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A review of more than 30 years of cytogenetic studies of Tephritidae in support of sterile insect technique and global trade

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Abstract

Cytogenetics of Tephritidae has contributed for more than 30 years to the efficient control of pest members of this family and the species delimitation among them. The sterile insect technique (SIT) is a species-specific and environmentally friendly method for the population control of insect pest species, always as a component of integrated pest management approaches. The construction of polytene chromosome maps facilitated the development, characterization, and improvement of genetic sexing strains, which have boosted the effectiveness of SIT and are considered as models for developing genetic sexing methodologies in other target species. In parallel, characterization of mitotic karyotypes and the availability of polytene chromosomes in Diptera have been and are being used as supplementary evidence in taxonomic studies. Lately, under the common understanding that speciation can be achieved via various pathways and under the umbrella of integrative taxonomy, cytogenetic studies have contributed to multidisciplinary taxonomic approaches. Such studies can shed light on the borders between closely related species and/or incipient speciation, and this is crucial both for the implementation of SIT and the revision/improvement of quarantine policies. In this review, we summarize the cytogenetic status of Tephritidae and discuss the contribution of cytogenetics to the development of genetic sexing strains in this family.

Introduction

Tephritidae is a Diptera family that includes almost 4 000 species, classified in ca. 75 genera, with some of them representing agricultural pests of major economic importance. The availability of polytene chromosomes in Diptera, including tephritids, has enabled the development and application of cytogenetics in support of basic and applied research. The sterile insect technique (SIT), an environment-friendly pest control method and a major component of integrated pest management (IPM) approaches, has been developed and applied for the population control of several tephritid species. For more than 30 years, cytogenetics of Tephritidae has played a catalytic role in the development of key tools in support of SIT as well as of species delimitation. The Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann), has been the model species for the development and utilization of appropriate cytogenetic tools.

In this review, we summarize the contribution of cytogenetics to the development of Tephritidae genetic sexing strains (GSSs) and we discuss the cytogenetic status of other tephritids. Moreover, we demonstrate the importance of cytogenetics for studying chromosome evolution and for improving species resolution and, finally, we outline the role of cytogenetics in the next-generation sequencing (NGS) era. All these aspects combined are

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important contributions of cytogenetics to the environmentally friendly control of pest insects, to the improvement of crop production, and to the free worldwide trade of crops.

Ceratitis capitata as a model for Tephritidae: historic background and progress of medfly genetics and cytogenetics

Ceratitis capitata is one of the most destructive pests of fruits and vegetables worldwide. It attacks fruits, flowers, vegetables, and nuts of more than 260 species (Liquido et al., 1991). It has a high dispersive ability, a very large host range, and a tolerance of both natural and cultivated habitats over a comparatively wide temperature range (White & Elson-Harris, 1992). For these reasons, it has successfully established in many parts of the world (White & Elson-Harris, 1992; Bonizzoni et al., 2001, 2004). It has a high economic impact, negatively affecting production, increasing control costs, and restricting market access.

In the first 70 years of the 20th century, insect control methods were based mainly on synthetic insecticides. Since its foundation in 1964 and the establishment of the Insect Pest Control Subprogramme, the Joint FAO/IAEA Division has been involved in the development and application of SIT for the population control of *C. capitata* and other pest insect species. SIT involves the laboratory rearing of the pest target, its sterilization (usually through irradiation), and the release of sterile insects in the field (Dyck et al., 2005). This leads to infertile crosses and, through the continuous release of overflooding numbers of sterile insects, to population suppression (Dyck et al., 2005). By using sterile bisexual releases, *C. capitata* was eradicated in Mexico, Chile, and parts of Guatemala and Argentina, plus some small areas in the USA (FAO/IAEA, 1990; Enkerlin, 2005). During these efforts, it was recognized that the cost-effectiveness and the efficiency in action could be significantly improved by releasing only males. This could be achieved through the development of genetic sexing methods (Robinson & van Heemert, 1982; Robinson et al., 1986, 1999; Franz, 2005). Within this frame, in 1983, the Joint Division initiated activities to develop GSSs through a Coordinated Research Project (CRP). At the beginning of this initiative, very little was known about the genetics and cytogenetics of the *C. capitata*. The mitotic karyotype (2n = 12) and the chromosomal organization of the species had been published few years before (Radu et al., 1975). In this first study, mitotic chromosomes were numbered from 1 to 6, where 1 stands for the sex chromosomes and 2–6 represent the five pairs of autosomes, in descending order of length. This nomenclature was followed by other researchers from there on (Zacharopoulou, 1990) (Figure 1A). Moreover, genetics of some morphological mutants was available (Rössler & Kotlin, 1976; Rössler, 1979a) and the construction of the first GSS was reported (Rössler, 1979b; Robinson & van Heemert, 1982). Finally, genetic markers for the autosomal chromosomes of *C. capitata* were also described (Saul & Rossler, 1984). Several research teams collaborated for more than 5 years, and at the end of this CRP (1988), many of the required genetic tools such as mutations, polytene chromosomes, and chromosomal rearrangements, including both Y–autosome (Y;A) and autosome–autosome (A;A) translocations, were available. These results clearly showed that development of GSSs was a feasible goal (FAO/IAEA, 1990).

The contribution of genetics and cytogenetics in the improvement and the evaluation of GSSs in *Ceratitis capitata*

Polytene chromosomes have been an excellent tool for the genetic analysis of Diptera insects. Especially in *Drosophila* and mosquito species, they have been used for almost a century for the analysis of chromosomal rearrangements, understanding organization and evolution of chromosomes, mapping of traits of interest, and numerous other applications (Ashburner et al., 1982; Coluzzi, 1982; Ashburner, 1989a; Ayala & Coluzzi, 2005). Starting from 1986, polytene chromosomes of *C. capitata* were reported for two tissues, the trichogen cells from male pupae (Bedo, 1986, 1987) and the salivary glands of the third instars (Zacharopoulou, 1986, 1987, 1990). However, a comparative analysis showed that their banding patterns were different to such a degree that even the tips could not be matched. As this phenomenon was not common among Diptera, a parallel analysis of polytene chromosomes from male pupae trichogen cells and larval salivary glands of an available *C. capitata* Y;A translocation strain (Rössler, 1979b) was done (Bedo & Zacharopoulou, 1988). This analysis confirmed the banding pattern differences between the two tissues of the species. In addition, considerable differences were found in the structure that represents the sex chromosomes. In trichogen cells, the X chromosome is found as an association of condensed large granules, whereas the Y chromosome appears as a black spherical body. Both sex chromosomes are located near, or associated with, the nucleolus. In salivary gland nuclei, the X chromosome is represented by scattered groups of small granules but the Y chromosome cannot be observed in polytene chromosome slides prepared through routine protocols (Zacharopoulou, 1990; Zacharopoulou et al., 1992; Rosetto et al., 2000). The different banding patterns of polytene chromosomes in the two tissues may be attributed to the differential gene activity at different stages of development (Zacharopoulou, 1990; Zacharopoulou

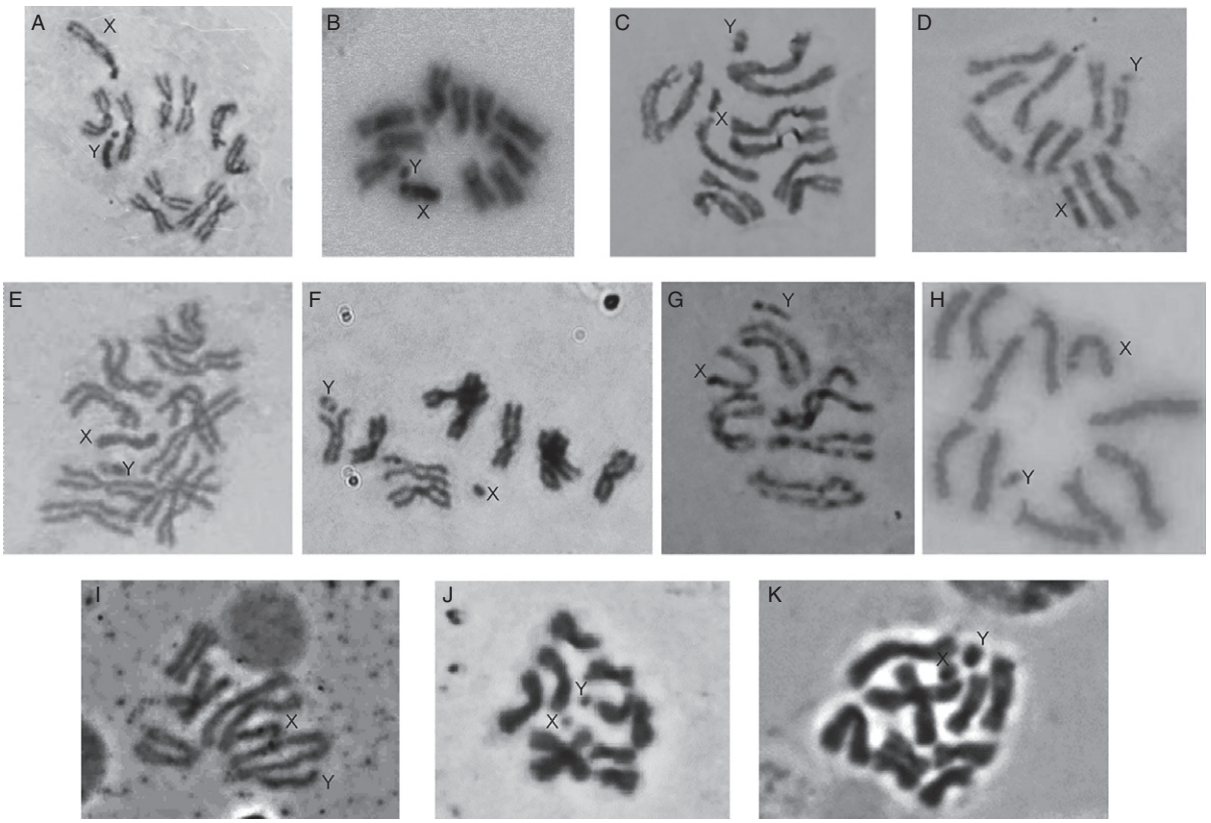

Figure 1 Male mitotic karyotype of Tephritidae species. X and Y chromosomes are indicated. (A) *Ceratitis capitata*, (B) *Bactrocera oleae*, (C) *B. tryoni*, (D) *B. dorsalis*, (E) *Zeugodacus cucurbitae*, (F) *Dacus ciliatus*, (G) *Anastrepha fraterculus*, (H) *A. ludens*, (I) *Rhagoletis cerasi*, (J) *R. completa*, and (K) *R. cingulata*. Mitotic karyotypes presented here are either new photographs (B, C, F, I, J, K) or previously published in (A) Mavragani-Tsipidou et al. (2014), (D) Zacharopoulou et al. (2011a), (E) Zacharopoulou et al. (2011b), (G) Gariou-Papalexiou et al. (2016), and (H) Garcia-Martinez et al. (2009).

et al., 1991a,b). Furthermore, the polytene chromosomes derived from salivary glands exhibit extensive ectopic pairing and breakage, contrary to the chromosomes of the trichogen cells. A comparative electron microscope analysis indicated that the polytene chromosomes of orbital bristle cells present a lower degree of polytenization compared to that of larval salivary glands (Semeshin et al., 1995). This is consistent with the increased thickness of bands and the absence of weak points and breakage of the chromosomes in trichogen cells, which could be attributed to the intercalary heterochromatin (Zhimulev et al., 1982). These differences have some practical consequences. As the Y chromosome is not visible in salivary glands in routinely prepared slides, only the trichogen cells can be used for the accurate mapping of the Y;A translocations that are used in the construction of the GSSs.

The availability of polytene chromosomes in *C. capitata* as well as of several Y;A and A;A translocations has given the opportunity to align the mitotic complement with the polytene chromosomes and with the genetic linkage groups by the cytogenetic analysis of these strains (Zacharopoulou, 1990). After the correlation of polytene and mitotic chromosomes and following the numbering system established for mitotic chromosomes (Radu et al., 1975), the polytene elements were numbered 2–6 (Zacharopoulou, 1990). Moreover, the analysis of autosomal breakpoints in several translocation strains allowed the alignment of polytene chromosome maps from salivary gland and trichogen cells (Kerremans et al., 1990; Zacharopoulou et al., 1991a,b). In addition, detailed comparative analysis was achieved by in situ hybridization with several cloned DNA sequences available for the *C. capitata* (Zacharopoulou et al., 1992; Kritikou, 1997; Gariou-Papalexiou et al., 2002) (Tables 1 and 2). These studies clearly indicated that the five polytene elements found in the polytene complement correspond to the five autosomes of the mitotic ones. The sex chromosomes are largely heterochromatic and thus underreplicated in polytene tissues (Bedo, 1986; Bedo & Webb, 1989; Zacharopoulou, 1990; Zacharopoulou et al., 1992; Rosetto et al., 2000).

Table 1 Availability of in situ hybridization data on polytene chromosomes for the various tephritid species. Numbers and types of markers and the literature sources are indicated

		Type of markers			
Genus	Species	Genes	Anonymous clones	Microsatellites	ESTs
Ceratitis	*capitata*	20[1]	17[2]	44[3]	–
Bactrocera	*oleae*	18[4]	2[5]	13[6]	35[7]
	dorsalis	6[8]	–	–	–
	tryoni	5[9]	–	6[10]	–
Dacus	*ciliatus*	5[11]	–	–	–
Rhagoletis	*cerasi*	1[12]	–	–	–

[1]Gariou-Papalexiou et al. (2002), Zacharopoulou et al. (1992); [2]Zacharopoulou et al. (1992); [3]Stratikopoulos et al. (2008, 2009); [4]Zambetaki et al. (1999, 2000); Kakani et al. (2012); Drosopoulou et al. (2014); [5]Zambetaki et al. (1999); [6]Augustinos et al. (2008); [7]Tsoumani et al. (2011); [8]Augustinos et al. (2014); [9]Zhao et al. (1998); [10]Zhao et al. (2003); [11]Drosopoulou et al. (2011a,b); [12]Present study.

Genetics and cytogenetics have also helped significantly in unraveling sex determination in *C. capitata*. Early studies using chromosome aneuploids, Y;A translocations, and Y chromosome length polymorphisms indicated that, unlike *Drosophila*, the Y and not the X/autosome ratio is responsible for the male sex determination (discussed in Zapater & Robinson, 1986; Lifschitz & Cladera, 1989). The cytogenetic analysis of naturally occurring mitotic karyotypes harboring Y polymorphisms (smaller Y chromosome) pointed to the fact that only a part of the Y chromosome is required to determine the male sex (Lifschitz & Cladera, 1989). The hypothesis that a relatively small portion of this chromosome, named the Maleness factor, is sufficient to determine the male sex was verified with the availability and analysis of aneuploid viable offspring of several Y;A translocation strains through in situ hybridization (FISH) with two probes on mitotic spreads (Willhoeft & Franz, 1996). One probe was a Y-specific *C. capitata* clone containing repetitive DNA isolated by Anleitner & Haymer (1992), and the second probe contained a part of the ribosomal DNA from *Drosophila hydei* Sturtevant. In this way, it was possible to determine the length of the Y fragment present in aberrant males, resulting in the mapping of the male-determining region (the Maleness factor) at the proximal part of the long arm representing about 15% of the entire Y chromosome (Willhoeft & Franz, 1996).

From these studies, it became evident that a reciprocal translocation between the Y chromosome and the autosome carrying the dominant wild-type allele of a recessive selectable marker would link the wild-type phenotype to the male sex. In this case, the males are heterozygous (wild type) and the females are homozygous (mutant) for the marker. For the construction of such GSSs, several selectable markers (visible mutations) were evaluated, such as the dark pupae (*dp*), niger (*nig*), or the slow development (*sw*) mutation that could be used for the self-separation of male and female pupae (Rössler, 1979b; Cladera & Delprat, 1995; Cladera et al., 2002; Delprat et al., 2002). However, none of them was used in SIT applications. The strains currently used in SIT applications are based on Y;A translocations involving the fifth autosome (Kerremans et al., 1990, 1992; Kerremans & Franz, 1994; Franz, 2005). The first generation of these GSSs that was used in operational SIT programs was based on the selectable marker white pupae (*wp*) located on the right arm of this autosome (Robinson & van Heemert, 1982). However, this sex separation method was neither accurate nor cost-effective enough for large operational programs. A second mutation, a temperature-sensitive lethal (*tsl*) linked to *wp*, enabled the construction of the second generation of GSSs. The combination of these two markers allows the early, easy, and reliable separation of males and females (Franz, 2005). In these GSSs, males emerge from brown pupae and are resistant to elevated temperatures, whereas females emerge from white pupae and are killed at early embryonic stages after a treatment with elevated temperatures.

During these efforts, numerous Y;A translocations were induced through radiation and more than 30 of them were analyzed in detail (Kerremans et al., 1990, 1992; Franz et al., 1994; Kerremans & Franz, 1994, 1995; Franz, 2005) (Figure 2). Using the aforementioned strains and classical genetic approaches, such as transpositions and deletion mapping, six mutations – including *wp* and *tsl* – were cytologically mapped and enabled the construction of an integrated cytogenetic map (Kerremans & Franz, 1994), incorporating the available genetic linkage map for this chromosome (Rössler & Rosenthal, 1992). Based on these analyses and on a thorough evaluation in semi-scale mass rearing in the Insect Pest Control Laboratory (IPCL),

Table 2 In situ localization of unique genes that facilitated the correspondence of Tephritidae polytene elements with each other and with *Drosophila melanogaster* Muller elements

Gene	Element	*D. melanogaster*	*Ceratitis capitata*	*Bactrocera oleae*	*B. tryoni*	*B. dorsalis*	*Dacus ciliatus*	*Rhagoletis cerasi*
$PS_2\alpha$	A	15A1 (X)	64B (5L)	12 (IL)	68C (5L)			
w		3C2 (X)	65C (5L)	13 (IL)	71B (5L)			
Pgd		2D (X)	68B (5L)					
S_{36}, S_{38}		7F1-2 (X)	70B (5L)	10 (IL)				
V_{g1}, V_{g2}		8E-9B (X)	72A (5L)					
ovo		4E (X)		2 (IL)		63 (5L)		
*CkII*β		10E (X)		3 (IL)				
Sxl		6F4-7B3 (X)	79B (5R)	20 (IR)		78 (5R)		
Zw		18D12-13 (X)	79C (5R)					
Hsp70	B	–	24C (3L)	96 (VR)	28B (3L)	26 (3L)	96 (VR)	81 (IVR)
Adh		35B3 (2L)	–					
Sod_2	C	53D (2R)	42B (4L)					
DHR3		46F (2R)	44B (4L)					
EcR		42A (2R)	53B (4R)					
e2			57-58 (4R)	61 (IIIR)				
e3			59 (4R)	65 (IIIR)				
Hsp23, *26*, *27*	D	67B (3L)	81A (6L)	71 (IVL)				
scarlet		73A3-4 (3L)	83A (6L)	78 (IVL)	82A (6L)	82 (6L)		
tra		73A (3L)		71 (IVL)		86 (6L)		
$S_{15, 16, 18, 19}$		66D (3L)	88B (6L)					
prm		66D (3L)	88B (6L)	74 (IVL)				
Hsp83		63B-C (3L)	94C (6R)	68 (IVL)				
Sod_1		68A8-9 (3L)	99A (6R)	86 (IVR)	97B (6R)			
$Adh_{1,2}$ Adh_2	E	–	2C-3A[1] (2L)					
Gld		84D (3R)	4C (2L)			6 (2L)		
Ache		87E (3R)	11C (2L)	34 (IIL)				
dsx		84E (3R)		44 (IIR)				
β-*tub*		97E (3R)	18A (2R)	38 (IIR)				
Hsp70		87A;87C (3R)	–					

[1]Deletion mapping (Zacharopoulou et al., 1991b; Malacrida et al., 1992).

initially one strain, VIENNA 4, with improved stability was selected for a large-scale evaluation in an active SIT program (Petapa, Guatemala).

The structure of the Y;A translocation affects the productivity (through parameters such as egg hatch and adult emergence) and the stability of the GSS. Primarily two genetic mechanisms are of importance: the segregation behavior during male meiosis and the recombination in males. The presence of a Y;A translocation in GSS males leads to adjacent-1 segregation during male meiosis and, therefore, to offspring with either deletions or triplications of parts of the genome. The location and size of these aberrations are determined by the position of the translocation breakpoint on the autosome, whereas the sex of the genetically unbalanced offspring resulting from the mating with a normal female is determined by the Y-chromosomal breakpoint (Willhoeft & Franz, 1996). Unless the aberration is very small, the genetically unbalanced offspring show a reduced viability at various stages of development, thereby reducing the productivity of a strain. The ratio of alternate to adjacent-1 segregation determines what proportion of the offspring is genetically unbalanced, whereas the type (deletion or triplication) and the length of the aberration in combination with the sex of the resulting unbalanced offspring determines to what extent these flies are viable and to which stage of development they can survive. This has not only important consequences for the overall productivity of a strain but also affects its mass production characteristics (quality control parameters) such as percentages of egg hatch, larval survival and adult emergence, percentage of deformed flies, flight ability, and even mating performance.

The stability of a sexing system is threatened either by homologous recombination between the translocated and

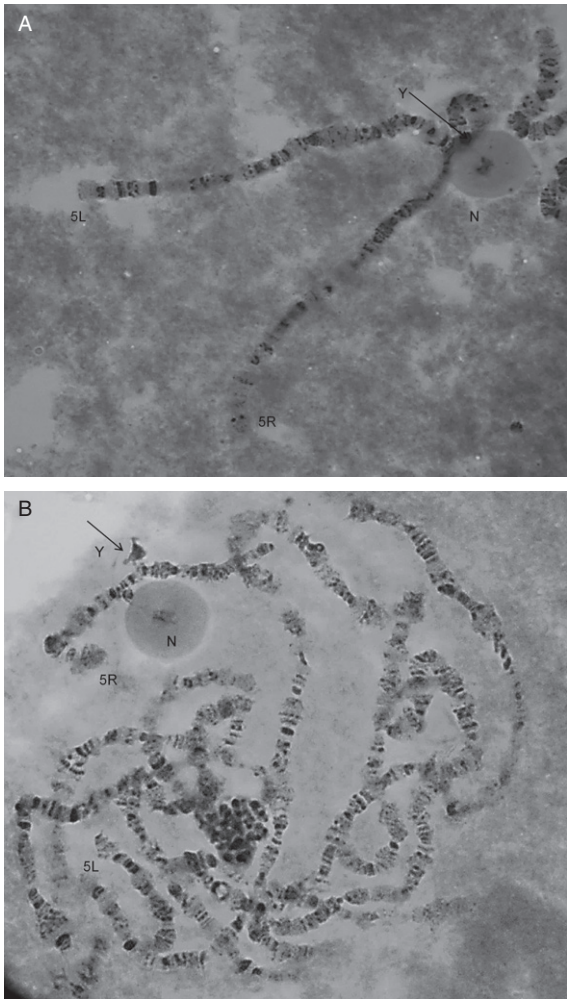

Figure 2 Polytene chromosomes of trichogen cells of *Ceratitis capitata* GSSs. The two arms of chromosome 5 are indicated (5L, 5R). The autosomal breakpoint can be connected with the Y chromosome (arrow), seen as the dense black sphere in Figure 2A, or with the nucleolus (N) in Figure 2B. (A) Strain Vienna 8 without the D53 inversion, T(Y;5)52A. (B) Strain Vienna 7 without the D53 inversion, T(Y;5)58B. Figure 2B has been published before in Mavragani-Tsipidou et al. (2014). Both photos are also included in Augustinos et al. (this issue).

the free autosome (type 1) or by recombination between the two translocated Y fragments (type 2) (Franz, 2002, 2005). Autosomal recombination is of relevance if the exchange takes place in the region between the autosomal breakpoint and the location of the selectable marker(s). Although recombination in males occurs at very low frequencies (Cladera, 1981; Rössler & Rosenthal, 1992; Franz, 2002, 2005), the resulting wild-type female recombinants pose a threat to a mass-rearing colony because they have a selective advantage over their mutant sisters and, therefore, accumulate in the colony leading to gradual breakdown of the sexing system because more and more females cannot be separated from the males and would have to be released with them. To generate GSSs that are stable even under the harsh conditions of mass rearing, two genetic strategies have been used: Y;A translocations were selected where the autosomal breakpoint is as close as possible to the selectable marker(s) thereby reducing the male recombination frequency, or chromosomal inversions were added on the autosome carrying the selectable marker(s) rendering the recombinants inviable.

Following a large screen based on the reduced recombination on chromosome 5, a pericentric homozygous viable inversion (D53) was detected. Recombination tests with several markers of chromosome 5 showed that this inversion has a strong recombination-reducing effect. This inversion was combined with the translocation T(Y;5) 101, and a new strain was developed, named VIENNA 8^{D53+}. Currently, either the VIENNA 7^{D53} (in few mass-rearing facilities) or the VIENNA 8^{D53+} (in more facilities) are used in SIT programs (Franz, 2005; Augustinos et al., 2017; IAEA-NAIPC, 2017) (Figure 2).

The construction of the first balancer chromosome for chromosome 5 of *Ceratitis capitata*

Balancer chromosomes in *Drosophila* were introduced to genetics by HJ Muller for maintaining lethal mutations, to avoid any selection. For this reason, he introduced an inversion in one of the chromosomes, as a crossover suppressor (Muller, 1918; Altenburg & Muller, 1920). He also marked the balancer chromosome with a visible dominant mutation for an easy identification of heterozygous flies. Following that, balancer strains were constructed for all *Drosophila* chromosomes, contributing to the establishment of *Drosophila melanogaster* Meigen as a model organism for all Diptera.

In *C. capitata*, several efforts were made to induce inversions, but contrary to the frequent recovery of translocations (Kerremans et al., 1992; Gourzi, 1996), none had been reported until 1997 (Busch-Petersen & Southern, 1987; Gourzi, 1996). Inversions are very common in natural populations of *Drosophila* (Krimbas & Powell, 1992) but not in *C. capitata* where analysis of natural populations failed to reveal any. These results showed that a translocation-based balancer might be a more feasible target in *C. capitata*. In fact, during the first attempts, a marked translocation strain was recovered *T(3;5)Sb*, *y* (Gubb et al., 1998). However, translocation strains are not ideal, as adjacent-1 segregation and recombination events may give aneuploidy progeny, thus reducing the fertility of the strain. For this reason, the construction of an inversion-based balancer chromosome was pursued. Following

several efforts, the first balancer for the fifth chromosome, *FiM1* (fifth inversion multiple one), was constructed (Gourzi et al., 2000). It carries three overlapping pericentric inversions induced during three successive irradiation experiments followed by genetic screens for crossover suppression in single-pair crosses. In all these irradiation experiments, several translocations were also recovered. *FiM1* was marked by the dominant mutation Sr^2, which is homozygous lethal (Gourzi, 2000). Several screens to isolate recessive lethal or visible mutations on chromosome 5 following an ethyl methanesulfonate (EMS) treatment were undertaken to assess the usefulness of the strain. These experiments showed that the *FiM1* strain is a suitable balancer for the recovery of recessive lethal alleles as well as visible mutations on chromosome 5 (Gourzi, 2000; Rapti, 2000).

Polytene chromosome maps in *C. capitata* contributed to the development of this species as a model organism in the Tephritidae family. In addition to their important role in the construction of stable GSSs, the first in situ hybridization study of polytene chromosome of the species was performed with nine characterized transcription units and 17 anonymous molecular probes. These first results supported the homology of the chromosome arm 5L with the X chromosome of *Drosophila* (Table 2). Six of the 10 polytene arms were marked with molecular probes, and a repetitive element was identified at multiple sites in banded autosomes and the heterochromatic X chromosome (Zacharopoulou et al., 1992). By additional in situ hybridization experiments, a remarkable conservation of linkage groups between *Drosophila* and *C. capitata* was identified (reviewed in Gariou-Papalexiou et al., 2002) (Table 2), confirming the earlier comparative studies using biochemical and morphological markers (Malacrida et al., 1986). However, despite the advances in the genetics and population genetics of the species, the availability of molecular markers was still very limited. A significant number of microsatellite clones for the species (Bonizzoni et al., 2000; Stratikopoulos et al., 2008, 2009) was isolated and used to enrich the cytogenetic maps through in situ hybridization (FISH) (Stratikopoulos et al., 2008, 2009) (Table 1). This integrated cytogenetic map recently allowed the initiation of the physical genome mapping in *C. capitata* by assigning 43 scaffolds linked to genes and microsatellite sequences distributed on the five autosomes 2–6 (Papanicolaou et al., 2016). Three scaffolds seem to be Y-linked by in situ hybridization on the mitotic chromosomes (Anleitner & Haymer, 1992). Moreover, the *ceratotoxin* genes previously mapped on the X chromosome by in situ hybridization on mitotic chromosomes as well as to the heterochromatic network in polytene nuclei (Rosetto et al., 2000) were linked to a single scaffold, representing the X chromosome (Papanicolaou et al., 2016).

Cytogenetic status of the other tephritids

Although cytogenetics in other tephritids is not as advanced as in *C. capitata*, there are some species with remarkable progress. The olive fruit fly, *Bactrocera oleae* (Rossi), can be considered the second best-studied species of this group. Currently, there is cytogenetic knowledge for six genera – *Ceratitis*, *Bactrocera*, *Zeugodacus*, *Dacus*, *Anastrepha*, and *Rhagoletis* – taking into account the recent taxonomic revision suggesting that the *zeugodacus* subgenus of *Bactrocera* should be elevated to genus level (*Zeugodacus*) (De Meyer et al., 2015b; Virgilio et al., 2015).

Bactrocera species

Bactrocera oleae, the only representative of the genus found in Europe and the only member of the *B. daculus* subgenus studied so far, is the major pest of the olive fruit, causing extremely high annual losses in the olive crops. Its great economic importance for the olive-producing countries and its recent expansion in new olive tree cultivation areas (http://www.cabi.org/isc/datasheet/17689) raised considerable interest in its biology, genetics, and cytogenetics toward the development of SIT applications.

Early cytogenetic reports described the mitotic karyotype of *B. oleae* (Frizzi & Springhetti, 1953; Krimbas, 1963), whereas a more detailed analysis including polytene chromosome maps from three tissues – fat body, salivary glands, and Malpighian tubules – was performed in the 1990s (Mavragani-Tsipidou et al., 1992; Zambetaki et al., 1995). The mitotic complement of *B. oleae* consists of six pairs of chromosomes (2n = 12) (Figure 1B) that are labeled following the *C. capitata* labeling system (Zacharopoulou, 1990). The polytene complement consists of five long, well-banded chromosomes (10 polytene arms) labeled from I to V in order of descending size. This labeling system has no correlation with the mitotic chromosomes. Comparison of the polytene complement among the three somatic tissues revealed good correspondence of the banding pattern and the centromeric heterochromatic mass of each chromosome element as well as similarities in the ectoping pairing of the chromosome ends (Zambetaki et al., 1995). Like *C. capitata*, no inversions have been observed, neither in the laboratory nor in the natural populations of *B. oleae* tested.

Based on the lack of differences in the number of the polytene elements between the two sexes, it was proposed that the sex chromosomes do not form polytene elements,

but a heterochromatic network, as in *C. capitata*. Later, chromosome painting FISH experiments proved that indeed the X chromosomes form a heterochromatic granular network spread among the polytene arms, whereas the Y chromosome is restricted to a small compact body close to the heterochromatic network (Drosopoulou et al., 2012), resembling the black spherical body formed by the Y chromosome in the *C. capitata* pupal trichogen cells (Bedo, 1986, 1987).

The availability of the *B. oleae* polytene chromosome maps enabled the in situ localization of a considerable number of molecular markers (Table 1). More specifically, 18 gene sequences (Zambetaki et al., 1999, 2000; Kakani et al., 2012; Drosopoulou et al., 2014), two anonymous genomic clones (Zambetaki et al., 1999), 13 microsatellites (Augustinos et al., 2008), and 35 ESTs (Tsoumani et al., 2011) have provided molecular markers for all *B. oleae* chromosomes (nine out of 10 chromosome arms). Based on the chromosome distribution pattern of the above sequences, the correspondence of the polytene arms and the significant synteny conservation between *B. oleae* and *C. capitata*, *B. tryoni* (Froggatt), and *D. melanogaster* have been revealed (Zambetaki et al., 1999, 2000; Tsoumani et al., 2011; Drosopoulou et al., 2014) (Tables 2 and 3). Furthermore, the localization of one mini-satellite repeat on the centromeres of chromosomes 4 and 5 of the mitotic complement and III and IV of the polytene complement enabled the first correlation between two autosomes of the mitotic and polytene complements in *B. oleae* (Tsoumani et al., 2013).

The Queensland fruit fly, *B. tryoni*, is a serious pest in eastern Australia, causing significant damages in agricultural crops each year (Sutherst et al., 2000). In contrast to extensive studies related to physiology and ecology (Bateman, 1972; Meats, 1989), genetic and molecular studies in this species were limited until the late 1990s. Zhao et al. (1998) presented the mitotic karyotype (Figure 1C) and the polytene chromosome maps of this species as well as the first comparative studies with *C. capitata* and *B. oleae* polytene chromosome maps. Significant similarities but also differences in banding patterns were found between *C. capitata* and the two *Bactrocera* species. One pericentric inversion relative to *C. capitata* was identified in both *Bactrocera* species, on chromosome 5 (Zhao et al., 1998). Moreover, in situ hybridization of molecular markers on polytene chromosomes, previously used for *C. capitata* (Zacharopoulou et al., 1992), was performed on *B. tryoni* polytene chromosomes, and their location supported the chromosomal homology between the two species at the molecular level. The localization of markers on *B. tryoni*'s chromosomes 3L and 5L, in respect to the homologous *C. capitata* chromosomes, confirmed the chromosome rearrangements in *B. tryoni* chromosomes in relation to *C. capitata* (Zhao et al., 1998). Later, the establishment of the five linkage groups of the species was achieved using visible and microsatellite markers (Zhao et al., 2003).

The oriental fruit fly, *Bactrocera dorsalis* (Hendel), is among the most destructive pests of agriculture (Clarke et al., 2005). It is a polyphagous species attacking fruits and vegetables of more than 170 species of significant economic importance. The species is widespread in various countries of South-East Asia and the Pacific regions (White & Elson-Harris, 1992). It was accidentally introduced into Hawaii (USA) during 1944–1945 (Mau, 2007). Several infestations have been also reported in California, USA, but these were eradicated.

Bactrocera dorsalis is considered as a large species complex, including more than 90 species which are difficult to distinguish based on morphological characteristics (Drew & Hancock, 1994; Drew & Romig, 2013; Boykin et al., 2014). Hybridization between its members has been reported based on laboratory studies (McInnis et al., 1999;

Table 3 Polytene chromosome element correspondence within Tephritidae and with the *Drosophila* Muller elements

Genus	Species	Polytene elements				
Ceratitis	*capitata*	2(L/R)	3(L/R)	4(L/R)	5(L/R)	6(L/R)
Bactrocera	*oleae*	II(L/R)	V(R/L)	III(L/R)	I(L/R)	IV(L/R)
	dorsalis	2(L/R)	3(L/R)	4(L/R)	5(L/R)	6(L/R)
	tryoni	2(L/R)	3(L/R)	4(L/R)	5(L/R)	6(L/R)
Zeugodacus	*cucurbitae*	2(L/R)	3(L/R)	4(L/R)	5(L/R)	6(L/R)
Dacus	*ciliatus*	II(L/R)	V(R/L)	III(L/R)	I(L/R)	IV(L/R)
Anastrepha	*ludens*	III	VI	II	IV	V
	fraterculus	III	VI	II	IV	V
Rhagoletis	*cerasi*	I	IV	II	III	V
	completa	IV	V	II	I	III
	cingulata	IV	V	II	I	III
Drosophila	*melanogaster*	E(3R)	B(2L)	C(2R)	A(X)	D(3L)

Tan, 2000) or in nature (Wee & Tan, 2005), making species delimitation a difficult task.

At the cytological level, mitotic chromosomes were used to distinguish entities within the complex (Hunwattanakul & Baimai, 1994; Baimai et al., 1995, 1996, 1999a,b, 2000). Moreover, several morphological recessive mutations were described for the species belonging to five linkage groups (McCombs & Saul, 1992). More recently, a wild-type strain from Thailand (Institute of Radiation for Agriculture Development) and a Y;A translocation strain (McCombs & Saul, 1995) maintained at IPCL were used for mitotic and polytene chromosomes analysis (Zacharopoulou et al., 2011a). The mitotic karyotype referred to as form A (Baimai et al., 1995) represents the ancestral form of *B. dorsalis* complex species. It consists of five pairs of autosomes and a pair of sex chromosomes. The autosomes are differentiated by size and arm ratio and are labeled from II to VI in descending order of their size (Figure 1D). Sex chromosomes (pair I) are the smallest of the set; the X is longer than Y, which is a dot-like. Both sex chromosomes seem to be mostly heterochromatic with an exception of a part of X chromosome that is lighter and shares characteristics of both heterochromatin and euchromatin as indicated by the degree of staining and chromatid separation compared to autosomes.

The polytene complement of *B. dorsalis* sensu stricto consists of five banded elements that correspond to the five autosomes of the mitotic karyotype. The mostly heterochromatic sex chromosomes are not represented in polytene nuclei, consistent with the previously analyzed tephritids. An interesting finding during the analysis of this species was the detection of several polymorphic inversions contrary to previously analyzed species. These rearrangements seem to be concentrated on two chromosome arms (2R and 4L). However, we cannot exclude that such rearrangements occur in other chromosomes as well.

Comparative analysis of *B. dorsalis* s.s. polytene chromosomes with *C. capitata* revealed significant similarities, sufficient for the establishment of their homology, as well as differences. These differences were restricted mainly on two chromosome arms 3L and 5L. Moreover, the pericentric inversions observed in *B. tryoni* chromosome 5 relative to *C. capitata* were also found in *B. dorsalis* (Zacharopoulou et al., 2011a).

Zeugodacus species

Zeugodacus cucurbitae (Coquillett) is a species with a long history regarding its classification within the family Tephritidae. A recent review describes all the available information regarding the species, and the new generic classification of the species is proposed (De Meyer et al., 2015b; Virgilio et al., 2015). The species is a major agricultural pest in many Asian and Oceanian countries (Drew & Romig, 2013; De Meyer et al., 2015b). It was also introduced into Africa and several islands in the western Indian Ocean. The species is of significant agricultural importance, attacking more than 120 plant species, and it is a major pest of Cucurbitaceae and Solanaceae. Despite its wide distribution and importance as an agricultural pest, knowledge of the genetics of the species is still limited. Fifteen morphological markers have been described and assigned to five autosomal linkage groups (McCombs et al., 1996). A cytogenetic analysis of the species was undertaken (Zacharopoulou et al., 2011b) using two laboratory colonies: a wild-type strain from Bangladesh and a GSS constructed by McInnis et al. (2004) using a wild-type strain from Hawaii, USA. The mitotic karyotype consists of six pairs of chromosomes including one pair of heteromorphic XX/XY sex chromosomes (Figure 1E). Like all analyzed tephritids, five polytene elements, corresponding to the five autosomes, were found and no typical chromocenter was found. Comparative analysis with *C. capitata* polytene chromosomes revealed significant banding pattern similarities that supported the proposed homology of the chromosomes in the two species. This analysis points to the presence of intrachromosomal rearrangements, mainly inversions, and transpositions between the two species. One pericentric inversion in chromosome 5, previously described for *Bactrocera* species, is also present in *Z. cucurbitae*, suggesting that the fixation of this inversion occurred before the divergence of *Zeugodacus* and *Bactrocera*.

Dacus species

The Ethiopian fruit fly, *Dacus ciliatus* Loew, is the only representative of the *Dacus* genus, for which cytogenetic information is available. It is a species of economic importance in several countries of sub-Saharan Africa and the Middle East, attacking mainly plants of Cucurbitaceae (White & Elson-Harris, 1992; Kapoor, 1993). Its mitotic karyotype consists of six pairs, including one pair of small heteromorphic sex chromosomes (Figure 1F). Its polytene complement shows a total number of five long chromosomes (10 polytene arms) (Drosopoulou et al., 2011a). Chromosome homologies between *D. ciliatus* and *B. oleae* polytene complements based on the banding pattern similarities and the localization of the *hsp70* gene have been proposed (Drosopoulou et al., 2011a). A recent comparison of its polytene complement to those of *Z. cucurbitae* showed extensive similarities among them and common features that differentiate them from *Bactrocera* species (Augustinos et al., 2015). The availability of a reference polytene chromosome map along with the documented application of in situ hybridization in this species is expected to facilitate future genome projects as well as

efforts for developing and characterization of GSSs toward the support of SIT control methods.

Anastrepha species

The genus *Anastrepha* includes about 200 described species of frugivorous fruit flies which are restricted to the American continent, distributed in 17 intrageneric groups. The *A. fraterculus* group includes 29 species and most of them occur in Brazil (White & Elson-Harris, 1992; Norrbom et al., 1999). Currently, the nominal *Anastrepha fraterculus* (Wiedemann) is considered as a complex species and seven members have been described based on multidisciplinary approaches (Hernández-Ortiz et al., 2012, 2015). The karyotypes of the seven entities show the same number of chromosomes but can be distinguished by the size of sex chromosomes (Goday et al., 2006; Hernández-Ortiz et al., 2012) (Figure 1G). All members of the *A. fraterculus* intrageneric group are characterized by acrocentric chromosomes (Selivon et al., 2005; Goday et al., 2006; Cáceres et al., 2009). However, out of this group, there are *Anastrepha* species with (1) total chromosome number 2n = 12 with submetacentric or a combination of submetacentric and rod-shaped chromosomes and (2) different number of total chromosomes such as *Anastrepha pickeli* Lima with 2n = 8 (XX/XY), *Anastrepha leptozona* Hendel with 2n = 10 (XX/XY) or different numbers of sex chromosomes, such as *Anastrepha bistrigata* Bezzi and *Anastrepha serpentina* (Wiedemann) with a karyotype of 2n = 11 for males and 2n = 12 for females ($X_1X_2Y/X_1X_2X_1X_2$) (Selivon et al., 2005, 2007).

Photographic maps of the polytene chromosomes have been presented for *Anastrepha ludens* (Loew) (Garcia-Martinez et al., 2009) and an Argentinian population of *A. fraterculus* spec. 1 (Gariou-Papalexiou et al., 2016). The polytene complements of the above species consist of five long-banded chromosomes representing the five acrocentric autosomes of the metaphase karyotype. Like all tephritids, the sex chromosomes are visible as a granular heterochromatic network. Comparative analysis of the banding pattern between the two *Anastrepha* species, as well as between *A. ludens* and *C. capitata*, allowed the establishment of chromosomal homologies among the above species (Garcia-Martinez et al., 2009; Gariou-Papalexiou et al., 2016). The comparison between the two *Anastrepha* species, although difficult and still preliminary, revealed differences in the banding patterns for the chromosome VI including an inversion and a transposition.

The two *Anastrepha* species for which polytene chromosome maps are available have acrocentric chromosomes contrary to the metacentric or submetacentric chromosomes (Figure 1G and H) in the other tephritids analyzed so far. Despite this difference, polytene chromosome homologies can be established, mainly due to the conservation of chromosomal ends and specific chromosomal regions. The chromosome ends in all tephritids analyzed so far are similar, supporting the notion that the chromosome ends in all Diptera are highly conserved (Zdobnov et al., 2002).

Rhagoletis species

Cytogenetic information is available for several pest species from the genus *Rhagoletis*. Early studies were limited to mitotic chromosomes, presenting the typical Tephritidae karyotype (2n = 12) for most *Rhagoletis* species, although deviations were also reported (Bush, 1966; Bush & Boller, 1977; Frias, 2002). Procunier & Smith (1993) confirmed the mitotic karyotype (2n = 12) of *Rhagoletis pomonella* (Walsh) originally proposed (Bush, 1966) and presented a crude description of its polytene chromosomes together with the localization of the *rDNA* genes. More recently, the mitotic karyotypes and detailed photographic maps of the salivary gland polytene chromosomes of three *Rhagoletis* pests, namely the European cherry fruit fly, *Rhagoletis cerasi* (L.), the walnut husk fly, *Rhagoletis completa* Cresson, and the American cherry fruit fly, *Rhagoletis cingulata* (Loew), were presented (Kounatidis et al., 2008; Drosopoulou et al., 2010, 2011b). The mitotic complements of the above species present the typical number found in most tephritids (2n = 12) with the sex chromosomes being very similar in length which makes the discrimination of female and male karyotypes difficult (Figures 1I, J, and K). The polytene complement consists of 10 polytene chromosome arms similarly to all tephritids. An interesting characteristic of the polytene chromosomes of *R. cerasi* and *R. cingulata* is the high number of asynaptic regions routinely found at specific regions. This phenomenon was correlated with the presence of stable *Wolbachia* infections in the populations tested and possible gene transfer events between Wolbachia and host species (Kounatidis et al., 2008; Drosopoulou et al., 2011b).

Comparison of the banding pattern of the polytene chromosomes of *R. cerasi*, *R. cingulata*, and *R. completa* indicates extensive homology of certain polytene regions among the three species. The most apparent homologies are found between *R. cingulata* and *R. completa* suggesting a closer phylogenetic relationship which is also supported by the similarity of their mitotic karyotype and by molecular data (Smith & Bush, 1997; Drosopoulou et al., 2010, 2011b). Based on observed similarities, it is proposed that chromosome I of *R. cingulata* and *R. completa* is homologous to chromosome III of *R. cerasi* and that chromosomes II is homologous in all three *Rhagoletis* species (Drosopoulou et al., 2011b).

Rhagoletis show lower polytene chromosome banding quality, and this makes a direct comparison with the other tephritids more difficult. In situ hybridization of the *hsp70* gene on the polytene arm IVR of *R. cerasi* allows the alignment of this element with those of the other tephritids (Tables 2 and 3). Moreover, the conservation of chromosome tips also allows the correlation of additional polytene elements of *Rhagoletis* with those of other tephritids (Table 3). Gene mapping data are also needed to confirm the suggested correspondence and reveal further chromosome homologies among them.

Cytogenetic analysis of GSSs in *Bactrocera dorsalis*, *Zeugodacus cucurbitae*, and *Anastrepha ludens*

For several major insect pests, SIT is being applied as a key component of areawide IPM programs. Cytogenetic analysis is important to understand both the behavior and stability of GSSs in pest species. The analysis of GSSs in *C. capitata* is based on polytene chromosomes from trichogen cells of male pupae in which the Y chromosome is evident. *Ceratitis capitata*'s Y chromosome is quite large, as revealed in mitotic karyotypes (smaller but comparable with the autosomes), and this facilitated both the construction of Y;A translocation-based GSSs and their cytogenetic analysis. The fact that (1) in the other tephritids analyzed so far no polytene cells were found where the Y chromosome is visible like in the male trichogen cells of *Ceratitis* and (2) the Y chromosome is very small, even dot-like in some of them, suggests that in these species, both the construction of GSS and their analysis may not be as straightforward as in *C. capitata*.

Despite these drawbacks, two more GSSs based on white pupae mutations are available for other Tephritidae species. The first was constructed for *B. dorsalis* (McCombs & Saul, 1995) and the second for *Z. cucurbitae* (McInnis et al., 2004). Genetic analysis of both strains was performed based on the approach used for *C. capitata*. The egg-to-adult recovery rate for the *B. dorsalis* GSS was 47%, indicating that a simple Y;A translocation is present. On the other hand, the *Z. cucurbitae* GSS showed a very low egg-to-adult recovery rate of 22%, an indication that more than one autosome is involved. Cytogenetic analysis using mitotic and polytene chromosomes showed that both Y;A translocations involve the chromosome arm 5R (Figures 3 and 4). In *Z. cucurbitae*, a second translocation between chromosomes 2 and 5 was found, accounting for the lower egg hatch and adult emergence observed (Zacharopoulou & Franz, 2013) (Figure 4).

The first GSS for *Anastrepha* species was developed for *A. ludens*, based again on Y;A translocations, using the *black pupae* (*bp*) genetic marker. Several GSSs were isolated, most of them showing low egg hatch rates (Zepeda-Cisneros et al., 2014). The strain with the highest egg hatch was cytogenetically analyzed, using both mitotic and polytene chromosomes. The analysis of mitotic chromosomes showed that the Y;A translocation involves the longest autosome (Figure 5). Polytene chromosome analysis using male larvae revealed that polytene element III is involved in the translocation, based on the characteristic ectopic pairing between the telomeres. In polytene nuclei from samples without the Y;A translocation, the ectopic pairing of chromosomes III and V is complete. However, in the GSS analyzed, only one homolog of chromosome III is involved in this ectopic pairing. This suggests that chromosome III is the autosome involved in the Y;A translocation (Figure 5).

Evolution, speciation, and resolution of species complexes

Chromosomal rearrangements are considered important players in speciation. Especially in Diptera, earlier studies using polytene chromosomes in *Drosophila* and mosquitos

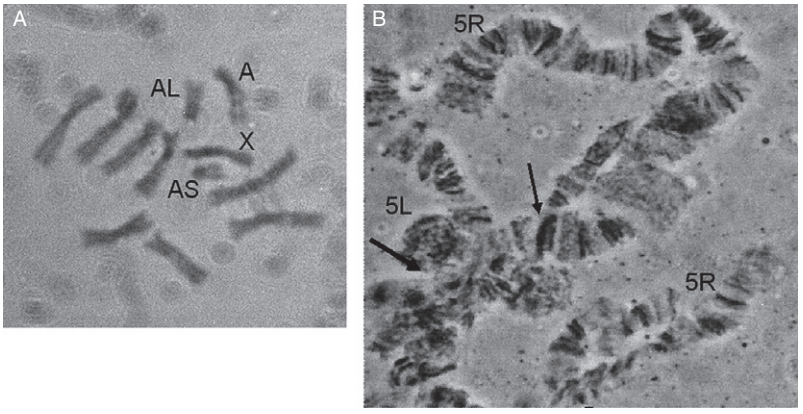

Figure 3 The *Bactrocera dorsalis* GSS. (A) Mitotic karyotype derived from a male larva. X, the X chromosome; A, normal chromosome 5; AL-AS, the two translocated chromosomes. (B) Polytene chromosomes derived from a male larva. The thin arrow indicates the translocation breakpoint on the 5R chromosome arm, and the thick arrow indicates the centromere of chromosome 5. This figure was first published in Zacharopoulou & Franz (2013).

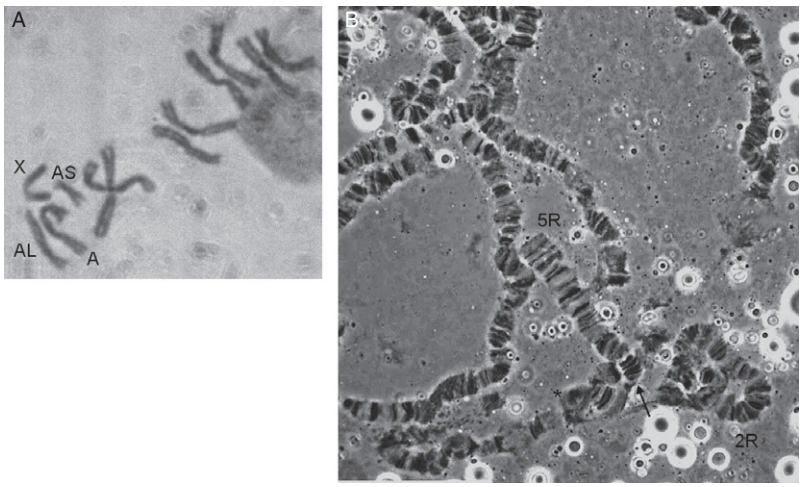

Figure 4 The *Zeugodacus cucurbitae* GSS. (A) Mitotic karyotype derived from a male larva. X, the X chromosome; A, normal chromosome 5; AL-AS, the two translocated chromosomal fragments. (B) Polytene chromosomes derived from a male larva, showing the T(Y;2;5) translocation. The arrow indicates the translocation breakpoint on the 5R chromosome arm, and the asterisk indicates the small part of chromosome 2 that has been translocated to chromosome 5. This figure was first published in Zacharopoulou & Franz (2013).

indicated that inversions usually accompany speciation and are even more crucial for speciation in sympatry (Ashburner et al., 1982; Ashburner, 1989b; Krimbas & Powell, 1992). Recent high-resolution whole-genome data are in accordance with classical cytogenetic studies, proving their accuracy and importance (Rieseberg, 2001; Schaeffer et al., 2008; Lee et al., 2013; Love et al., 2016; Sharakhov et al., 2016).

In tephritids, the available cytogenetic data suggest the presence of specific chromosomal rearrangements that may have contributed or at least have accompanied speciation events in this family. These data include comparative analysis of polytene chromosome banding pattern and in situ hybridization with selected genes (Zacharopoulou et al., 1992; Zhao et al., 1998; Gariou-Papalexiou et al., 2002; Mavragani-Tsipidou, 2002; Drosopoulou et al., 2010; Augustinos et al., 2014). Recent advances in the tephritid genome projects, such as those of *C. capitata* and *B. tryoni* (Gilchrist et al., 2014; Papanicolaou et al., 2016; Sved et al., 2016), support the proposed chromosomal homologies among them (Zacharopoulou et al., 1992; Zhao et al., 1998; Gariou-Papalexiou et al., 2002; Mavragani-Tsipidou, 2002; Drosopoulou et al., 2010; Augustinos et al., 2014). As shown in the past, evolution in Diptera in general involves mainly intrachromosomal rearrangements and can be used to unravel phylogenetic relationships (Ashburner et al., 1982; Coluzzi, 1982; Krimbas & Powell, 1992; Coluzzi et al., 2002). This is clearly shown by the suggested homologies of Tephritidae chromosomes with Muller elements (*Drosophila*) and the

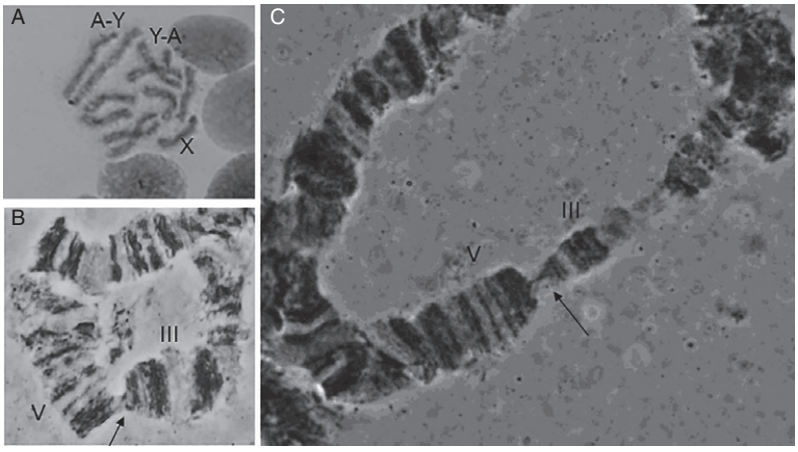

Figure 5 The *Anastrepha ludens* GSS. (A) Mitotic karyotype of a male third instar. X, the X chromosome; A-Y, the translocated chromosomal fragment that carries the autosomal centromere; Y-A, the translocated chromosome that carries the Y centromere. (B) Polytene chromosomes of a third instar. The arrow indicates the ectopic pairing among the tips of the III and V polytene elements. (C) Polytene chromosomes of a male third instar. The arrow indicates the ectopic pairing among the tips of the III and V polytene elements. Note that only the normal homologue of the III element is connected to element V. This figure was first published in Zepeda-Cisneros et al. (2014).

extended synteny among them (Gariou-Papalexiou et al., 2002; Mavragani-Tsipidou, 2002). As an example, numerous genes deriving from the recently obtained genome of the *B. tryoni* were assigned to specific linkage groups and polytene chromosomes based on in situ hybridization data derived from earlier studies (Zhao et al., 1998; Gilchrist et al., 2014; Sved et al., 2016). Comparison of these data with genome data from *D. melanogaster* verifies the high degree of synteny in Diptera, since the clear majority of these genes was assigned to the proposed Muller elements (Sved et al., 2016). The fact that few genes are present in non-homologous chromosomes, as shown either by in situ hybridization studies (such as the *Adh* and *Hsp70*; see Table 2) (Gariou-Papalexiou et al., 2002) or by recent genomewide studies (Sved et al., 2016) cannot contradict the argument regarding the overall conservation of Muller elements within Diptera. These recent findings support the absence of extended interchromosomal rearrangements but only partly address the extent and degree of intrachromosomal rearrangements (Sved et al., 2016). Based on comparative analysis of polytene chromosome banding patterns and the available in situ hybridization results within tephritids, there is strong evidence for the presence of specific intrachromosomal rearrangements, concentrated mainly on chromosomes 3, 5, and 6, following the *C. capitata* numbering system (Zhao et al., 1998; Drosopoulou et al., 2010; Zacharopoulou et al., 2011a; Augustinos et al., 2014). These rearrangements include at least two intrachromosomal transpositions on chromosomes 3 and 5 (Figures 6 and 7) and two pericentric inversions on chromosomes 5 and 6 (Figure 8). The pericentric inversion on chromosome 5 differentiates *Ceratitis* from *Bactrocera*, *Zeugodacus*, and *Dacus* species, whereas the pericentric inversion on chromosome 6 differentiates *Bactrocera* from *Ceratitis*, *Zeugodacus*, and *Dacus* species. The combined use of banding pattern comparative analysis and additional in situ hybridization of appropriate probes can reveal chromosomal rearrangements that either account for the differentiation at species level or at least can be used as diagnostic characters to distinguish them.

Nowadays, there is accumulating evidence that different factors (or a combination of factors) may contribute to speciation. This makes it even more difficult to 'dissect' the causal factors of speciation, especially as there can be an interplay between them. Taking all this into account, the current trend in taxonomy, known as integrative taxonomy, suggests the utilization of multidisciplinary, independent approaches for addressing potential speciation events (Schlick-Steiner et al., 2010). This is even more important for the resolution of closely related species (complexes of species, cryptic species, or taxa under incipient speciation). In this sense, cytogenetics is considered as an important tool which, in combination with others, can shed light on the phylogenetic relationships and species delimitation.

The clear definition and identification of species as well as the accurate resolution of species limits within species complexes are of paramount importance for decision making regarding quarantine policies and, therefore, can have a severe impact on trade. The same is also true for the incorrect synonymization of species or the unjustified split of species under different names, because this can lead to false quarantine restrictions and therefore can have a very negative impact on the economy of a country or even of a whole continent. The clear and reliable identification of species is also important for application of species-specific control methods, like SIT that depends on the effective mating of the released flies with flies of the target population. Only if the pest species in the target area is identified with certainty, the most appropriate strain can be selected for the mass rearing and release in a SIT program.

A recent example that incorporates various approaches for species resolution is the analysis of the *B. dorsalis* species complex. More than 10 years of research from different laboratories around the world, coordinated by IAEA (Hendrichs et al., 2015), proposed the synonymization of four economically important taxa of the complex – *B. dorsalis* s.s., *Bactrocera papaya* Drew & Hancock, *Bactrocera philippinensis* Drew & Hancock, and *Bactrocera invadens* Drew, Tsura & White – under the common name of '*B. dorsalis*' (Schutze et al., 2014). This work is expected to influence quarantine applications, as these taxa are dispersed in Asia, Africa, and the Americas, and they have a very high invasion and establishment potential. The understanding that they constitute a single biological entity is expected to reduce quarantine restriction and facilitate world trade (Schutze et al., 2014). Cytogenetics supported the resolution of these taxa through the analysis of colonies established at the IPCL of the Joint FAO/IAEA Division, representing different taxa from different geographic regions (Zacharopoulou et al., 2011a; Augustinos et al., 2014, 2015). These colonies were used also by other research groups, collecting data from various research fields (molecular, genetic, symbiotic, mating compatibility and competitiveness, pheromone profiles, etc.), thus providing independent data from the same biological material.

Tephritidae is considered as a family with a dynamic evolutionary history that has led to many recent taxonomic revisions. The presence of complex species is common in other genera as well, such as the *A. fraterculus* complex and the *Ceratitis FAR* complex (*Ceratitis fasciventris* Bezzi, *Ceratitis anonae* Graham, *Ceratitis rosa* Karsch). For both complexes, multidisciplinary approaches had

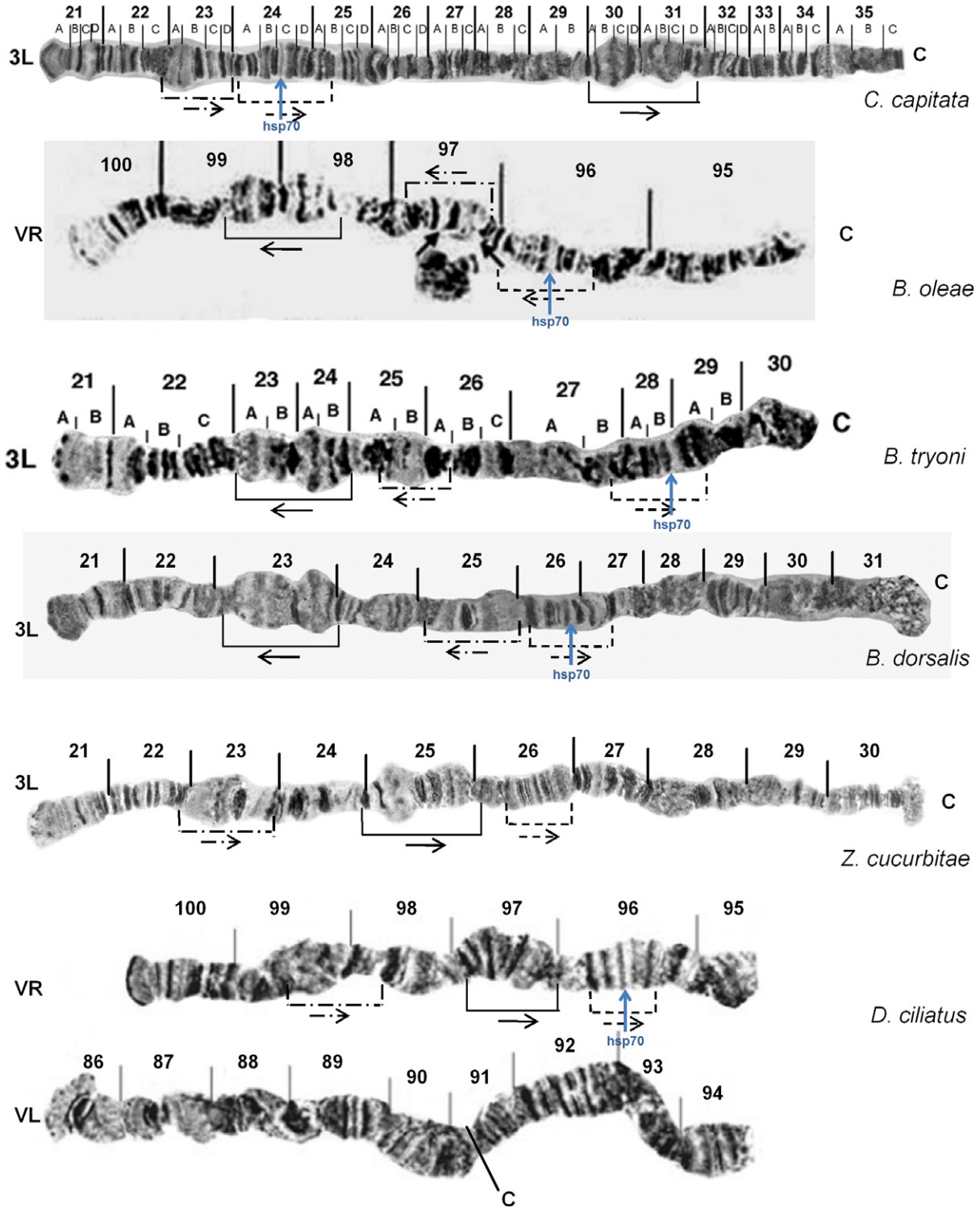

Figure 6 Comparison of the 3L polytene chromosome arm between *Ceratitis capitata*, *Bactrocera oleae*, *B. tryoni*, *B. dorsalis*, *Zeugodacus cucurbitae*, and *Dacus ciliatus*. Chromosomal rearrangements are indicated by lines and arrows. C, centromere. Blue arrows indicate in situ hybridization. This figure has been constructed for the present study, using polytene arms of the previously published polytene chromosome maps of the species. [Colour figure can be viewed at wileyonlinelibrary.com]

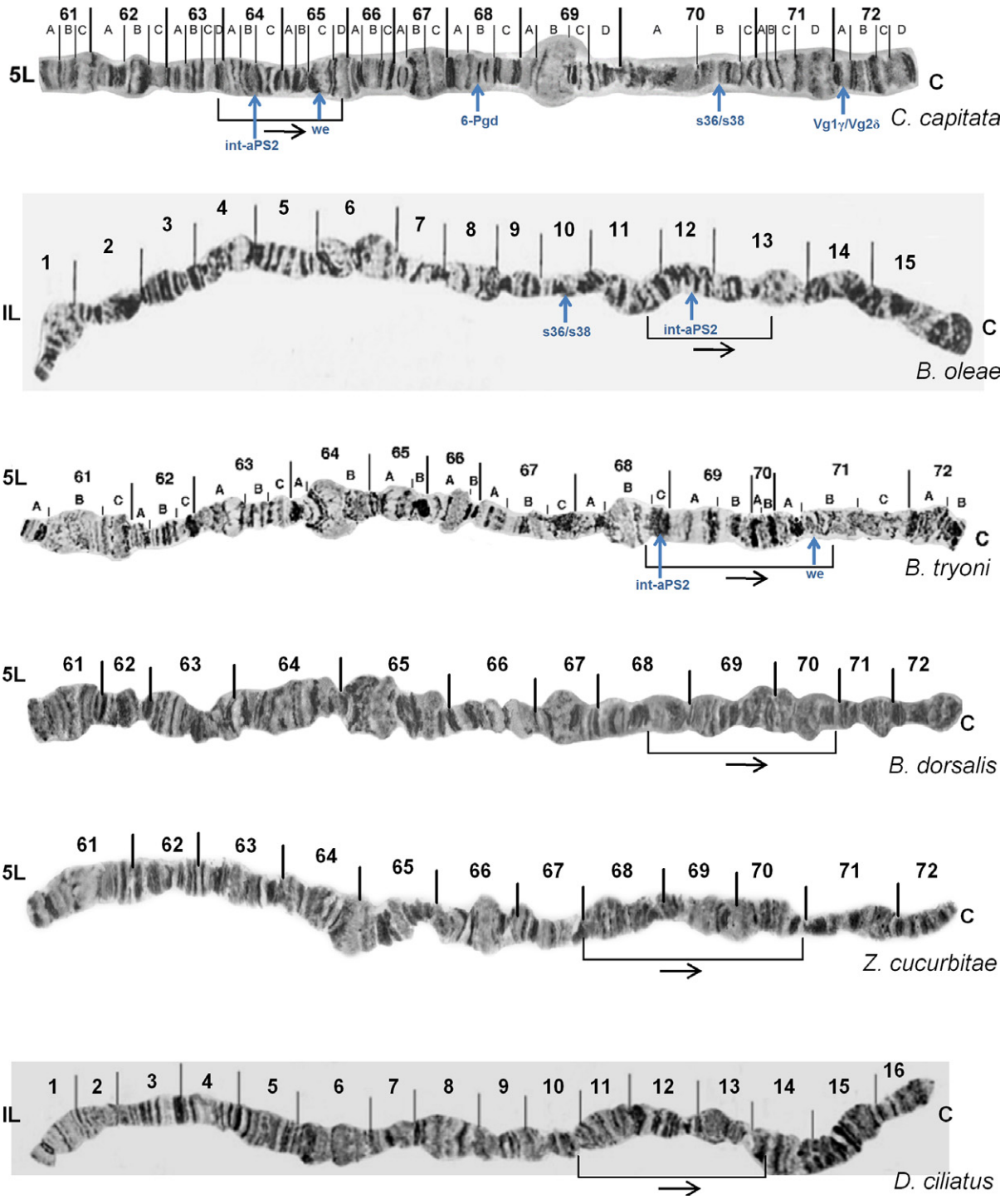

Figure 7 Comparison of 5L polytene chromosome arm between *Ceratitis capitata*, *Bactrocera oleae*, *B. tryoni*, *B. dorsalis*, *Zeugodacus cucurbitae*, and *Dacus ciliatus*. Chromosomal rearrangements are indicated by lines and arrows. C, centromere. Blue arrows indicate in situ hybridization results. This figure has been constructed for the present study, using polytene arms of the previously published polytene chromosome maps of the species. [Colour figure can be viewed at wileyonlinelibrary.com]

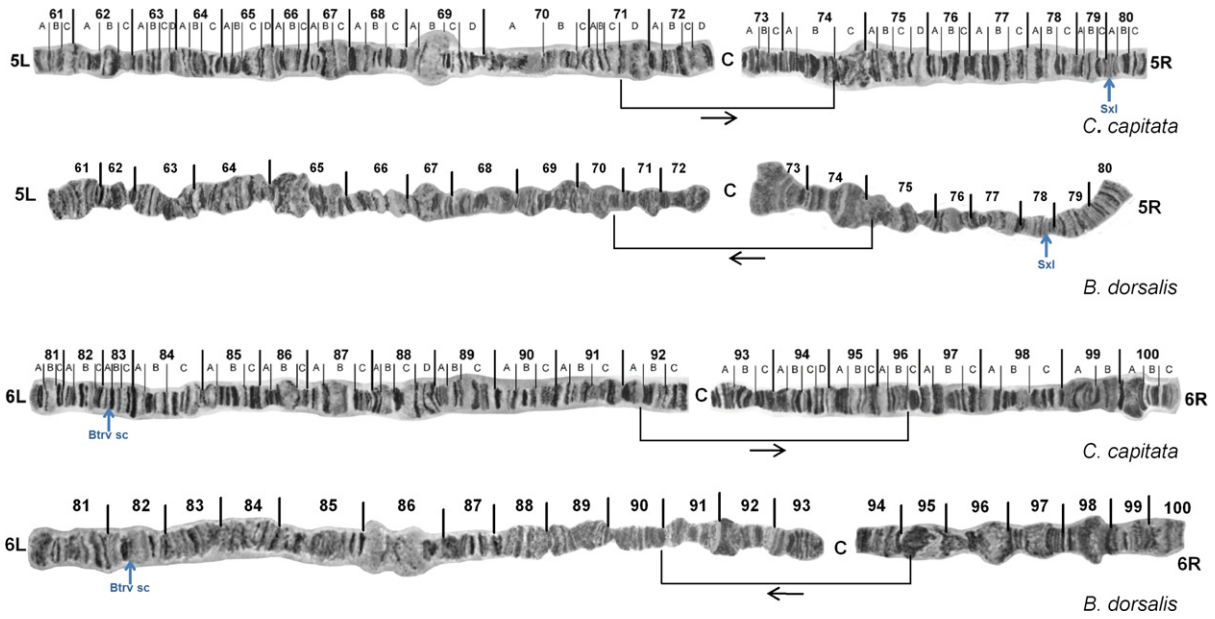

Figure 8 Two pericentric inversions between *Ceratitis capitata* and *Bactrocera* species. These inversions in respect to *C. capitata* are shared among all *Bactocera* species analyzed so far; therefore, only one (*B. dorsalis*) was selected for the comparison. (A) The pericentric inversion between chromosome 5 of *C. capitata* and *B. dorsalis*. (B) The pericentric inversion between chromosome 6 of *C. capitata* and *B. dorsalis*. C, centromere. Blue arrows indicate in situ hybridization results. This figure has been constructed for the present study, using polytene arms of the previously published polytene chromosome maps of the species. [Colour figure can be viewed at wileyonlinelibrary.com]

been and are being used (Hernández-Ortiz et al., 2012; De Meyer et al., 2015a; Dias et al., 2015). The recent publication of polytene chromosome maps of a member of the *A. fraterculus* complex (Gariou-Papalexiou et al., 2016) and the forthcoming publication of mitotic karyotypes and polytene chromosomes maps of a member of the *C. FAR* complex (Drosopoulou et al., 2017) are expected to facilitate taxonomic studies in both complexes.

Contribution to genome sequencing projects

From the start of sequencing efforts of complex organisms, it was evident that the ability to map sequenced clones, contigs, or scaffolds greatly improved the resolution of the whole sequence result. The more tools one had at hand, such as RFLPs, ESTs, or VNTR markers, the better the outcome was. An unrivaled tool in the hands of dipteran geneticists was the presence of characterized polytene chromosomes in several species. Such chromosomes give the opportunity to physically map with unsurpassed accuracy a given clone by in situ hybridization (Schaeffer et al., 2008; Fierst, 2015). Therefore, one can localize a sequenced scaffold in silico by virtue of known loci it may contain. Alternatively, one can easily generate by PCR new probes corresponding to scaffold segments and then localize those by in situ hybridization. If such probes are designed at distant parts of a long scaffold, then both the scaffold's position and orientation can be accurately determined on chromosomal regions. This kind of physical localization of scaffolds on polytene maps can render gap-closing efforts more targeted and efficient. Thus, the exploitation of molecular landmarks to align scaffolds to high-resolution polytene maps is a powerful way to achieve high-quality genomic assembly.

A case in proof is evident in the recent publication of the whole genome of *C. capitata* (Papanicolaou et al., 2016). The preexisting integrated cytogenetic map containing a large number of microsatellite loci along with several gene loci (Zacharopoulou et al., 1992; Gariou-Papalexiou et al., 2002; Stratikopoulos et al., 2008, 2009) was used to anchor sequenced scaffolds on the insect's polytene chromosomes. Among others, 43 autosomal and one X-linked scaffold were physically mapped, totaling more than 212 Mb or ca. 45% of the genome. The integration of all tools used resulted in an assembled genome of only 1 806 scaffolds, with 50% of total sequence length contained in a mere 34 sequences (L50) of ≥4 Mp (N50) (Papanicolaou et al., 2016).

Cytogenetic mapping is also being utilized for the assembly of the recently sequenced genome of the olive fruit fly, *B. oleae* (https://i5k.nal.usda.gov/Bactrocera_

oleae). Scaffolds are being anchored onto the polytene chromosomes of the species by already mapped molecular markers they contained or via in situ hybridization of newly generated probes from their ends. In this way, mapped scaffolds cover more than 50% of the *B. oleae* chromosomes (H Djambazian, A Bayega, K T Tsoumani, E Sagri, M Gregoriou, E Drosopoulou, P Mavragani-Tsipidou, K Giorda, G Tsiamis, K Bourtzis, A Papanikolau, S Oikonomopoulos, K Dewar, D Church, K D Mathiopoulos & J Ragoussis, unpubl.). Further, chromosomal hybridizations of annotated genes should improve the established physical map as well as the assembly verification, allowing more advanced genome organization analyses. The genome of *B. tryoni* has also been published (Gilchrist et al., 2014). As a matter of fact, it was the first tephritid genome to be sequenced. Sequencing was solely based on regular Illumina libraries, mate-pair libraries, and 454 FLX Titanium pyrosequencing. However, as no mapping information was utilized to further link scaffolds, sequencing statistics are much inferior to those of the other two tephritids. Finally, the genomes of two other tephritids – *Z. cucurbitae* (https://i5k.nal.usda.gov/content/bactrocera-cucurbitae) and *B. dorsalis* (https://i5k.nal.usda.gov/content/bactrocera-dorsalis) – have also been sequenced, but no mapping data have been integrated.

Conclusions and future challenges

Taken together, cytogenetics has played a catalytic role in the development and application of SIT for the population control of major tephritid species. This has been achieved either through the construction and characterization of GSSs or via its contribution in integrative taxonomic studies, which have clarified relationships between closely related species and/or incipient speciation phenomena. Thus, cytogenetics has been a key tool for species-specific, highly efficient, and cost-effective SIT applications against insect pest populations. In addition, cytogenetics has significantly contributed in recent genome sequencing projects of tephritid pest species by facilitating the production of better-assembled genomes.

Despite its great potential, tephritid and insect cytogenetics in general face significant challenges. Insect species with low-quality or completely lacking polytene chromosomes may not be amenable to the cytogenetic characterization summarized in this review. However, with the advent of molecular cytogenetics as well as the potential of creating targeted mutations and highly precise chromosomal rearrangements using novel genome-editing tools such as CRISP/Cas9 may greatly facilitate both the production and the characterization of GSSs and hence the implementation of SIT applications. Moreover, molecular cytogenetics can significantly contribute to studies for species delimitation and accelerate high-quality assembly of the many insect genomes which are to be sequenced through the i5k project. But the most important challenge the field of insect cytogenetics has to face is the lack of a new generation of cytogeneticists! Urgent actions are needed by the Departments of Biology and Entomology worldwide to put more emphasis on insect cytogenetics in their undergraduate and postgraduate courses.

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