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A comparative analysis of the chromosomes of three FARQ species complex members, *Ceratitis rosa*, *C. quilicii*, and *C. fasciventris* F2 (Diptera: Tephritidae)

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Abstract

The *Ceratitis* FARQ species complex consists of four highly destructive agricultural pests of Africa, namely *C. fasciventris*, *C. anonae*, *C. rosa*, and *C. quilicii*. The members of the complex are considered very closely related and the species limits among them are rather obscure. Their economic significance and the need for developing biological methods for their control makes species identification within the complex an important issue, which has become clear that can only be addressed by multidisciplinary approaches. Chromosomes, both mitotic and polytene, can provide a useful tool for species characterization and phylogenetic inference among closely related dipteran species. In the current study, we present the mitotic karyotype and the polytene chromosomes of *C. rosa* and *C. quilicii* together with *in situ* hybridization data. We performed a comparative cytogenetic analysis among the above two species and *C. fasciventris*, the only other cytogenetically studied member of the FARQ complex, by comparing the mitotic complement and the banding pattern of the polytene chromosomes of each species to the others, as well as by studying the polytene chromosomes of hybrids between them. Our analysis revealed no detectable chromosomal rearrangements discriminating the three FARQ members studied, confirming their close phylogenetic relationships.

Introduction

Tephritidae is a speciose family of Diptera, with a great number of species characterized as serious agricultural pests (Bickel *et al.*, 2009). In particular, the genera of *Anastrepha*, *Bactrocera*, *Ceratitis*, *Dacus*, *Rhagoletis*, and *Zeugodacus* include some of the most destructive fruit flies that cause severe economic losses due to crop damaging of commercial fruit and vegetable and restrictions to global trade (White and Elson-Harris, 1992; De Meyer *et al.*, 2015*b*). From the genus *Ceratitis*, the Mediterranean fruit fly, *Ceratitis capitata*, is the best studied species used as a model pest organism, because of its almost global distribution and its enormous economic impact (Malacrida *et al.*, 2007). In the recent years, attention has been also drawn to the *Ceratitis* species of the African FARQ complex. Until 2016, the complex was known as FAR species complex and was considered to consist of three closely related species, *C. fasciventris*, *C. anonae*, and *C. rosa* (Virgilio *et al.*, 2008). However, accumulating evidence from studies on molecular genetics (Virgilio *et al.*, 2013), morphometrics (Van Cann *et al.*, 2015), developmental physiology (Tanga *et al.*, 2015), behavior and sexual compatibility (De Meyer *et al.*, 2015*a*), chemoecology (Vaníčková *et al.*, 2015), and environmental preferences (Mwatawala *et al.*, 2015) lead to the conclusion that *C. rosa* was, in fact, consisting of two entities, one of which has been described as a new species within the complex, *C. quilicii* (De Meyer *et al.*, 2016). Microsatellite analysis indicated the existence of two genotypic groups in *C. fasciventris*, as well, referred to as types F1 and F2 (Virgilio *et al.*, 2013). This was confirmed also by morphological data, however, in the absence of integrative evidence, *C. fasciventris* is still considered as one species (De Meyer *et al.*, 2015*a*).

The four members of the FARQ complex are highly polyphagous attacking plants from more than 25 different families and are considered a major threat to the agricultural production and economy of many countries of the African continent, as well as species of quarantine significance (White and Elson-Harris, 1992; Smith *et al.*, 1997; De Meyer *et al.*, 2002). *C. fasciventris* and *C. anonae* are distributed mainly through Western and Central Africa, found sympatrically in several regions, while *C. rosa* and *C. quilicii* present overlapping distribution

in Eastern and Southern Africa (De Meyer *et al.*, 2002, 2016; Copeland *et al.*, 2006). It has been reported that *C. rosa* occupies mainly lower altitude areas, while *C. quilicii* predominates in cooler highland regions (Mwatawala *et al.*, 2015), probably reflecting differences in the developmental and survival rates of the two species in respect to climate variation (Tanga *et al.*, 2015). It should be noted that *C. quilicii* is the only one of the two sibling species found in the southernmost parts of Africa where the climate is more temperate (De Meyer *et al.*, 2015*a*). However, because of the recent separation into different species, the specific distribution patterns for *C. rosa* and *C. quilicii* may need reevaluation. Among the FARQ pests, *C. rosa* and *C. quilicii* are the most aggressive ones causing significant destruction to a large variety of crops and presenting high expansion potential. Already, *C. quilicii* has been introduced in the Islands of Mauritius and La Réunion (White *et al.*, 2000; De Meyer *et al.*, 2016) and a great concern has arisen about their possible expansion to more temperate climates outside Africa, since they can survive in a wide temperature and altitude range (Duyck and Quilici, 2002; Copeland *et al.*, 2006; Geurts *et al.*, 2012; de Villiers *et al.*, 2013; Tanga *et al.*, 2018).

The species of the FARQ complex are extremely similar in morphology; males are hardly identified by subtle differences in the setal ornamentation and pigmentation of mid femur and tibia, while females are practically indistinguishable (De Meyer *et al.*, 2015*a*, 2016). Species delimitation and phylogenetic relationships among the four taxa are not fully resolved. Several approaches have been undertaken toward this direction including morphometrics (Van Cann *et al.*, 2015), interspecies hybridization and estimation of developmental stability (Erbout *et al.*, 2008), biochemical characterization of pheromones and cuticular hydrocarbons (Vaníčková *et al.*, 2014, 2015; Břízová *et al.*, 2015), and molecular/genetic data of nuclear and mitochondrial sequences (Douglas and Haymer, 2001; Barr and McPheron, 2006; Barr *et al.*, 2006, 2012; Virgilio *et al.*, 2008, 2012), with the analysis of a specific microsatellite set conferring better resolution among populations of the complex (Delatte *et al.*, 2013; Virgilio *et al.*, 2013, 2019). Recently, a phylogenomic study based on genome-wide SNP analysis provided consistent resolution and better insights into the phylogenetic relationships of the FARQ members (Zhang *et al.*, 2021). A good understanding of the evolutionary relationships and the development of accurate, simple, and fast diagnostic tools for the sibling species of the FARQ complex is of great importance for the implementation of quarantine measures, as well as for biological control applications, including the sterile insect technique (SIT), against these pests.

The number and structure of chromosomes are fundamental genetic characteristics of species, while chromosome rearrangements are considered to play a major role in speciation. In Diptera, the occurrence of polytene nuclei in several juvenile tissues has greatly facilitated the study of chromosomes due to their enormous size and consistent banding pattern (Zhimulev and Koryakov, 2009). Numerous cytogenetic studies in Drosophila but also in mosquitoes have explored the evolutionary changes of chromosome structure among related species and, together with modern genomic data, substantiated that chromosome rearrangements and especially paracentric inversions promote speciation, mainly through suppressing recombination and, thus, preserving sets of co-adapted alleles, and suggested that they could be used as phylogenetic markers (Sturtevant and Dobzhansky, 1936; Coluzzi *et al.*, 1979; Krimbas and Powell, 1992; Noor *et al.*, 2001; Rieseberg, 2001; Kirkpatrick and Barton, 2006; Kulathinal *et al.*, 2009; Faria and Navarro, 2010; McGaugh and Noor, 2012; Lee *et al.*, 2013). In Tephritidae, as well, differences in the size and structure of mitotic sex chromosomes have been descripted as diagnostic characters among closely related species (Hunwattanakul and Baimai, 1994; Baimai *et al.*, 1995, 2000; Baimai, 1998; Goday *et al.*, 2006; Cáceres *et al.*, 2009; Hernández-Ortiz *et al.*, 2012; Giardini *et al.*, 2015). Furthermore, comparative analyses of polytene chromosomes have identified specific rearrangements that could distinguish between genera, subgenera, or species (Augustinos *et al.*, 2015; Zacharopoulou *et al.*, 2017; Gouvi *et al.*, 2022). Cytogenetic information on tephritid pests has also been proved valuable for the development and characterization of genetic sexing strains essential for the implementation of certain control methods, such as SIT (Augustinos *et al.*, 2015; Zacharopoulou *et al.*, 2017; Gouvi *et al.*, 2022). Even so, taking into consideration that speciation is a complex procedure driven by variable factors one can understand that chromosome structure and cytogenetics could only be one of multiple tools for species delimitation. Especially in cases of recent or ongoing speciation, pools of independent data in the context of 'integrative taxonomy' (Schutze *et al.*, 2017*a*, 2017*b*) and modern genome-wide analyses (Zhang *et al.*, 2021) are necessary for clearer perception.

In this study, we describe the mitotic and polytene chromosome of *C. rosa* and *C. quilicii* and we conduct a comparative polytene chromosome analysis among the above species and *C. fasciventris* F2 by observation of polytene nuclei of each species as well as of F1 hybrids between them. Furthermore, we localized the *hsp70* gene on the polytene chromosomes of the above species, since rearrangements which include the chromosome region where the *hsp70* locus resides on the 3L polytene arm seem to be common among several tephritid species (Drosopoulou *et al.*, 2017; Zacharopoulou *et al.*, 2017), some of them closely related (Gouvi *et al.*, 2022). Our aim is to reveal possibly existing chromosome rearrangements that could be informative toward the better understanding of the phylogenetic relationships of the species and could be used as discriminating characters for species identification within the FARQ complex.

Materials and methods

Insects from five colonies maintained at the Insect Pest Control Laboratory (IPCL), Seibersdorf, Austria were used in the present study. The above colonies were established from insects originating from confirmed colonies of *Ceratitis fasciventris* F2 (hereafter *C. fasciventris*), *C. rosa* and *C. quilicii* maintained at ICIPE, Kenya and of *C. rosa* and *C. quilicii* maintained at CRI, South Africa. The colonies were reared under controlled temperature, humidity, and light conditions, as previously described (Drosopoulou *et al.*, 2017).

Mitotic chromosome preparations were spread from nerve ganglia of third instar larvae following the air-drying technique described in Mavragani-Tsipidou *et al.* (2014). Brain tissue was dissected in physiological solution, treated with hypotonic solution (1% sodium citrate) for about 15 min and fixed in fresh fixation solution (methanol/acetic acid 3:1) for 3 min. Samples were macerated in a small drop of 60% acetic acid, dripped onto a clean slide and placed on a hotplate (40–45 °C). After air-drying, preparations were stained in Giemsa solution (5% Giemsa in 10 mM phosphate buffer, pH 6.8) and observed with 100×

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magnification objective lens, using a phase contrast microscope (Leica DMR). Well spread nuclei were photographed using a CCD camera (ProgResCF^{cool}; JENOPTIK Jena Optical Systems, Jena, Germany). About ten chromosome preparations from individual larvae from each strain and at least ten well spread nuclei per preparation were analyzed.

Polytene chromosome preparations for banding pattern analysis were made from salivary glands of third-instar larvae as described in Mavragani-Tsipidou *et al.* (2014). Salivary glands were dissected in 45% acetic acid, transferred to 3N HCL for 1 min, and fixed in fixation solution (3 parts glacial acetic acid: 2 parts water: 1 part lactic acid) for about 5 min. Staining was performed in lacto-acetic-orcein for 5–7 min. After excess stain was removed, the glands were squashed in lacto-acetic acid. About 50 chromosome slides from each strain were prepared and well spread nuclei and/or isolated chromosomes were observed at 63× and 100× objectives in a phase contrast microscope (Leica DMR) and photographed using a CCD camera (ProgResCF^{cool}; JENOPTIK Jena Optical Systems).

Polytene chromosome preparations for *in situ* hybridization were made following the procedure described by Mavragani-Tsipidou *et al.* (2014). A genomic fragment of the *hsp70* gene of *Ceratitis capitata* (Papadimitriou *et al.*, 1998) was used as probe. Labeling of the probe and detection of the signal was performed using the 'DIG-DNA Labeling and Detection kit' purchased by ROCHE, Mannheim, Germany and following the protocol described in Mavragani-Tsipidou *et al.* (2014). Hybridization was performed at 65 °C. Five preparations and at least ten well spread nuclei per preparation were observed at 100× magnification with a Leica DMR phase contrast microscope equipped with a CCD camera (ProgResCF^{cool}, JENOPTIK Jena Optical Systems).

Results and discussion

Mitotic chromosomes

The karyotypes of *C. rosa* and *C. quilicii* ($2n = 12$) appear identical to each other consisting of five pairs of autosomes and one pair of heteromorphic sex chromosomes (XX/XY) (fig. 1). The largest metacentric (chromosome 2), as well as the only submetacentric (chromosome 3) autosome pair can be easily identified (fig. 1). The remaining three autosomes (namely 4, 5, and 6) being all metacentric of similar size cannot be easily distinguished by our analysis. The two sex chromosomes differ significantly in size: the X chromosome is submetacentric of medium size, while Y is a small metacentric chromosome (fig. 1b, d). The karyotypes of *C. rosa* and *C. quilicii*, presented after Giemsa

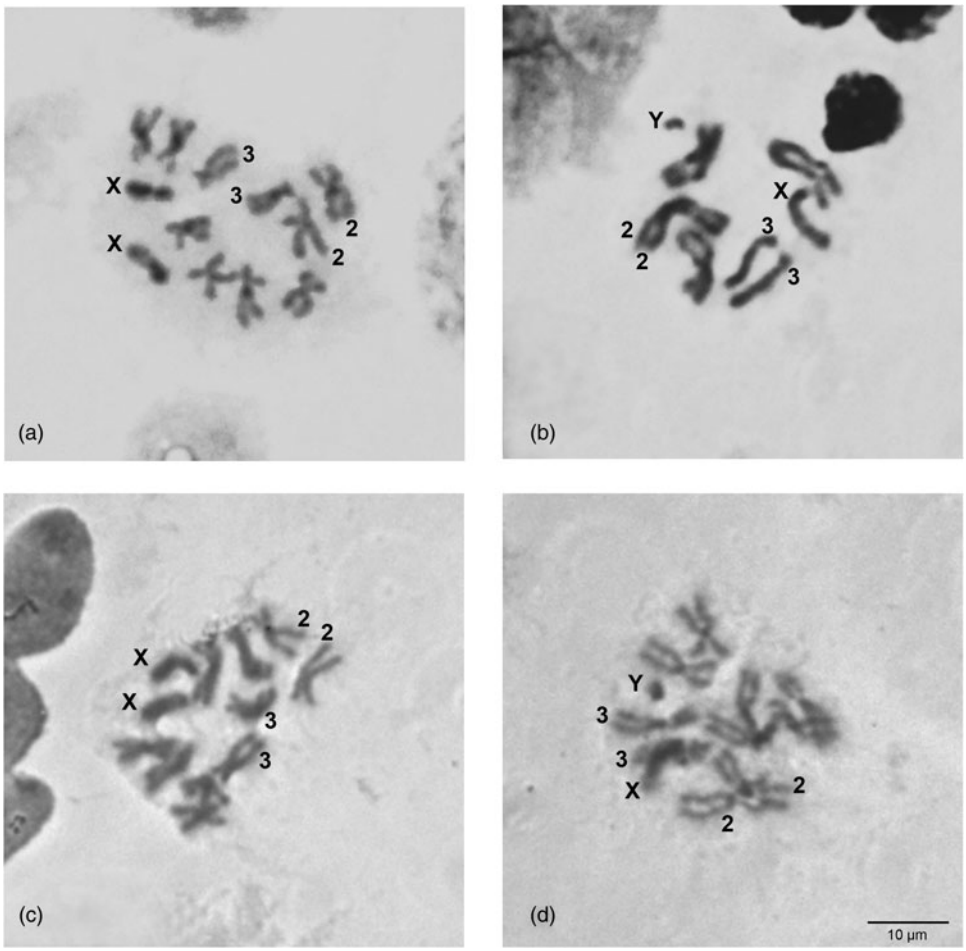

Figure 1. Mitotic karyotypes of *C. rosa* (a and b) and *C. quilicii* (c and d). (a, c) Female; (b, d) male. The sex chromosomes, X and Y, as well as the autosomes 2 and 3 are shown.

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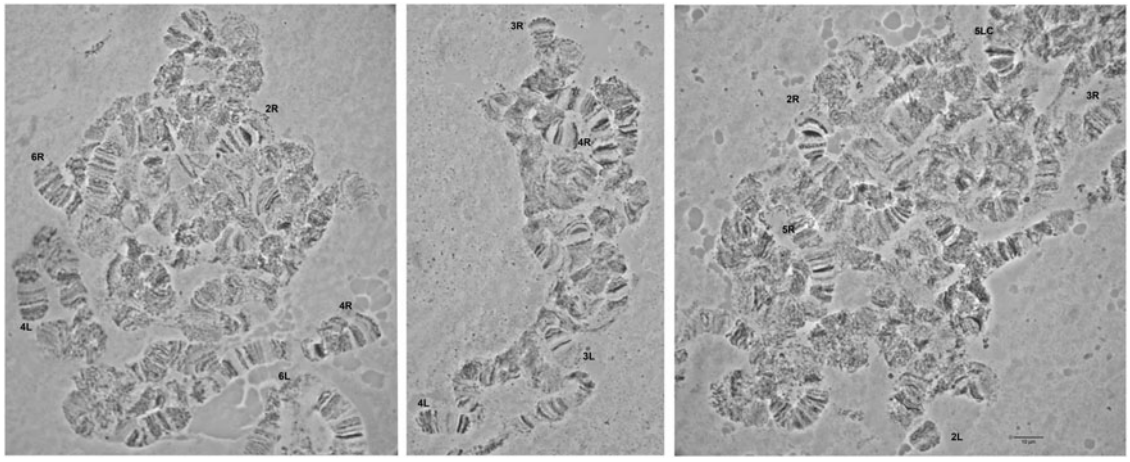

Figure 2. Polytene nuclei of F1 hybrids between *C. rosa* and *C. quilicii*. The telomeres of the polytene elements are indicated. 5LC indicates the 5L centromere. No asynapses are observed.

staining, are in agreement with the *C. rosa* karyotype described by Willhoeft and Franz (1996). They also appear very similar to the mitotic karyotype of the closely related member of the FARQ complex, *C. fasciventris* (Drosopoulou *et al.*, 2017), in which the X chromosome seems to be of slightly smaller size (relatively to the autosomes). Similarly, the main difference of all FARQ karyotypes to *C. capitata* is the considerably shorter X and Y chromosomes. Such variation in the size of the sex chromosomes, reflecting differences of the amount of heterochromatin, can be commonly observed among very closely related, e.g. within a complex (Baimai *et al.*, 1995, 2000; Selivon *et al.*, 2005*a*; Cáceres *et al.*, 2009) or a bit more distantly related, e.g. within a genus, (Hunwattanakul and Baimai, 1994; Frias, 2004; Selivon *et al.*, 2005*b*; Zacharopoulou *et al.*, 2017) species of tephritids.

Polytene chromosomes

The salivary gland polytene nuclei of two *C. rosa* colonies and two *C. quilicii* colonies have been studied. Analysis showed that the polytene complement of the above species consists of ten long polytene arms with distinct banding pattern, corresponding to the five autosomes, while a dispersed heterochromatic network represents the under-replicated sex chromosomes (Supplementary figs 1 and 2), similarly to other Tephritidae species (Zacharopoulou *et al.*, 2017; Gouvi *et al.*, 2022). Although no typical chromocenter was observed, the centromeric region of different chromosomes could be found loosely connected (Supplementary fig. 1a). Chromosomes were numbered from 2 to 6, chromosome arms were designated as L or R (Supplementary figs 1 and 2) based on the similarities to *C. fasciventris* polytene chromosome maps (Drosopoulou *et al.*, 2017) and following the numbering system proposed for the polytene chromosomes of the medfly, the first tephritid species analyzed cytogenetically (Zacharopoulou *et al.*, 2017).

Detailed comparison of the polytene chromosome banding pattern failed to reveal differences either among the analyzed strains of *C. rosa* and *C. quilicii* nor between each analyzed strain and *C. fasciventris* (Supplementary figs 3 and 4). Aiming to confirm the identical banding pattern of the analyzed species, the polytene chromosomes of F1 hybrids between *C. rosa* and *C. quilicii*, as well as between *C. rosa* and *C. fasciventris* and *C. quilicii* and *C. fasciventris* were also examined. The analysis of the polytene nuclei of the hybrids did not reveal any chromosome rearrangements between the parental strains. Synapsis of the homologous chromosomes was almost perfect in the hybrids between *C. rosa* and *C. quilicii* (fig. 2), while in the hybrids with *C. fasciventris* minor polymorphic asynapses were observed (Supplementary figs 5 and 6). Asynapses were mainly located at or close to the telomeric and the centromeric regions of the polytene arms and their extent was limited although it could vary among different nuclei (figs 3 and 4). The number and frequency of minor asynaptic sites were higher in the hybrids between *C. rosa* and *C. fasciventris* compared to the ones between *C. quilicii* and *C. fasciventris*. The most evident asynapses were the ones at the tips of chromosome arms 2L, 2R, 3L, 4R, and 6R (figs 3 and 4).

The above observations indicate that the chromosomes of *C. rosa* and *C. quilicii*, at least at the banding pattern level, can be considered as homosequential to each other and to *C. fasciventris* and the available polytene chromosome maps of *C. fasciventris* (Drosopoulou *et al.*, 2017) is suggested to be used as reference map for the three FARQ species.

The lack of detectable differences in the mitotic and polytene chromosomes of the three FARQ species indicates that they are very close genetically. This is also supported by previous molecular genetic studies, including analysis of nuclear and mitochondrial fragments, DNA barcoding and analysis of complete mitogenomes, that couldn't resolve phylogeny or provide robust discriminating tools for the members of the complex (Virgilio *et al.*, 2008, 2012; Barr *et al.*, 2012; Drosopoulou *et al.*, 2017, 2021). The limitations of the above approaches seem to be overcome only by genome-wide sequencing data succeeding to provide a robust phylogenetic inference within the complex (Zhang *et al.*, 2021). Absence of chromosomal rearrangements has also been observed between the two members of the *B. dorsalis* complex, namely *B. dorsalis* and *B. carambolae* (Augustinos *et al.*, 2015), however, within other species complexes of Tephritidae chromosome differences have been used as differentiating characters and revealed incipient speciation (Selivon *et al.*, 2005*b*, 2005*a*; Goday *et al.*, 2006; Cáceres *et al.*, 2009; Hernández-Ortiz *et al.*, 2012).

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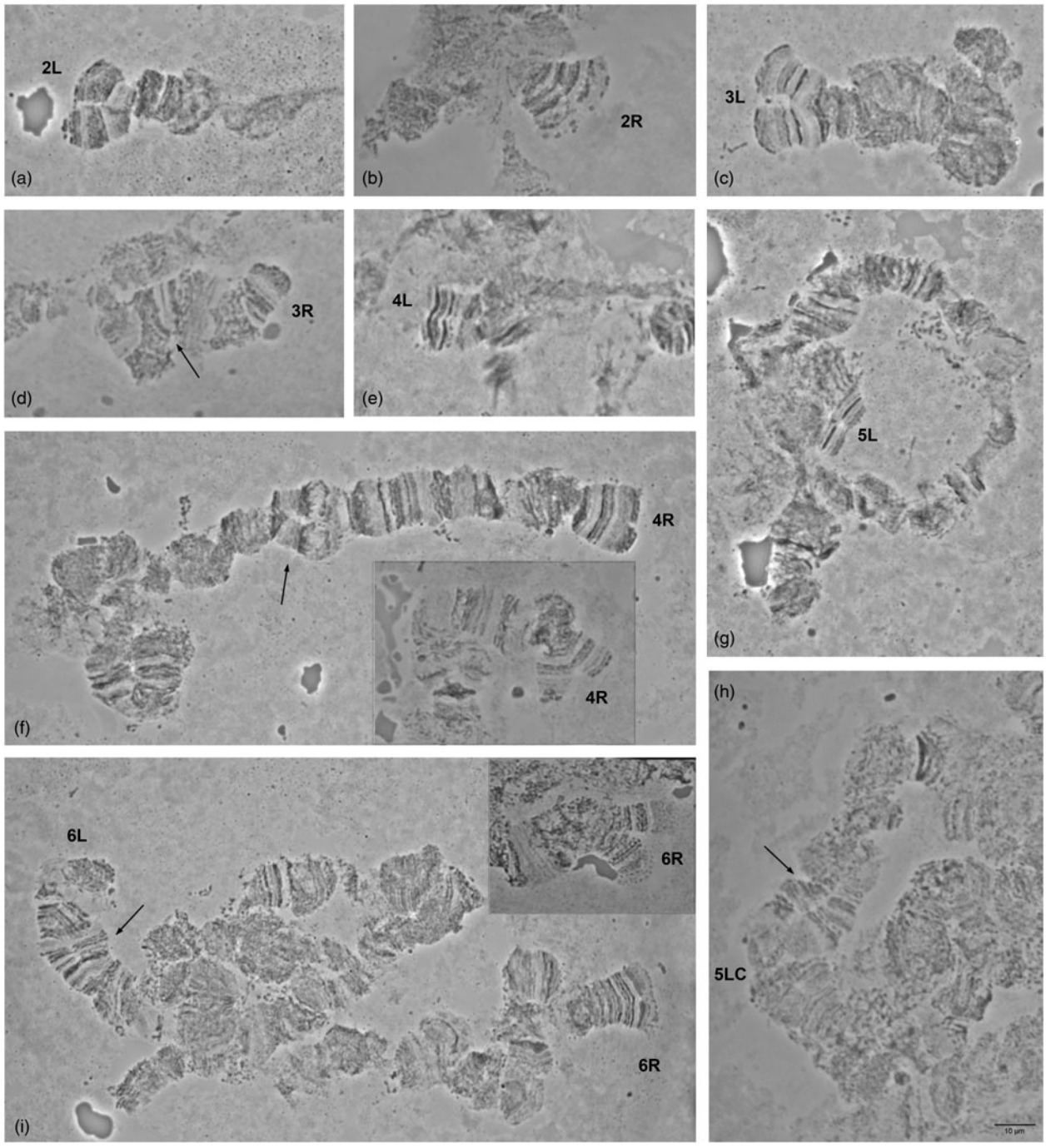

Figure 3. Asynapses frequently observed in the nuclei of F1 hybrids between *C. rosa* and *C. fasciventris*. The asynaptic telomeres of the polytene elements are indicated. Variable extent of asynapsis observed for 4R and 6R telomeres is presented in (f) and (i), respectively. Arrows indicate asynapses in the inner parts of the polytene elements. 5LC indicates the 5L centromere.

Chromosome localization of the hsp70 *gene*

The *hsp70* gene has been localized on the polytene chromosome of *C. rosa* and *C. quilicii*. A unique hybridization signal has been identified on the same chromosomal position (3L polytene chromosome arm, region 27) in all strains tested (fig. 5). The localization site of the *hsp70* gene on *C. rosa* and *C. quilicii* is identical to the one observed in *C. fasciventris* (Drosopoulou *et al*., 2017) (fig. 5), supporting the homosequentiality of the chromosomes of the three members of the FARQ complex. Nevertheless, it is acknowledged that the localization of a much greater number of probes is required to draw conclusions about genomic synteny among the studied species.

In comparison to *C. capitata*, the site of the *hsp 70* gene is different in the FARQ complex species indicating intrachromosomal rearrangements (Drosopoulou *et al*., 2017) that have differentiated the structure of the 3L chromosome arm of the above species. The presence of rearrangements, such as translocations and inversions,

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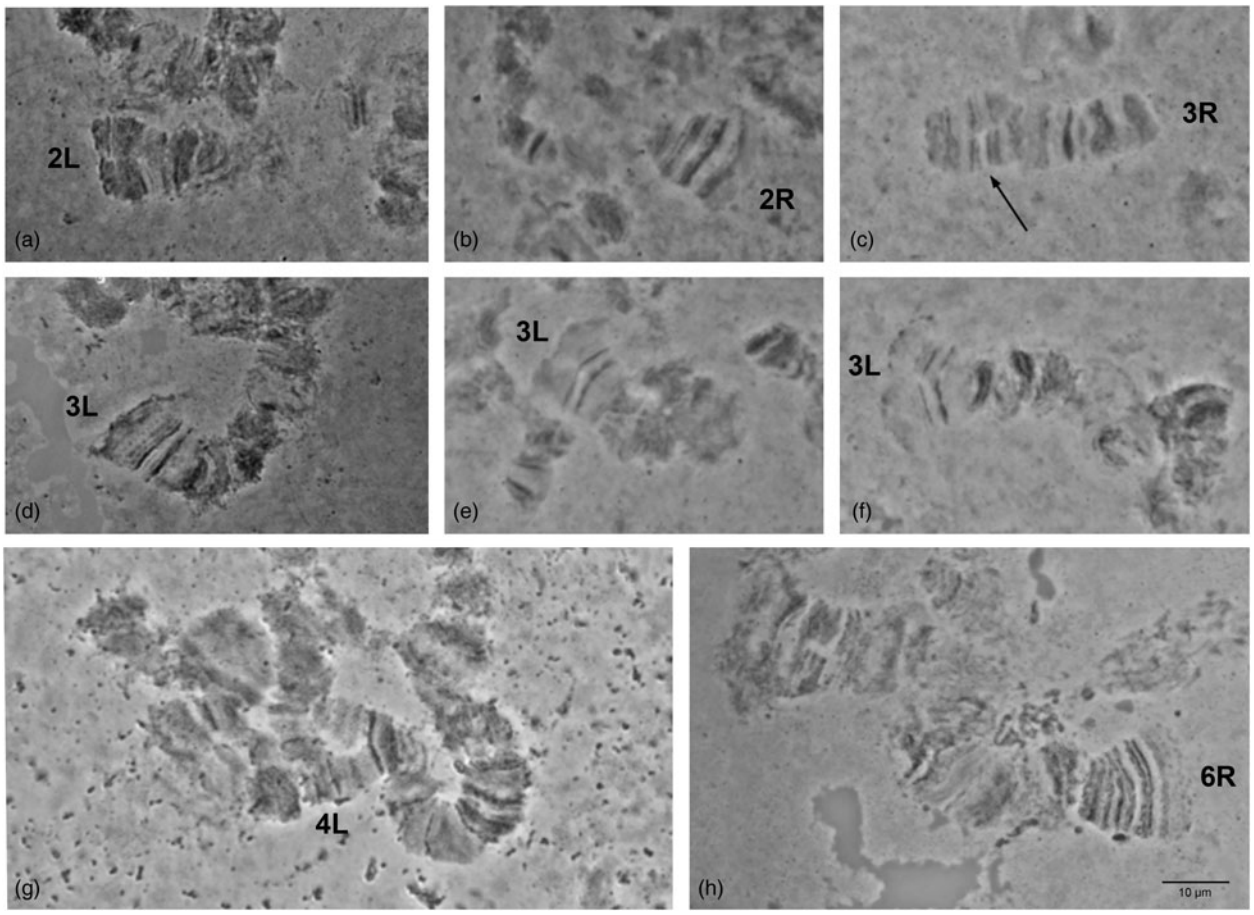

Figure 4. Asynapses frequently observed in the nuclei of F1 hybrids between *C. quilicii* and *C. fasciventris*. The asynaptic telomeres of the polytene elements are indicated. Variable extent of asynapsis observed for 3L telomere is presented in (d–f). Arrows indicate asynapses in the inner parts of the polytene elements.

in the 3L polytene arm has also been revealed by previous comparative analyses among species of several Tephritidae genera (Zacharopoulou *et al.*, 2017; Gouvi *et al.*, 2022), supporting the role of chromosome rearrangements in speciation (Noor *et al.*, 2001; Rieseberg, 2001; Kirkpatrick and Barton, 2006; Faria and Navarro, 2010; McGaugh and Noor, 2012; Lee *et al.*, 2013) and

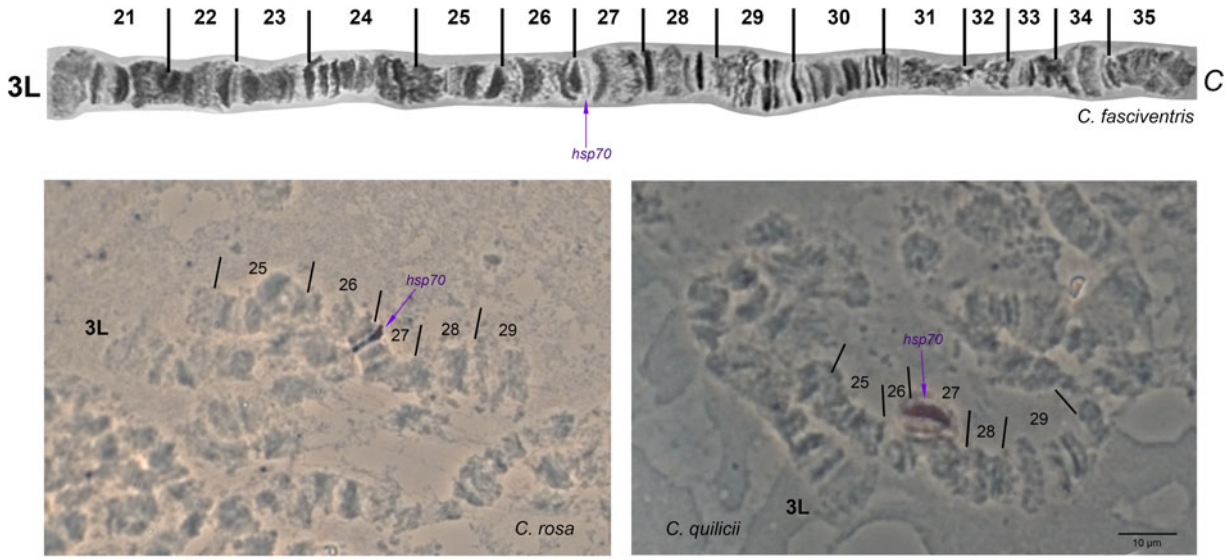

Figure 5. *In situ* hybridization of the *hsp70* gene probe on the salivary gland polytene chromosomes of *C. rosa* and *C. quilicii*. Arrows indicate the hybridization signals. The telomere of the 3L polytene arm is indicated. Numbered divisions are shown, separated by lines. The reference map of the 3L arm and the hybridization locus of the *hsp70* gene of *C. fasciventris* (Drosopoulou *et al.*, 2017) are presented on the top.

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their potential informativeness for phylogenetic inference among related tephritid species (Mavragani-Tsipidou *et al.*, 2014; Zacharopoulou *et al.*, 2017; Gouvi *et al.*, 2022).

Conclusions

Our comparative mitotic and polytene chromosome analysis of the colonized material of *C. rosa* and *C. quilicii* from two different African locations (Kenya and South Africa) and of *C. fasciventris* from Kenya did not unravel any detectable fixed chromosome rearrangements among the three members of the FARQ complex. The above emphasizes the need for multidisciplinary modern approaches when addressing sensitive issues of species designation within complexes of important insect pests, as only by the accumulation and evaluation of data coming from different aspects of the insect biology we can be led toward a more solid phylogenetic resolution and reliable species identification.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0007485323000214.

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Author contributions. E. D., A. A., K. B., and A. Z. designed the study. G. G., E. D., A. G.-P., and A. Z. performed the experiments. G. G., A. G.-P., and A. Z. took the pictures. G. G., E.D., A. G.-P., A. A., and A. Z. interpreted and analyzed the data. A. G.-P. prepared the figures. E. D. wrote the original draft manuscript. A. A. and K. B. critically revised the manuscript. All authors reviewed and approved the final version of the manuscript.

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Competing interest. None.

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