *et al.*, 2013) (fig. 3). Surprisingly, the overall genetic diversity of the specimens from colonies was relatively high, as shown by the patterns of

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multivariate dispersion in fig. 3. This suggests that the material used for the analyses was not particularly subjected to inbreeding depression, probably because the gene pool of the laboratory colonies was periodically renovated through the addition of wild individuals.

Discussion

The CHCs identified in *C. fasciventris*, *C. anonae*, *C. rosa* and *C. capitata* are branched and unsaturated alkanes. These compounds have been proven to be suitable chemotaxonomical markers in Diptera (Sutton & Carlson, 1993; Horne & Priestman, 2002; Caputo *et al.*, 2005; Ye *et al.*, 2007; Blomquist & Bagnères, 2010; Everaerts *et al.*, 2010), Hymenoptera (Dahbi *et al.*, 2008; Martin & Drijfhout, 2009; Guillem *et al.*, 2012; Pokorny *et al.*, 2014), Hemiptera (Gemeno *et al.*, 2012), Isoptera (Haverty *et al.*, 1990), Orthoptera (Chapman *et al.*, 1995) as well as in other insect orders (Everaerts *et al.*, 2008). Carlson & Yocom (1986) showed qualitative and quantitative species- and sex-specific CHC differences among six fruit-fly species (*A. ludens*, *A. suspensa*, *C. capitata*, *C. rosa*, *D. cucurbitae*, *D. dorsalis*) and identified straight chain hydrocarbons (*RI* 2900 for C_{29}, 3100 for C_{31}), methyl branched hydrocarbons (*RI* 2865 for 2-MeC_{28}, *RI* 3065 for 2-MeC_{30} and *RI*s 3125–3140 for 11-/13-/15-MeC_{31}) and alkadienes (*RI* 3250 for tritriacontadiene $C_{33:2}$, *RI* 3450 for pentatriacontadiene $C_{35:2}$ and *RI* 3650 for heptatriacontadiene $C_{37:2}$). Monomethyl alkanes were previously used to distinguish between the morphologically similar larvae of *C. capitata* and *A. suspensa* (Sutton & Steck, 1994).

Our results show that *C. anonae*, *C. fasciventris* – genotypic cluster F2 and *C. rosa* – genotypic cluster R2 (see Virgilio *et al.*, 2013) have markedly different CHC profiles and that the main compounds responsible for these differences are methyl-branched alkanes (4-MeC_{28}, 7-MeC_{29}, 4-MeC_{29}, 3-MeC_{29}, 3-MeC_{30}, 11,13-/13,17-/12,14-DiMeC_{31} and branched C_{35}), alkenes (hexatriacontene, branched heptatriacontene) and alkatriene (heptatriacontatriene). The configurations of the double-bond positions still remain to be resolved; further chemical analyses are scheduled for this purpose.

To a lesser extent, the CHC profiles also differ between sexes within each species (with more pronounced differences in *C. fasciventris* and *C. rosa* as compared to *C. anonae*). Thus, the variation of the CHC profiles seems to be hierarchically organized, with interspecific differentiation being higher than the gender-related differences. The CHC compounds that contributed the most to the separation of sexes within species were monomethyl alkanes and unsaturated alkanes. These types of CHCs were also identified in other fruit-fly species (Carlson & Yocom, 1986; Goh *et al.*, 1993; Rouault *et al.*, 2004; Vaníčková *et al.*, 2012) and were responsible for the quantitative and qualitative CHC differences observed between sexes in a number of dipteran species (Caputo *et al.*, 2005; Everaerts *et al.*, 2010; Oliveira *et al.*, 2011; Suarez *et al.*, 2011). Fruit-fly courtship behavior is a relatively complex process and includes chemical, auditory and visual stimuli (Wicker-Thomas, 2007). The importance of CHCs in insect mate choice is well established (e.g., Lahav *et al.*, 1999; Thomas & Simmons, 2008; Blomquist & Bagnères, 2010). Accordingly, cuticular signals have been shown to play an important role in the mate choice of fruit flies (Thomas & Simmons, 2008; Blomquist & Bagnères, 2010; Kather & Martin, 2012). As suggested for *Drosophila* spp., sexual selection might promote inter- and intra-specific differences in CHC patterns (Shirangi *et al.*, 2009; Takahashi *et al.*, 2012; Curtis *et al.*, 2013; Havens & Etges, 2013; Jennings *et al.*, 2014).

These results suggest that CHC profiles might be a suitable tool for the chemotaxonomical identification of the tephritid species that are difficult to recognize based on morphological or molecular characteristics. The methodology of the extraction and identification of the CHC markers described here might be useful when a large number of samples are to be determined. For an easy CHC extraction, pentane, hexane or dichloromethane can be used, and the subsequent chemical analyses may be performed using a variety of GC/MS setups equipped with a non-polar column (DB-5). The time needed for the analysis is much shorter when compared to molecular genetic approaches, and the overall cost of the analysis is significantly lower.

The present study shows that the presence or absence of particular CHCs might be used in the chemical diagnosis of the FAR complex. As such, our results represent an important first step in the development of a useful chemotaxonomic tool for cryptic species identification. Validation of the method would require further analyses that include samples from a larger geographic area. In the near future, additional analyses of the CHC profiles, including the other two morphotypes, *C. fasciventris* (F1) and *C. rosa* (R1), will be performed.

The supplementary materials for this paper can be found at http://www.journals.cambridge.org/BER

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