

Host specialisation in Tephritidae fruit flies in La Réunion : preference – performance – host phylogeny relationships

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Abstract:

Among invasive species, phytophagous insects have repeatedly become pests for agriculture. Such insects can differ widely in their degree of specialisation to their host plants. While host specialisation occurs as a natural process structuring food webs, it also represents a major factor determining the importance of insect pest potential damages. Predicting on which crops a given insect species may become a pest therefore requires (i) a good knowledge of both the realised (*i.e.*, resource used by the species in the field) and fundamental (*i.e.*, potential resource that the species is capable of using) host ranges of the insect species and (ii) a deep understanding of the processes by which insects are able to adapt to new hosts.

The Tephritidae community in La Réunion presents numerous advantages for an ideal study of the preference – performance – host phylogeny relationships. La Réunion hosts eight species of tephritids infesting crops that highly overlap in their host breadth with the other species of the community. This community includes two indigenous species and six exotic species that have different degree of specialisation. Some are generalist (*Ceratitis rosa*) and some are specialist (*Neoceratitis cyanescens*).

The aim of this PhD is to study the relation between host phylogeny, host preference of the females and larval performance of the tephritid community of La Réunion. This objective is developed through four main parts:

- (i) Phylogeny reconstruction of all host plant species of the eight tephritid species present in La Réunion.
- (ii) Study of historical records and definition of the host range in the field of each Tephritidae species and the evolution of this host range before and after the latest invasion of *Bactrocera zonata* in 2000.
- (iii) Estimation of host preference by studying behavioural and physiological responses to plant odours and volatile compounds in the laboratory.
- (iv) Finally, the results of the three tasks and performance data will be combined to define the relationships between performance, preference and phylogeny of host plants.

Preference of *Bactrocera cucurbitae* Coquillett for three commercial fruit vegetable hosts in natural and semi natural conditions

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Abstract:

Host preference of *Bactrocera cucurbitae* in three vegetables was determined under choice and no-choice experiments, in both natural and semi-natural conditions. The trials were run for three seasons between March 2013 and July 2014 in Morogoro Region, Tanzania. In each season, two sets of plots of watermelon *Citrullus lanatus*, cucumber *Cucumis sativus* and pumpkin *Cucurbita* sp., were established in mono and mixed cropping systems. One set of plots was placed under individual cages and another set of plots was left open. Colonies of *B. cucurbitae* were established from wild cucurbits and the emerging adults were maintained on artificial diets. Trials were also conducted to determine the dominant cucurbit infester in cucumber. Infestation rates and incidences were determined and used to indicate preference of *B. cucurbitae* among the three vegetables. Highest infestation rate of *B. cucurbitae* was recorded in watermelon while lowest in pumpkin. Infestation rates were significantly high in fruits grown under no-choice compared to choice plots. More flies emerged from fruits grown in no-choice plots. However, infestation rates were not significantly different among fruits from natural and semi-natural conditions. Further results show that *B. cucurbitae* dominated *Dacus bivittatus* and *D. frontalis* in cucumber. We conclude that watermelon is the most preferred host of the dominant cucurbit infester *B. cucurbitae*.

Mating compatibility among within *Bactrocera dorsalis* complex from north and south of Thailand

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Abstract:

Two horticultural pest tephritid fruit fly species which are highly morphologically similar to the destructive pest, the Oriental fruit fly *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae), have been recorded the distribution in Thailand since 2001. *Bactrocera papayae* Drew & Hancock was presented in the lower south and *B. carambolae* Drew & Hancock was presented in the lower half of Thailand. A decade later, fruit survey detected a small number of both species far north but *B. carambolae* was not captured in trap. For resolving biological species limits to overcome constraints to pest management and international trade, mating compatibility tests among within *B. dorsalis* complex from northern and southern extremes of geographical distribution of Thailand were carried out in field cages. Wild colonies of *B. dorsalis*, *B. papayae* and *B. carambolae* from Chiang Mai (north) and Nakhon Si Thammarat (south) were collected and maintained. Six replicates of intra and inter-species mating compatibility were examined in intra and inter-regional level. *Bactrocera dorsalis*, *B. papayae* and *B. carambolae* males at 23, 28 and 28 day-olds and females at 21, 28 and 28 day-olds, respectively were used. Twenty individuals of each sex of two different color marked populations were released in field cages. A potted mango tree approximately 180 cm height in each octagonal field cage was set for the experiment. Sexual compatible mating was quantified with the following indices: the Index of Sexual Isolation (ISI), the Male Relative Performance Index (MRPI), and the Female Relative Performance Index (FRPI).

Intra-species inter-regional tests of *B. dorsalis*, *B. papayae* and *B. carambolae* from north and south showed a slightly tendency for homotypic matings in all species with mean ISI value = 0.13 ± 0.35 , 0.12 ± 0.16 and 0.24 ± 0.22 , respectively.

Inter-species intra-regional tests showed that *Bactrocera dorsalis* was unselectively species cross mate to *B. papayae* in the north (ISI = 0.07 ± 0.32) but positive assortative mating to *B. papayae* in the south (ISI = 0.44 ± 0.27). *Bactrocera dorsalis* showed positive assortative mating to *B. carambolae* in both region of north (ISI = 0.15 ± 0.36) and south (ISI = 0.37 ± 0.25). *Bactrocera papayae* showed unselectively species cross mate to *B. carambolae* in north (ISI = -0.02 ± 0.25) and south (ISI = -0.01 ± 0.75).

Inter-species inter-regional tests showed that north population of *B. dorsalis* were random mate with south population of *B. papayae* (ISI = 0.07 ± 0.38) and *B. carambolae* (ISI = 0.09 ± 0.25) while south population of *B. dorsalis* were highly positive assortative mating to north population of *B. papayae* (ISI = 0.43 ± 0.22) and *B. carambolae* (ISI = 0.39 ± 0.22). North population of *B. papayae* showed unselectively species mating to *B. carambolae* from

south (ISI = -0.01 ± 0.34) meanwhile *B. papayae* from south showed slightly heterotypic matings to *B. carambolae* from north (ISI = -0.11 ± 0.10). *Bactrocera carambolae* from both north and south mated earlier than other two species (64.91 ± 3.45 min, 66.50 ± 5.55 min).

For laboratory assesses, each ten pairs of six cross were studies with six replicates, the highest average number of eggs per female was in cross which mother was *B. carambolae* (cross with ♂ *B. dorsalis* = 591.78 ± 103.96 , cross with ♂ *B. papaya* = 584.25 ± 219.23) followed with *B. papayae* (cross with ♂ *B. dorsalis* = 548.33 ± 151.38 , cross with ♂ *B. carambolae* = 445.68 ± 78.43) while *B. dorsalis* produced lowest eggs (cross with ♂ *B. papayae* = 435.90 ± 96.91 , cross with ♂ *B. carambolae* = 339.85 ± 45.46). Pupae recovery were less than 10% in any crosses that father was *B. carambolae*, and less than 20% in any crosses which mother was *B. carambolae*, while pupae recovery in cross of *B. dorsalis* and *B. papayae* were more than 25%. More than 40% hybrids progeny of each cross demonstrated mix features of parent while not more than 10% of the rest progeny in crosses which *B. carambolae* was father had clearly feature of *B. carambolae*. In cross which *B. carambolae* was mother, 2 folds of the rest hybrids progeny showed clear feature of *B. carambolae* while cross with *B. papayae* but not difference in cross with *B. dorsalis*. In crosses of *B. dorsalis* and *B. papayae* either which species was father or mother, 3-4 folds of the rest hybrids progeny showed more clear feature of *B. dorsalis* than *B. papayae*.

Analysis of sexual compatibility among *Anastrepha fraterculus* populations from Brazil: pre and post-zygotic isolation tests between two brazilian morphotypes

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Abstract:

In Brazil several studies concerning egg morphology, genetics and post-zygotic reproductive isolation tests suggest that *Anastrepha fraterculus* is a complex of sibling species and not a single biological entity, with, at least, three different species in the country. The definition of their taxonomic status will support the implementation of a SIT program for *A. fraterculus* in Brazil, that is a major pest in apple orchards in south of the country.

In this paper we evaluated the pre-zygotic sexual compatibility and post-zygotic reproductive isolation between 2 brazilian morphotypes: Brazilian-1 and Brazilian-3, represented, respectively, by a wild population from Bento Gonçalves (southern Brazil) and a wild population from Parnamirim (northeast Brazil).

Both wild populations were collected during 2014 from infested guavas and were analyzed by morphometric parameters, being classified as the two different morphotypes.

Comparisons between these two populations concerning sexual compatibility showed some degree of mating incompatibility (ISI=0.66). Individuals of Brazilian-1 in general had more mating participation compared with Brazilian-3 individuals.

Post-zygotic isolation tests showed decreased of egg eclosion, not in F1, but in F3 progeny derived from heterotypic crossings. These results are compatible with hybrid breakdown mechanism. Sexual ratio data of heterotypic crossings were compatible with Haldane's rule (deviation towards females), since F1.

The calling behavior analysis showed some different patterns between the two morphotypes and among them and the hybrids from the heterotypic crossings. However, both populations had predominant calling behavior during the morning.

The frequency and sequence of courtship behavior of the two populations were also compared. The results showed that both populations have complex sequence graphs and are similar concerning the degree of complexity and graphs properties. However, they are different in size of sequences and in frequencies of behavioral units. These differences in the courtship behavior may contribute to explain the reproductive isolation data obtained in pre-zygotic tests.

Effects of laboratory colonization on *Bactrocera dorsalis* (Diptera: Tephritidae) mating behaviour: ‘what a difference a year makes’

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Abstract:

Laboratory-reared insects are widely known to have significantly reduced genetic diversity in comparison to wild populations; however, subtle behavioural changes between laboratory-adapted and wild (or ‘wildish’ i.e. within one or very few generations of field collected material) populations are less well understood. Quantifying alterations in behaviour, particularly sexual, in laboratory-adapted insects is especially important for pest species that are mass-reared for use in pest management strategies that have a sterile insect technique component. We report subtle changes in sexual behaviour between ‘wildish’ *Bactrocera dorsalis* flies (F1 and F2) from central and southern Thailand and the same colonies 12 months later when at six generations from wild. Mating compatibility tests were undertaken under standardised semi-natural conditions, with number of homo/heterotypic couples and mating location in field cages analysed via compatibility indices. The two ‘wildish’ populations of *B. dorsalis* exhibited slight differences in time of mating (latency) and mating-site preference resulting in increased sexual incompatibility. This difference was lost with full mating compatibility obtained after 12 months’ colonisation. Relative participation also changed with time. These results reveal how subtle changes in sexual behaviour, as driven by laboratory rearing conditions, may significantly influence mating behaviour between laboratory-adapted and recently colonised tephritid fruit flies over a relatively short period of time.

Temperature tolerance, predictive models and mating compatibility studies among two populations of *Ceratitis rosa*

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Abstract:

During the period under review, the following program of work was undertaken - *Activity 1*: assessment of the development and survival of R1 and R2 populations of *Ceratitis rosa* at different temperatures; *Activity 2*: develop predictive model for the two *C. rosa* populations; *Activity 3*: assess the mating compatibility of the two populations and *Activity 4*: share materials with CRP participants.

Activity 1: Assessment of the development and survival of R1 and R2 populations of *Ceratitis rosa* at different temperatures. Comparative analysis of development and survivorship of two ecological divergent populations of the Natal fruit fly *Ceratitis rosa* Karsch designated as *C. rosa* R1 and *C. rosa* R2 from Kenya were studied at seven constant temperatures (10, 15, 20, 25, 30, 33, 35°C). Temperature range for development and survival of both populations was 15 - 35°C. The developmental duration was found to significantly decrease with increasing temperature for *C. rosa* R1 and *C. rosa* R2. Survivorship of all the immature stages of *C. rosa* R1 and *C. rosa* R2 from Kenya was highest at the range of 20-30°C (87-95%) and lowest at 15 and 35°C (61-76%). Results from temperature summation models showed that *C. rosa* R1 tolerated more heat compared to *C. rosa* R2. Total degree-day (DD) accumulation was estimated at 302.75 and 413.53 for R1 R2, respectively. These results demonstrate that *C. rosa* R1 and R2 were physiologically distinct in their response to different temperature regimes and support the existence of two genetically distinct populations of *C. rosa* and this calls for taxonomic revision of *C. rosa*. However, additional information on morphological characterization of *C. rosa* R1 and R2 is needed.

Activity 2: Develop predictive model for the two *C. rosa* populations. Life table parameters, using the data generated in Activity 1, were calculated using Insect Life Cycle Modelling (ILCYM) software. Results showed that at the lethal high temperature (35°C), the life span of female flies was approximately 6 times (R1) and 13 times (R2) less than that of their counterparts at the lowest temperature (15°C). Fecundity increased with increasing temperatures up to 30°C and significantly decreased thereafter for both populations. Several non-linear models were fitted to the data for developmental time, mortality and reproduction of both populations of *C. rosa*. Cohort updating algorithm and rate summation approach were stochastically used for simulating age and stage structure populations and generate life-table parameters. Both populations attained a maximum net reproductive rate (28.3–100.7 females/female/generation) and total fecundity (84.2 – 300.4 individuals/female/generation) at 25°C. The highest value of intrinsic rate of increase (0.04 – 0.12 females/female/day), shortest doubling time (5.9 – 26.4 days) and maximum finite rate of increase (1.02 – 1.12 females/female/day) were observed at 30°C for both populations. These findings demonstrate the species' capacity to adapt to different a wide range of temperatures and the present model can be simulated spatially for estimating the pest risk and undertaking agro-ecological specific pest management strategies.

Activity 3: Assessment mating compatibility of R1 and R2 populations of *C. rosa*. Additional replications were undertaken during the period under review. As with previous observations, results from mating compatibility studies showed a high degree of mating incompatibility between the 2 populations of *C. rosa* and also between the 2 populations and that of *C.*

fasciventris. The ISI values ranged from 0.78 to 0.92 with the R1 *C. rosa* x R2 *C. rosa* showing the highest degree of isolation. All matings were achieved by males of the R1 population. Latency to mate among the mating combinations was not significantly different. In general mating duration was also similar for all mating combinations. Over 80% of the mating occurred on the tree canopy for all the combinations.

Activity 4: Shipment of materials to CRP participants. During the period under review, different populations of *C. rosa* specimens of different developmental stages and quantities were shipped to Dr. Gary Steck (USA), Dr. Marc De Meyer (Belgium) and Dr. Lucie Vanickova (Czech Republic) for various activities related to the CRP.

***Ceratitis rosa* along an altitudinal transect in Central Tanzania: niche partitioning among the two Natal fruit fly morphotypes and other *Ceratitis* pest species**

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Abstract:

Two standard parafferomones, trimedlure (routinely used for *Ceratitis rosa* and *C. capitata*) and terpinyl acetate (routinely used for *C. cosyra*) were compared with enriched ginger root oil (EGO) lure for detecting and monitoring the presence and relative population abundance of these particular pest species. Standard yellow fruit fly traps were used for the comparison, which was conducted at 10 sites along an altitudinal transect ranging from 540 to 1650 masl on the Uluguru mountains, in Morogoro Region (Central Tanzania). A gradual change of relative occurrence of the two *C. rosa* morphotypes was clear from the EGO lure trapping. The morphotype R1 was predominant at lower altitudes while morphotype R2 was predominant at higher altitudes. Further experiments are needed to confirm if temperature thresholds in the developmental physiology of both morphotypes cause this difference. The mango fruit fly, *Ceratitis cosyra*, shows a distinct predominance at altitudes below 800 masl as shown in both the EGO lure and the terpinyl acetate trapping. The catches of all three target species were higher in traps with the EGO lure compared to the conventional lures trimedlure and terpinyl acetate. It is argued that for these species EGO lure can act as a suitable and more effective alternative for trimedlure and terpinyl acetate parafferomones. In addition, EGO lure has the added advantage that it combines the taxon spectrum for the two latter substances, thus requiring the use of only a single attractant.

Female attraction towards male sexual pheromone in *Anastrepha fraterculus*

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Abstract:

Elucidation of the mechanisms that underlie reproductive incompatibility among species within species complexes is relevant to delimit species accurately for biogeography, ecology, conservation and pest management. In the case of the sterile insect technique (SIT), one of the main concerns when dealing with cryptic species complexes is to determine the extension over which one population/species can be used for sterile releases in more than one region. In this sense, evaluating the occurrence of mating incompatibility and assessing the underlying mechanisms should be done under situations that resemble natural conditions as much as possible. Here we present an experimental approach that allows determining the role of chemical communication between sexes on the recognition of mates of the same origin in which field cages are set up with 2 potted citrus trees to provide the arenas for males to attract females. Populations/origins are tested in pair-wise combinations. Males are encaged in small containers made of a metal mesh (artificial leks). Males from one origin are placed in one tree and males from the other origin in the other tree. Females are allowed to visit artificial leks and their position is recorded at regular intervals during their time of sexual activity. Results are discussed under the context of SIT as well as its applicability as quality control tests.

Chemical ecology of genera *Anastrepha* and *Ceratitis* (Diptera: Tephritidae)

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Abstract:

Pheromone communication *inter alia* plays an important role in fruit-fly courtship behavior. In genera *Anastrepha* and *Ceratitis* (Diptera: Tephritidae) males use plant volatiles to locate partners and hosts and produce long-ranging sex pheromone to attract females for mating. Close-range mate recognition signals, such as the cuticular hydrocarbons, may influence the rejection or acceptance of a male by female during short-ranging evaluation.

Here we present an overview of our research conducted during the period of the CRP project, from 2010 up to today, focusing on pheromone and cuticular hydrocarbons of the two above-mentioned fruit fly genera. We characterised the chemical composition of the cuticular hydrocarbon mixtures of males and females from *C. capitata*, *A. fraterculus* and *Ceratitis* FAR cryptic species complexes. We elucidated the possible use of these semiochemicals as taxonomic markers for the putative species delimitation. Furthermore, we described (i) differences in pheromone composition of *C. capitata* and *Ceratitis* FAR complex males, (ii) the key pheromone components of courtship, their role as attractants of *A. fraterculus* females and (iii) the variability of these compounds among the geographically distinct populations of *A. fraterculus* cryptic species complex.

Rectal gland volatiles and phylogenetic relationships of pest sibling species within the *Bactrocera dorsalis* complex - Incipient species

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Abstract:

There are eight major putative cryptic pest species within the *Bactrocera dorsalis* complex - *Bactrocera dorsalis sensu stricto*, *B. papayae*, *B. philippinensi*, *B. invadens*, *B. carambolae*, *B. caryeae*, *B. kandiensis*, and *B. occipitalis*. The first four have been synonymized to be the same biological species, *B. dorsalis*. The remaining four may be incipient species belonging to the same phylogenetic clade, particularly since *B. carambolae* and *B. occipitalis* have been shown to be able to naturally hybridize with *B. dorsalis* in Malaysia and Philippines, respectively. Males of these taxa are known to be attracted to, and compulsively feed on methyl eugenol (ME). ME is biotransformed into two ME-metabolites - (E)-coniferyl alcohol (ECF) and 2-allyl-4,5-dimethoxyphenol (DMP) and sequestered in the rectal gland. These two compounds have been shown to be the male sex pheromone components in *B. dorsalis*. However, in *B. carambolae* consumed ME is converted to only ECF, which attracts and induces conspecific females to extrude their ovipositor. Additionally, *B. carambolae* males also endogenously synthesized four other rectal volatiles of which a major component is 6-oxo-1-nonanol [OXO], and three minor components - 1,6-nonanediol [NDL], N-3-methylbutyl acetamide [MBA] and ethyl benzoate. While laboratory hybrids of *B. carambolae* and *B. dorsalis* produced various combinations of rectal gland volatiles of the two parental species, natural hybrids that showed the combinations of those glandular volatiles have also been captured in the wild. Preliminary chemical analyses of male *B. kandiensis* rectal gland further revealed that besides producing ECF after ME consumption, they also produced OXO, NDL and MBA along with olean and ethyl 2-hydroxy-3-methylbutanoate, very similar to *B. carambolae*, thereby providing evidence that it is also an incipient species.

Emission of male sex pheromone by four cryptic species, *Bactrocera dorsalis*, *B. invadens*, *B. papayae* and *B. philippinensis*, following Methyl Eugenol consumption)

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Abstract:

Four cryptic pest species of the *Bactrocera dorsalis* complex - *Bactrocera dorsalis sensu stricto*, *B. papayae*, *B. philippinensis* and *B. invadens*, were known to be strongly attracted to, and compulsively fed on methyl eugenol (ME). Following consumption of ME, the four species were shown to biotransform ME into two identical ME-analogues - (*E*)-coniferyl alcohol and 2-allyl-4,5-dimethoxyphenol which were then transported via hemolymph from the crop to the rectal gland for temporary storage. It was known that calling *Bactrocera* males emitted sex pheromone to entice conspecific females during courtship at dusk. As chemical signals involved in insect sexual communication are often highly species-specific, we investigated, both qualitatively and quantitatively, the sex pheromone production and utilisation, i.e. before (volatile chemical components stored in the male rectal gland) and during (volatile chemicals emitted by calling males) courtship for sexual communication, by each of the cryptic male species following ME consumption. The results of comparative quantification of sex pheromone production in the rectal gland and air-borne emission in terms of composition and ratio for the four cryptic species will be presented and discussed during the meeting.

Analysis of cuticular hydrocarbon composition in *Bactrocera dorsalis* complex)

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Abstract:

Cuticular hydrocarbons are components of insect epicuticle. Their primary function is protection against desiccation. Secondly they are involved in intra- and inter-specific communication, such as species and gender recognition, fertility signaling, mating status, chemical mimicry and many others. Due to their species-specificity they became an excellent identification tool in insect taxonomy. For extractions were used males and females of *B. philippinensis* Guimaras, *B. papaya*, *B. dorsalis* Saburi, *B. invadens*, *B. carambolae*.

Male and female flies 9th day after emergence were individually placed in small vials and washed by 200 μ l of *n*-hexane with 1-bromundecan as an internal standard (5 μ g/ml). The washing lasted 5 minutes. The identification and quantification of cuticular hydrocarbons were performed on the GC \times GC/TOFMS. During the measurement were used not only one dimensional modes, but also two dimensional, which provide us better resolution of long-chained hydrocarbons. The hydrocarbons were identified by a comparison of their MS fragmentation patterns and retention indices-RI. Semi-quantitative analyses showed both qualitative and quantitative differences especially between males and females profiles in studied complex. Female profiles showed high amount of short-chained hydrocarbons, which are not present in male profiles.

Pheromone communication in *Ceratitis* FAR complex)

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Abstract:

Ceratitis fasciventris, *Ceratitis anonae* and *Ceratitis rosa* are fruit flies originating from the African continent. *C. fasciventris* and *C. anonae* occur sympatrically in both East and West Africa, while *C. rosa* is more restricted to southern and eastern Africa where its distribution partially overlaps with that of *C. fasciventris* but not with *C. anonae*. The nominal species show quite subtle morphological differences. Recent large scale genetic comparison of allelic variations at 16 microsatellite loci in different African *Ceratitis* FAR populations indicates a possible presence of five taxonomic entities.

Insect pheromones are species-specific and represent an important part of pre-mating isolation associated with speciation. In fruit flies, the pre-mating behavior is initiated by male sex pheromones that attract females to mate. To determine the taxonomical relationships within *Ceratitis* FAR complex, we studied male sex pheromones of four available genetically different populations, originated from Kenya. Specifically we investigated two populations of *C. rosa* from highlands and lowlands, one population of *C. anonae* and one strain of *C. fasciventris*. Pheromones were collected by headspace technique from adult males and were analyzed using comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry (GC×GC-TOFMS) and gas chromatography combined with electroantennographic detection (GC-EAD).

GC×GC-TOFMS analyses followed by multivariate statistics showed that male sex pheromones are complex mixtures that significantly differ both qualitatively and quantitatively among the FAR complex species studied. GC-EAD analyses revealed that only a relatively small set of identified compounds elicited antennal responses suggesting their prominent roles in pheromone communication. GC-EAD experiments with equal concentrations of synthetic standards proved that antennae of different populations were differentially sensitive to individual substances suggesting different abilities to detect these compounds.

Our data suggest that sex pheromone communication in all studied fruit fly populations is highly specific and may thus contribute to reproduction isolation.

Third instar larvae of *Ceratitis* FAR complex and *C.* (Diptera: Tephritidae)

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Abstract:

Third instar larvae of members of the *Ceratitis* FAR complex, including *Ceratitis fasciventris* (Bezzi), *Ceratitis anonae* Graham, and *Ceratitis rosa* Karsch are described and compared with those of *Ceratitis capitata*. Diagnostic characters, such as presence vs. absence of a secondary tooth on the mouthhooks, previously used to separate *C. capitata* from *C. rosa*, are shown to be variable in each species. Significant variation in diagnostic morphological characters among populations of *C. rosa* from east and south Africa is documented; however the differences are not simply congruent with the R1 and R2 designations based on other studies. Quantitative measures of numerous larval morphological characters are consistently smaller in *C. fasciventris* and distinguish them from other species of the FAR complex. Larvae of *C. capitata* can be distinguished from those of the FAR complex by characters such as absence of accessory plates of the oral ridges, the shape of the anterior spiracle, and the pattern of dorsal spinules. Previous studies indicated that absence of accessory lobes separate the genus *Ceratitis* from *Bactrocera*, but this is shown to be incorrect, as accessory lobes are in fact present in several species of *Ceratitis*.

**Tools for studies on taxonomy and systematics of the *Anastrepha fraterculus* complex:
Third instar larvae and karyotypes**

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Abstract:

The South American Fruit Fly, *Anastrepha fraterculus*, is a complex of eight morphotypes. Every one of these morphotypes should exhibit a different pest and quarentenarian status. Biological and ecological data of them are important for pest management programs, but more accurately taxonomy and systematic relations among them are basic information for decisions on integrated pest management. The morphology of the third instar larvae and mitotic chromosomes were studied for improving the knowledge on taxonomy and systematic of the complex. Third instar larvae of 6, 7, 8, 9 and 10 days old of the Andean morphotype reared on fruits or artificial diet, were studied using outline-geometric and linear morphometry. The heterochromatic chromosome regions were studied in the cytogenetical work with Colombian, Ecuadorian and Mexican morphotypes. In the larvae, the shape of the left mouth hook of the cephalopharyngeal sclerite was studied by outline-geometric morphometry and 27 measurements of the cephalopharyngeal sclerite and size of the body were used in the lineal morphometry. The objective of the work was to find the suitable variables for use in the differentiation of the morphotypes. Karyotypes of the populations studied were described and differences could be found between Brazilian and Andean - Meso-Caribeann lineages, reinforcing the results of different origins of them.

Use of wing shape for delimitation of cryptic species of the *Anastrepha fraterculus* complex in Mesoamerica and Andean countries

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Abstract:

Previous studies based on linear measurements of the female aculeus, mesonotum, and wing revealed the existence of seven morphotypes within the nominal species *Anastrepha fraterculus* along the Neotropical Region. In this study we follow a geometric morphometric approach based on 40 populations of this species spread throughout Meso-America and the Andean countries (including Mexico, Central America, Venezuela, Colombia, Ecuador, and Peru). Eighteen homologous landmark coordinates were digitized on the wing of 628 females for further analyses. The main goal was to explore similarities among samples, and evaluate the reliability of this morphometric approach for delimitation of morphospecies. Our findings determined that morphotypes previously recognized via the linear morphometrics were also separated by the geometric morphometry of the wing shape, and a new Ecuadorian morph inhabiting highlands from Ecuador and Peru was identified. Moreover, comparisons of morphological similarities exhibited that morphotypes are grouped into three phenotypic lineages and would not be directly related each other, so it can be concluded they have dissimilar origins which are discussed in light of other evidence.

Wing morphometrics as a possible tool for the diagnosis of the *Ceratitis fasciventris*, *C. anonae*, *C. rosa* complex (Diptera: Tephritidae)

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Abstract:

Previous attempts to resolve the *Ceratitis* FAR complex (Diptera, Tephritidae) showed contrasting results and revealed the occurrence of five microsatellite genotypic clusters (A, F1, F2, R1, R2). In this paper we explore the potential of wing morphometrics for the diagnosis of morphospecies and genotypic clusters within the complex. We considered a set of 227 specimens previously morphologically identified and genotyped at 16 microsatellite loci. Seventeen wing landmarks and six wing band areas were used for morphometric analyses. Permutational multivariate analysis of variance detected significant differences among morphospecies and among genotypic clusters (for both male and females). Unconstrained and constrained ordinations could not resolve groups corresponding to morphospecies or genotypes. However, posterior group membership probabilities (PGMPs) of a Discriminant Analysis of Principal Components (DAPC) allowed the correct diagnosis of a relevant proportion of specimens (but with differences among morphospecies and among genotypic clusters). This study suggests that wing morphometrics and PGMPs might represent a possible tool for the diagnosis of species within the *Ceratitis* FAR complex. We propose a tentative diagnostic method and provide a first reference library of morphometric measures that might be used for the identification of unidentified specimens of the complex.

A review of the current knowledge on *Zeugodacus cucurbitae* (Diptera: Tephritidae) in Africa

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Abstract:

We hereby review all available information regarding the occurrence and biology of the melon fly, *Zeugodacus cucurbitae*, in the Afrotropical region. Its invasion history in Africa is briefly presented and the African populations compared with other populations worldwide. The African populations show very low intraspecific variability with regard to DNA barcoding and adult morphology. Although there are some differences observed in host range throughout the continent, these appear to be the result of other factors like interspecific competition, host availability, and ecological niche partitioning, rather than an indication of different cryptic species with specific host preferences. From the results obtained and reviewed in this study, there seems to be no indication that *Zeugodacus cucurbitae* represents a species complex. Its relationship with regard to other cucurbit feeding fruit flies in Africa is briefly discussed.

Routes of introduction and spread *Zeugodacus cucurbitae* (Diptera: Tephritidae) in Africa

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Abstract:

The possible worldwide colonization routes of the melon fly, *Zeugodacus cucurbitae* (Diptera: Tephritidae), are mainly inferred from sparse historical records. Here we evaluate possible colonization scenarios of *Z. cucurbitae* in Africa through the Bayesian approach implemented by DIYABC. Populations of *Z. cucurbitae* were sampled from 17 localities in East, West and Central Africa and genotyped at 19 microsatellite loci and 3 mitochondrial gene fragments (COI, COII, ND6). AMOVA suggests the occurrence of four main population groups colonizing Congo, West Africa, Sudan and East Africa. The Bayesian assignment of STRUCTURE shows further genetic structuring in East Africa, with populations from Uganda diverging from those of Tanzania and populations from Burundi and Kenya sharing admixture proportions with West African samples. The analysis of mitochondrial haplotypes suggests that the initial colonization and subsequent demographic expansion of *Z. cucurbitae* in Africa originated from a limited number of introduction events. The most plausible scenario resulting from Bayesian simulations further corroborates the Asian origin of African *Z. cucurbitae*. Invasions originated from a single or from a limited number of introductions (possibly in Tanzania) and resulted in range expansion from East to West Africa. Furthermore simulations suggest a relatively recent origin of *Z. cucurbitae* in Africa (1889-1992) roughly corresponding to the first historical records (1936 for East Africa, 1999 for West Africa).

Different speciation paths and multidisciplinary approaches to provide insight in tephritidae species complexes: a case study from the *B. dorsalis* complex

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Abstract:

Different pathways can lead to speciation in insects. Among them, chromosome rearrangements are recognized as key speciation factors, especially in sympatry. In recent years, symbiotic bacteria (mainly reproductive) are receiving much attention regarding their ability to enhance speciation. *Wolbachia* is probably the most ubiquitous of them in insects and is believed to be a putative speciation agent since it is known that it can cause barriers in gene flow through a variety of reproductive alterations including Cytoplasmic Incompatibility (CI). The selective sweeps that this bacterium can cause, along with its recently documented effects on the fitness of the host, indicate that it can be implicated in enhancement of barriers among differentiating taxa. Other bacteria species can also be involved but are less studied and characterized up to now. The variety of mechanisms leading to speciation, and the sometimes obscure limits among species, point to the need for multi-disciplinary approaches and well characterized material in studies addressing speciation. Such approaches should ideally utilize different available tools and provide independent lines of evidence such as morphometrics, molecular/genetic markers, cytogenetics and mating compatibility/competitiveness studies.

Accurate identification of species limits among economic important pests is critical for international trade and agriculture, both for implementation of environmental-friendly control methods, such as SIT and designing/imposing quarantine measures. In this respect, here we discuss the efforts to address the species status of a member of the *B. dorsalis* complex recently introduced in IPCL, as a wild population from infested fruits. Our approach includes: a) identification by experts using morphometric characters and standard identification keys; b) evaluation and/or development of mitochondrial DNA and nuclear DNA markers; c) cytogenetic characterization, based on mitotic nuclei and polytene chromosomes; d) mating compatibility studies and e) symbiotic characterization. Our data indicate that we are dealing with a member of the *B. dorsalis* complex exhibiting a unique combination of properties, both in genomic and symbiomic level, quite distinct from *B. dorsalis* s.s. and its close relatives.

Contribution of cytogenetics to taxa discrimination within species complexes toward SIT applications: the example of the *Bactrocera dorsalis* and results on the *Ceratitis FAR* and *Anastrepha fraterculus* complexes

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Abstract:

Numerous years of cytogenetic research in *Drosophila* and mosquito species have shown that speciation in Diptera is almost universally accompanied with chromosomal rearrangements (CRs). Especially inversions are considered as key players, since they can impose barriers in the gene flow among differentiating taxa, probably through the restriction in the gene flow within and near the inverted regions. Although mitotic chromosomes can provide information, polytene chromosomes are an excellent tool for resolution of CRs. The characteristic banding patterns of polytene chromosomes have long been used to infer the degree of relatedness of taxa and the phylogenetic relationships among members of species complexes. This kind of information is of great importance for the application of sterile insect technique (SIT) approaches for pest population control and also for the facilitation of the international trade of commodities.

In this context, we have previously attempted to detect chromosomal characteristics that could help to discriminate among five of the economic important members of the *Bactrocera dorsalis* complex (*B. dorsalis s.s.*, *B. papayae*, *B. philippinensis*, *B. invadens* and *B. carambolae*). The comparative analysis of the mitotic karyotypes and the polytene chromosomes of the above taxa as well as of the F1 bidirectional hybrids of *B. dorsalis s.s.* with *B. invadens* and *B. carambolae* did not reveal any chromosomal rearrangements that could act as diagnostic characters and as barriers to gene flow among them. In addition, *in situ* hybridization of several genes on the polytene chromosomes on the five members of the complex as well as on the two bidirectional hybrids did not reveal any differences on their mapping position. Therefore, all the taxa analysed can be regarded as homosequential to *B. dorsalis s.s.*

In order to further explore the cytogenetic status within the *B. dorsalis* complex and the limitations of comparative cytogenetics in the resolution of Tephritidae complex species, we analyzed the mitotic and polytene chromosomes of two more species that are currently colonized in the Insect Pest Control Laboratory (IPCL): *B. tryoni*, a species that does not belong to the *B. dorsalis* complex but is closely related to it and a recently colonized member of the complex which, however, seems to be distinct from the above five taxa. Additionally, F1 hybrids between *B. dorsalis s.s.* and the aforementioned species were cytogenetically characterized. Comparative cytogenetic analysis revealed that the mitotic karyotypes of the analyzed species differ from that of the reference species *B. dorsalis s.s.* Furthermore, their polytene chromosomes presented at least one inversion that differentiates each of the two species from *B. dorsalis s.s.* The above observations show that at least cytogenetically the

newly colonized member of the *B. dorsalis* complex is a clearly differentiated species entity within the complex.

Having the confidence that cytogenetic analysis can help in resolving boundaries within Tephritidae species complexes, we studied the chromosomes of members of the *Ceratitis* FAR and the *Anastrepha fraterculus* complexes. Specifically, the mitotic karyotype and the polytene chromosomes of a *Ceratitis fasciventris* colony established in IPCL have been analyzed. Comparison of the polytene chromosomes of *C. fasciventris* with the reference map of *C. capitata* revealed fixed rearrangements in two polytene arms, 3L and 5L. Additionally, in collaboration with Giardini C, Lanzavecchia SB and Cladera JL (Instituto de Genética, Instituto Nacional de Tecnología Agropecuaria, Argentina), the mitotic karyotype and the polytene chromosome map of *Anastrepha fraterculus* (morphotype Tucuman) have been developed also from a colony established in IPCL. Thus, the chromosome maps of *C. fasciventris* and *A. fraterculus* can form the reference for future cytogenetic analyses of other members of these complexes.

**Use of mtDNA and nuclear markers to delimit and diagnose Brazilian species
within the *Anastrepha fraterculus* complex**

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Abstract:

The South American fruit fly, *Anastrepha fraterculus* (Wiedemann), is among the most serious agricultural pests in South America. In Brazil, *A. fraterculus* has been reported to infest 81 host species in 20 plant families. A series of morphological and genetic studies have revealed that *A. fraterculus* actually comprises a complex of multiple species. The actual number of putative species within the *A. fraterculus* complex and their associated biogeography is yet uncertain.

Differences among cryptic species could have significant consequences for pest quarantine, management, and eradication. Therefore, it is paramount to study populations from a wide geographic range in Brazil using mtDNA and nuclear markers in order to capture genetic diversity of this complex. Analysis of con-specific variation among *Anastrepha fraterculus* samples and con-generic variation among *fraterculus* species group samples is critical to resolving evolutionary relationships of cryptic species.

The cooperative research program has obtained over 630 expertly identified *Anastrepha* samples representing nearly 70 species. This includes over 260 *A. fraterculus* specimens gathered from several states in Brazil, Argentina, and Mexico plus an additional set of 175 specimens representing 20 taxa within the *fraterculus* species group as well as from other *Anastrepha* species groups. To date, DNA has been isolated from 630 adult specimens.

We sequenced a portion of the cytochrome oxidase c subunit I for 262 *A. fraterculus* specimens from a total of 37 localities in Brazil (34), Argentina (1), Mexico (1), and Peru (1). A subset of the DNA samples has been screened for variation also with a nuclear marker (ITS 1), totaling 58 *A. fraterculus* specimens from 32 localities in Brazil. Genetic diversity estimates and phylogenetic analyses of the aligned sequences suggest structuring among populations.

Additional accomplishments included analysis of a subset of six distinct populations distributed from the Northeast to the South in Brazil using 10 microsatellite markers. The results of all analyses (*Fst*, phylogenetic analysis, Structure, and spatial analyses of molecular variance) revealed a pattern of genetic differentiation that overlaps with the distribution of the known morphotypes in Brazil.

Our collaborative efforts also included morphometric analyses of populations of *A. fraterculus* from Brazil. Seventeen Brazilian populations that were genotyped using mitochondrial and nuclear markers were also examined for morphometric differences. A total of 192 specimens from 17 populations was examined. Two groups of populations belonging to Brazilian morphotype 1 and Brazilian morphotype 3 were recovered in the analyses and two populations (Campos do Jordão and Campinas in the state of São Paulo) seem to belong to a distinct biological entity reported for the first time, since they are distinct from the three morphotypes already reported in Brazil.

We screened wild females of *A. fraterculus* from eight populations in three distinct geographic regions in Brazil for the presence of *Wolbachia*. All specimens were found to be infected and our *Wolbachia* sequences most likely belong to supergroup A. Further characterization of these *Wolbachia* strains using multilocus sequence typing and phylogenetic analyses might help shed some light on the role they might play in post-zygotic isolation among *A. fraterculus* populations.

Nuclear ribosomal internal transcribed spacer 1 (ITS1) variation in the *Anastrepha fraterculus* cryptic species complex (Diptera: Tephritidae) of the Andean region.

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Abstract:

As part of an ongoing project to improve the identification of *Anastrepha* immature, the nuclear ribosomal internal transcribed spacer 1 (ITS1) was sequenced for *Anastrepha fraterculus* (Wiedemann, 1830) originating from 65 localities in the northern and central Andean countries of South America including Bolivia, Perú, Ecuador, Colombia, and Venezuela. The ITS1 regions of additional specimens from Central America (Mexico, Guatemala, Costa Rica, and Panama), Brazil, and Argentina (Tucumán) were also sequenced, and together with published sequences (Paraguay) provided context for interpretation.

Anastrepha fraterculus ITS1 sequence patterns were generally consistent with putative morphological groupings. A total of six ITS1 sequence variants were recognized in the Andean region comprising four groups: type I from Argentina (Tucumán), the Chiquitano forests of eastern Bolivia into the eastern Andean dry valleys, and the higher elevations (~3,000-3,500m) of the Cusco-Ayacucho region of Perú; type II in the northern coastal regions of Colombia and Venezuela and the eastern foothills of the Cordillera Oriental of Colombia south to at least the Ecuador border; type III of the Central-Northern Andes which is the most complex group with at least 3 variants. The IIIA sequence variant is seen in the common *A. "fraterculus"* of the lower elevations of the Andean periphery and dry valleys of Perú continuing north along the Pacific slopes and coastal plain of Ecuador into at least southwestern Colombia. The IIIB and IIIC variants occur from at least the Cordillera de Mérida of Venezuela and Cordillera Oriental of Colombia south into Andean Ecuador; and type IV in the Cordillera de Mérida of Venezuela and Cordillera Oriental and Cordillera Central of Colombia south into at least northern Ecuador.

Development and use of population genotype databases for species resolution within *Anastrepha fraterculus* and *Bactrocera dorsalis*

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Abstract:

Our aim was the development and use of molecular markers in *Bactrocera dorsalis* and *Anastrepha fraterculus* to determine the intra/inter-population diversity, the species' range shifts, and the indigenous or invasive status of newly observed outbreaks.

a) for *B. dorsalis* we have developed an integrated database of SSR genotypes from populations across the species range, in order to

- 1) infer the demographic history and the degree of differentiation of the adventive populations,
- 2) perform traceability surveys, i.e. to unravel the geographic origin of flies that invade regions for the first time or that re-colonize a SIT treated area, thereby identifying the source of invasion.

The database also includes *B. dorsalis/B. invadens* genotypes from across Africa.

b) for *A. fraterculus* we have developed SSR markers that have allowed us to assess the presence of relevant genetic differentiation among Brazilian populations. The observed genetic differentiative patterns overlap the distribution of the known morphotypes in the Brazilian area. For *B. dorsalis/B. invadens* we have identified Y-specific sequences that permit sex sorting at the larval stages.

c) for *Ceratitis capitata*, using genomic/functional genomic approaches, we have identified sequences correlated to reproduction and chemoreception that may help explain some behavioural traits and provide species-specific markers.

Summary of studies on *Bactrocera dorsalis* and *B. carambolae* for improving SIT and international trades

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Abstract:

Population genetics of *B. dorsalis* and *B. carambolae*

B. dorsalis and *B. papayae* (a junior synonym) collected across Southern Thailand and West Malaysia were studied using microsatellite markers derived from both. Results revealed that no significant difference between intra- and inter-specific variations was detected. Moreover, weak population structure and recent gene flow were observed among them, suggesting no status for cryptic species between them in the given areas. These results were congruent to other studies using the different approaches. Therefore, both forms can be treated as a single target for SIT program in Southern Thailand and West Malaysia. (Aketarawong et al. 2014, BMC Genetics)

On the other hand, *B. carambolae* collected from known distribution in four different geographies (i.e., Indonesia, Malaysia, Thailand, and Suriname) were studied to compare intra-specific variation. We found that *B. carambolae* from West Java may be a genetic resource of Suriname. Moreover, genetic variation is significantly related to geographical distance. While, inter-specific variation between *B. carambolae* and *B. dorsalis* has been analysed to understand a species boundary.

The genetic sexing strains for *B. dorsalis* and *B. carambolae*

Genetic sexing strains (GSSs) are strains that can be sex-sorted based on stable sex-specific phenotypes of sterile insect. It can reduce a cost of female mass-rearing production and releasing, and also increase the effectiveness of SIT. GSS for *B. dorsalis* named Salaya1 was developed by Y and autosome translocation based on pupal color dimorphism (brown pupal males and white pupal females). The maintenance of genetic variability under mass-rearing conditions of the Salaya1 was evaluated using microsatellite markers. We found the Y-pseudo-linked markers that provide an opportunity to further develop sperm marking. Field cage test showed a comparable mating competitiveness between the Salaya1 and wild population. After one year of releasing of sterile Salaya1 in a pilot scale, fruit infestation rate was reduced from approximately 30% to 5%. This suggested that the Salaya1 is ready for use and evaluation in much larger area-wide control programs. (Isasawin et al. 2012, European Journal of Entomology)

However, the Salaya1 showed a low level of mating competitiveness with wild *B. carambolae*. Therefore, a new GSS for *B. carambolae* was developed by introgression of two components of genetic sexing *B. dorsalis* Salaya1 (a part of the Y-pseudo linked autosome carrying a dominant allele of a white pupae gene (Y-A^{WP+}) and a free autosome carrying a white pupae recessive allele (A^{WP}) of *B. dorsalis* Salaya1) to *B. carambolae* genome. Morphology, male pheromone profile and genetic relationship study were performed to confirm the characterization of the strain comparing to parental strain. The Salaya5 showed a close similarity with those features in the wild *B. carambolae* strain. Moreover, the mating

field cage tests suggested that the Salaya5 has a potential to be used in *B. carambolae* SIT programs based on male-only release (Isasawin et al. 2014, BMC Genetics)

Male-specific sequences of *B. dorsalis*

(Collaborative research project between Mahidol University and University of Pavia)

Sex specific sequences were isolated and characterized from *B. dorsalis* males. Amplification of four contigs specifically provided product only in male genome of several *B. dorsalis* strains. Moreover, this pattern was also observed in strains of junior synonyms (*B. papayae*, *B. philipinensis* and *B. invadens*). Sequences of four contigs will be analysed to study genetic variations among them.

Crosses and reciprocal crosses of *Bactrocera dorsalis* (Hendel) (Diptera, Tephritidae) from nine different regions of China

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Abstract:

The oriental fruit fly, *Bactrocera dorsalis* (Hendel), was first found infesting fruits and vegetables in Fujian, China, in the 1990s. It has since spread to most southern and central parts of mainland China and continues expansion into northern China. As in other parts of its global range it is a major pest of most horticultural crops. *Bactrocera dorsalis* is a member of species complex with other closely related and morphologically cryptic species. To ensure that the fly in China was exclusively *B. dorsalis*, and not a mix of two or more cryptic taxa, population crosses were made and offspring viability assessed to the F3 generation for nine Chinese *B. dorsalis* populations and 15 crossing types (Yunnan x Fujian; Yunnan x Guangdong; Yunnan x Hainan; Yunnan x Shanghai; Yunnan x Hubei; Fujian x Hubei; Fujian x Guangxi; Guangdong x Shanghai; Guangdong x Zhejiang; Guangdong x Hubei; Hainan x Hubei; Shanghai x Hainan; Shanghai x Hunan; Zhejiang x Hunan; Hubei x Guangxi).

The egg hatch rates, pupal weights, adult emergence rates and flight abilities of each generation were analyzed. All crosses and reciprocal crosses produced reproductively viable offspring with no evidence of reduced fitness traits. The results infer that the across mainland China all *B. dorsalis* populations belong to same species.

Key words: population crossing; reciprocal crossing; egg hatch rate; pupal weight; adult emergence rate; flight ability; Dacinae