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Chromosome studies in North-Western Sicily males of *Rhynchophorus ferrugineus*

Antonella LANNINO[1], Luca SINEO[2], Stefania LO BIANCO[2], Vincenzo ARIZZA[2], Barbara MANACHINI[2]
[1]*Dipartimento di Fisica e Chimica, University of Palermo, Italy*
[2]*Dipartimento Scienze e Tecnologie Biologiche Chimiche e Farmaceutiche "STEBICEF", University of Palermo, Italy*

Abstract

Rhynchophorus ferrugineus (Olivier), known as red palm weevil (RPW) was accidentally introduced and established in Sicily (Italy) since 2005. As like in other Mediterranean sites, RPW has been causing extensive damage to palm trees and on its new host *Phoenix canariensis* Chabaud (Canary Island palms), acquired concomitantly with the colonization of the area. RPW shows a good ecological plasticity and slightly but significant morphological polymorphism (colour patterns and length of the rostrum in male individuals) that can be appreciated within different geographical populations. The aim of the present work was to investigate if this ecological plasticity and phenotypic variability can be accompanied by features in chromosomes like changes in diploid number or chromosome morphology, when compared to other RPW populations. Literature data on karyotype analysis reveals a diploid number (2n = 22). In this paper, the karyotypes of different morphotypes of RPW Sicilian populations, collected from *P. canariensis*, were analysed using conventional staining, C-banding and sequential staining with the fluorochromes chromomycin-A_3/4-6-diamidino-2-phenylindole (CMA_3/DAPI). The analyses of metaphases obtained from the testes of adults showed that the species has 2n = 22 chromosomes, with 10 autosomal pairs and a sex chromosome pair. The eu-heterochromatic composition investigated with CTG banding, DAPI, CMA_3, and NOR do not indicate any peculiarity in the populations investigated. *R. ferrugineus* has a Coleoptera Curculionoidea chromosomal asset and functional compartimentalization. RPW presents a karyotype with intermediate characteristics between Dryophthorinae and Curculioninae such as micro Y chromosome and the typical "sphere-shape" of the sexual bivalent in prophase -I or after C-banding.

Key words: red palm weevil, polymorphisms, karyotype, alternative host plant, invasive specie.

Introduction

The role of genomic and chromosomal variation in adaptation and speciation is still highly debated (Seehausen *et al*., 2014). Our knowledge of plant and animal variability is still limited resulting in a slight possibility of theoretical and practical applications.

Nevertheless, over the years cytogenetic bought an increasingly important role in the analysis of kinship between species of the various animal groups. The study of chromosomes, in fact, allows a comparison of genomic DNA organization from different species, or geographical varieties, highlighting similarities or differences that can be interpreted in the light of the phylogenetic reconstruction and their behaviour ecology. Despite is abundance the Coleoptera, the most representative order in the animal kingdom, are still not adequately investigated. This is due also to technical difficulties in karyological studies in Insecta. Light on the complex chromosomal organization of the genome of the invertebrates have been achieved by different karyological techniques of chromosome staining and banding that have been applied during the last decades (Vitturi *et al*., 2003; Colomba *et al*., 2006, Cabral De Mello and Martins, 2010). Actually great improvement to insect cytogenetic arrived with air-drying techniques for chromosome spreads. Classical cytogenetic developed differential staining and sequence specific stains that helped in the analysis of such difficult and hostile chromosomes. Silver impregnation allowed to focus transcriptionally active areas involved in the organization of the nucleolus (Ag-NORs); the C-banding (CTG banding) locates the heterochromatic DNA; fluorochrome staining as DAPI (AT-specific) and chromomycinA_3 (CMA_3, GC-specific) permit hypothesis on the compartmentalization of DNA bases. Molecular cytogenetic identified ribosomal genes localizations of undoubtedly phylogenetic usefulness as rRNAs are highly conserved in eukaryotes (Lopez-Leon *et al*., 1999; Vitturi *et al*., 1999). The family Curculionidae (Coleoptera) has 4600 genera and 51000 described species, including almost 80% of all weevil species (Kuschel, 1995; Oberprieler, 2004a; Oberprieler *et al*., 2007; 2014). Species belonging to Curculionidae occur all over the world, and feed on virtually all plants, mainly angiosperms and in particular monocotyledons which are the principal hosts, of the basal subfamilies Dryophthorinae and Brachycerinae. Therefore monocotyledons are considered the ancestral hosts of Curculionidae and that major driver in the diversification of the family (Marvaldi *et al*., 2002; Oberprieler, 2004b). The Dryophthorinae, associated mainly with palms, are one of the few well-defined and evidently monophyletic curculionid subfamilies, identified by several clear synapomorphies (Kuschel, 1995; Oberprieler *et al*., 2007). Insect pest species present interesting cytogenetic challenges with potential practical applications, such as the insect sterile technique (SIT) an alternative pest control method to the chemical insecticides (Paoli *et al*., 2014).

The red palm weevil (RPW), *Rhynchophorus ferrugineus* (Olivier) (Coleoptera Dryophthorinae), is considered as one of the most damaging pest of palm trees (Faleiro, 2006). The geographic origin of RPW was claimed to be South East Asia and Melanesia (Viado

and Bigornia, 1949) but multiple introductions of RPW to the Middle East, the Mediterranean Basin and US have occurred since mid 1980's through movement of infested offshoots (Faleiro, 2006). In Sicily it was recorded for the first time in 2005 infesting *Phoenix canariensis* Chabaud (Longo *et al*., 2008). Likely, Middle-east populations and the Mediterranean ones are originating from different geographic populations of RPW (El-mergawy *et al*., 2011). Moreover RPW shows high adaptability to different plant hosts, shifting its preferences from native palm trees to other never met plants as the case of *P. canariensis*. Lefroy (1906) described this pest for the first time on coconut palm and it considered as a key pest on coconut palm *Cocos nucifera* L. and on *Phoenix dactylifera* L. (Viado and Bigornia, 1949; Nirula, 1956).

Differences were recorded also in RPW morphology (Longo, 2006) and in biology e.g. time and numbers of post-embryonic instars. Studies in other Curculionidae have shown that karyotype can be different in geographical populations of the same species (Dutrillaux *et al*., 2008) or in population that colonize different hosts (Da Silva *et al*., 2015).

Although many studies were addressed RPW biology and physiology no studies were specifically investigate their intraspecific genetic diversity and polymorphisms that can arose in this adaptable species.

In consideration of the wide geographical distribution of the species, its polymorphism and polyphagia, it is useful to verify the constancy of chromosomal features in RPW populations. This can lead to better understand the possible influence of geographical distribution, isolation, in the possible arose of chromosomal polymorphisms during geographical clusterization. The two already analysed population from Egypt and India RPW, collected on native host *P. dactylifera*, karyotype are organized in 10 autosomal pairs and one pair of heterochromosomes (Bartlett and Rananavare, 1983; Al-Qahtani *et al*., 2014). All chromosomes are metacentrics and no variation has been described. Here we report the karyotype of RPW, collected in Sicily on *P. canariensis*, which is a new host for this species acquired when RPW was accidentally introduced in the Mediterranean countries (Longo, 2006; Longo *et al*., 2008; Manachini *et al*., 2011; 2013) and with different morphotypes.

This paper aims to contribute to the knowledge of the cytogenetic of RPW collected from the new palm host *P. canariensis*, in new areas (Sicily) and having different morphotypes. This will be achieved using several different chromosomal techniques including C-banding for the study of DNA heterochromatic, the analysis of ribosomal transcriptionally active areas (Ag-NORs); and the compartmentalization in genomic DNA bases, with base-specific fluorochromes DAPI and CMA_3.

Materials and methods

Collection of examined individuals

Males of *R. ferrugineus* were collected in Palermo (North-Western Sicily, Italy) in occasion of *P. canariensis* L. cutting down in accordance to phytosanitary measures for control and eradication of pests. The collection was performed in obedience to specific laws and in collaboration with the Regional Phytosanitary Service (Unità Operativa 43 Osservatorio per le Malattie delle Piante di Palermo) and with the "Azienda Regionale Foreste Demaniali (ARFD) della Regione Siciliana". A total of 50 males were collected from different infested *P. canariensis* trees in August 2013. Different morphotype of RPW were considered. They were identified according to the distinct coloration on its pronotum and elytra and through variation in position of the prothoracic spots, as described by Longo (2006).

Slide preparation

After a preliminary examination of the male gonadal maturation (through microscope visual checking of gonad development and sperm motility), a suitable chromosome preparation was obtained from 15 RPW. Cells from gonads and intestines have been prepared according to the technique of the air-drying (Sumner, 1972; Vitturi, 1992).

In particular, the abdomen of insects was separated from the rest of the animal, exposed to four repeated hypotonic treatment with distilled water, for a total time of 20 minutes. Subsequently, the material was fixed in Carnoy's fixative (60% absolute C_2H_5OH; 30% $CHCl_3$; and 10% CH_3COOH.). After 15-20 minutes, the testicular follicles or, alternatively, the intestines were removed and immersed in acetic acid at 55% and comminuted with two very thin needles to obtain a cell suspension. A few drops of this suspension, via a Pasteur pipette, were dropped on the slide previously cleaned with 55% acetic acid and maintained to a temperature of about 50 °C. The liquid was immediately aspirated by holding the pipette perpendicular to the slide surface. The quality of chromosomal preparations were systematically observed under phase contrast microscope.

Banding techniques

Conventional Giemsa staining

The Giemsa staining was performed using a 7.5% solution of pure Giemsa diluted in tap water for about 15-20 minutes at room temperature; subsequently, the product was rinsed and dried to be observed under the microscope.

Giemsa stain allows to observe the spermatozoa with a magnification of 20× under a light microscope DLMB Leica, (Wetzlar, Germany) and the chromosomes to be easy to count and to determine the morphology, information necessary to build the karyotype.

Homologous have been paired, on the basis of the size and position of the centromere; the pairs thus obtained were arranged in descending order with short arms upwards.

Silver impregnation (Ag-NOR)

This technique is applied to highlight nucleolar organizer regions (NORs). The followed protocol that proposed by Howell and Black (1980).

On each slide, with the aid of a Pasteur pipette, we put 5 drops of highly concentrated silver nitrate solution

($AgNO_3$)(1 g of $AgNO_3$ in 2 ml of distilled water) and three drops of a solution of 2% gelatine (1 g of gelatine in 50 ml of distilled water, adding cold 0.5 ml of formic acid, so as to obtain a colloidal solution of "development"). After that the slides were covered with a coverslip and placed on a hot plate at a temperature of 45-50 °C for a time varying from 60 to 90 seconds. The slides were then washed with distilled water, dried and observed under the microscope using normal light. This method allows detection in black portions of chromosomal rDNA transcriptionally active, which are coloured in black, while the rest of Chromosome turns yellow-brown.

C - B a n d i n g

This type of chromosome banding allows to identify the amount and location of heterochromatic DNA (constitutive heterochromatin and heterochromatin associated with NORs) and was carried out by following the method proposed by Sumner (1972). The slides, not previously treated with other types of staining, were subjected to the action, at successive times, three different solutions:

1- in a 6% solution of HCl 12M (6ml of hydrochloric acid at 37% in 94 ml of distilled water) one hour at room temperature;
2- after rinsing the slides in distilled water (in each step of solution), were immersed in a second solution at 5% (saturated) of barium hydroxide octahidrate in distilled water, heated to 50 °C for about 20 seconds;
3- then finally, immersed in the third solution consists of 2×SSC (0.82 g of trisodium-citrate and 1.75 g of sodium chloride in 100 ml of distilled water) at 60 °C (constant) for approximately one hour.

After being washed in tap water, the slides were stained with Giemsa at 7.5% (pH7) for 20 min, then washed, dried and looked at under the microscope.

The heterochromatic DNA looked so more intensely coloured than the heuchromatic one.

D i f f e r e n t i a l s t a i n i n g b y t h e u s e o f b a s e - s p e c i f i c f l u o r o c h r o m e s (D A P I a n d C M A $_3$)

Fluorochromes are dyes that bind, more intensely to certain chromosomal regions rather than others, depending on their base composition (AT or GC). With the fluorescence microscope, equipped with special filters (E4 for CMA_3 and A3 for DAPI), can be seen chromosomal regions more or less fluorescent.

The fluorochromes used in this work are the DAPI (4'-6-diamino-2'-phenylindole) (blue colour) and the CMA_3 (chromomycin A_3) (green colour), respectively for selective DNA rich in bases AT and CG , the staining was carried out according to the protocol proposed by Schmid and Haaf (1984).

In particular, the DAPI staining was carried out by dissolving the fluorochrome in McIlvaine buffer at pH7 (0.5 mg/ml); 50 µl of the solution seats on prepared and adhered evenly using a cover slip. Before observation microscope slides were incubated in a humid chamber overnight.

The colouring fluorochrome CMA_3 requires a 1:20 dilution from a stock solution (1 mg CMA_3 in 500 µl of absolute ethanol) in McIlvaine buffer (pH 7); 50 µl of the preparation were placed on a slide and then incubated in a humid chamber in the dark for 25 minutes covered with a coverslip.

After removing the cover glass, the slide was washed in McIlvaine, dried and mounted in Vectashield antifade 2.5% (Vector Laboratories) for minimizing photobleaching of fluorochromes.

M i c r o s c o p i c o b s e r v a t i o n s , m i c r o p h o t o g r a p h y a n d d i g i t a l r e w o r k i n g

The observations of chromosome preparations were performed with Leica microscope equipped with the necessary for observation in normal light, contrast-phase and fluorescence using a different filter depending on the fluorochrome used. In particular, we used a

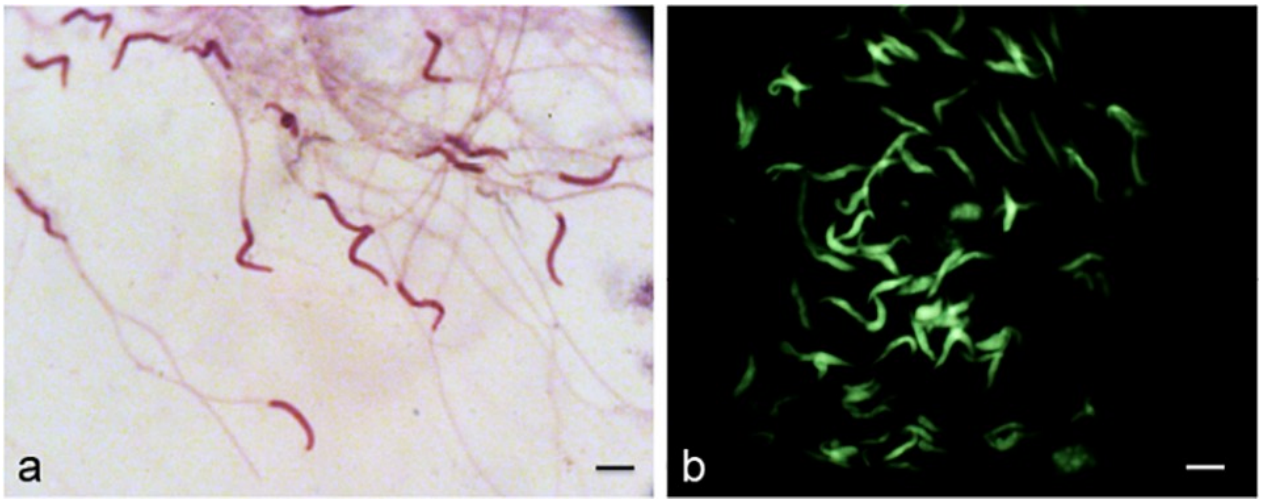

Figure 1. Spermatozoa (a: Giemsa staining; b: CMA_3) of RPW. Populations from Palermo (Italy) collected from infested palm trees of *P. canariensis*. Bar = 10 µm.

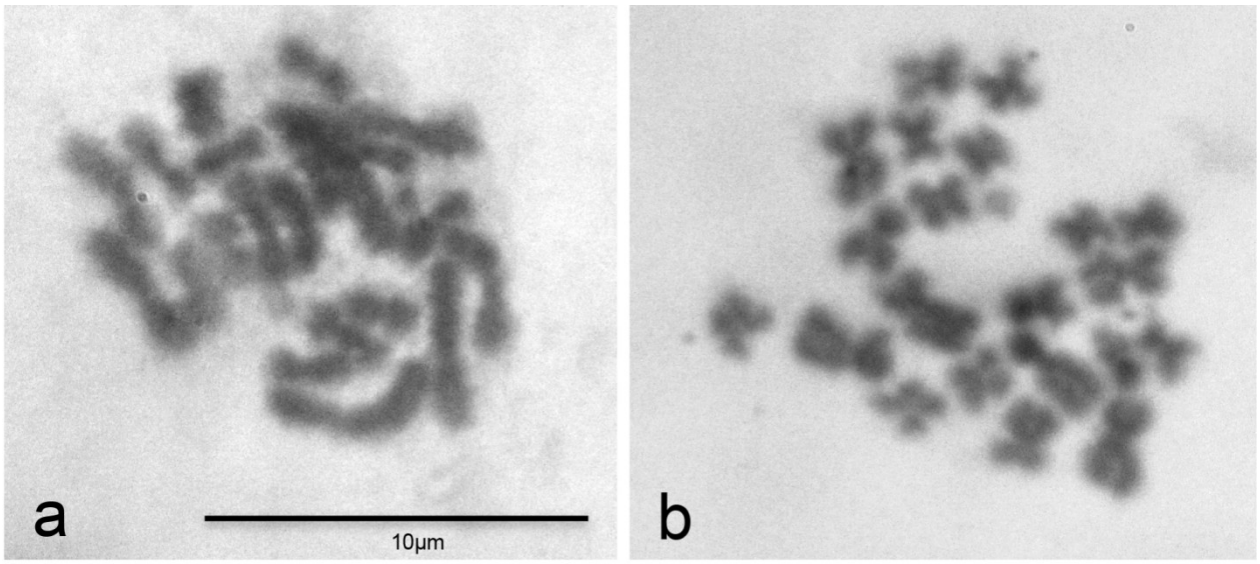

Figure 2. Karyotype (a: meiosis Metaphase-I; b: mitotic Metaphase-I) of males of RPW. Populations from Palermo (Italy) collected from infested palm trees of *P. canariensis*. Bar = 10 µm.

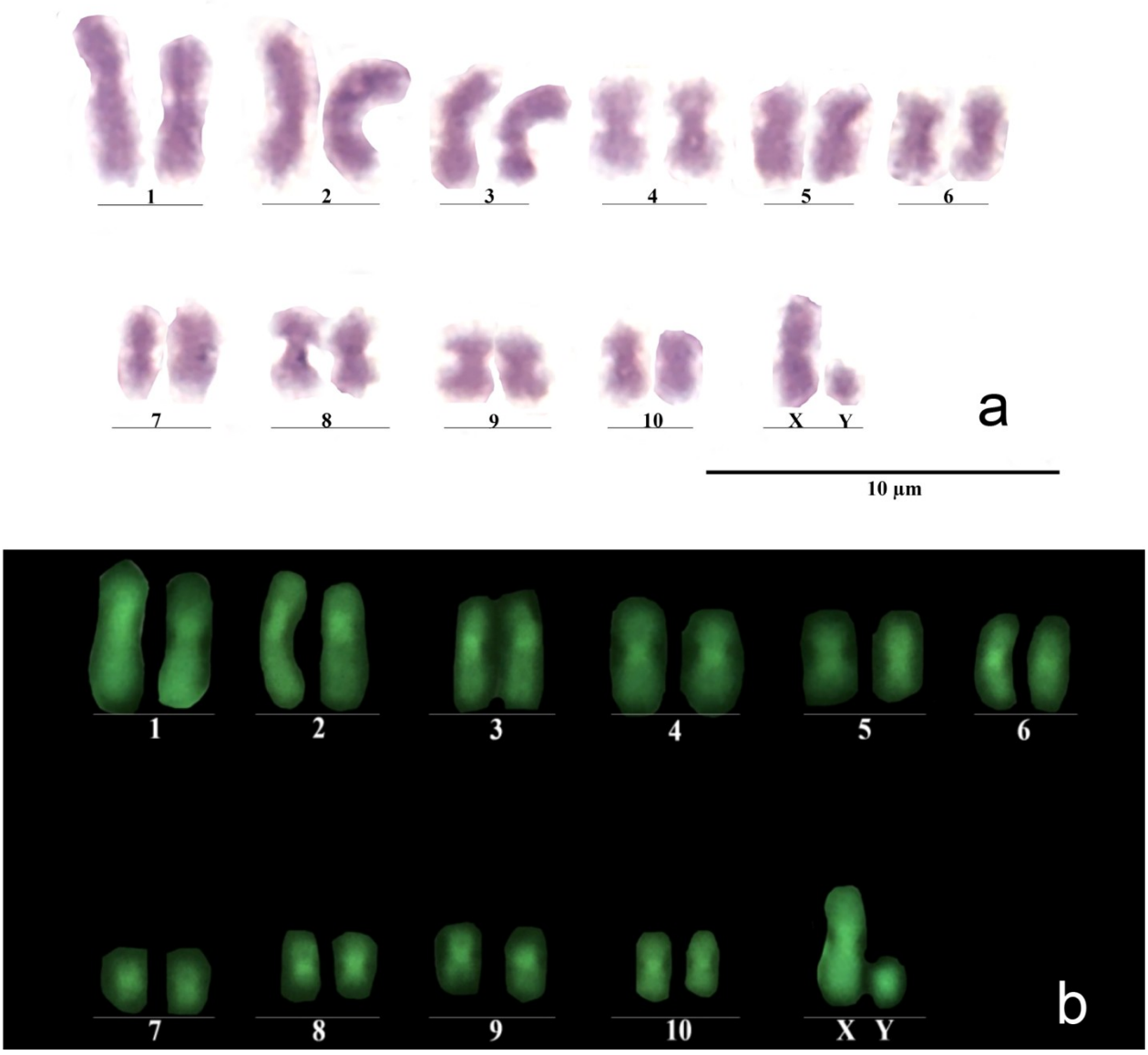

Figure 3. Karyotype found in different morphotypes of males of RPW. Populations from Palermo (Italy) collected from infested palm trees of *P. canariensis*. Chromosomes show Giemsa staining (a) and CMA_3 (b). Bar = 10 µm.

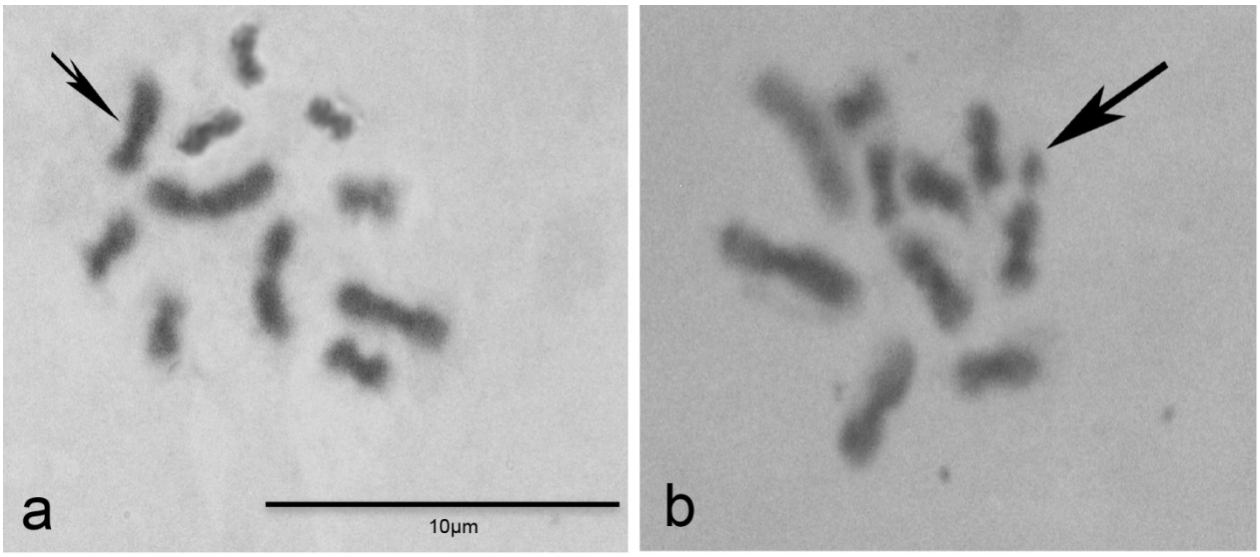

Figure 4. Metaphases-II and karyotypes of males of RPW from Palermo. The arrows indicate the X chromosome (a) and the Y chromosome (b). Bar = 10 µm.

set of filters Leica I3 (BP 450-490; LP 515) for the observation of the preparations treated with the fluorochromes. The photos were taken with camera Canon 600D and processed digitally through the program Adobe Photoshop CS 6.

Results

Chromosomes stained with Giemsa and karyotype

Spermatozoa (figure 1a-b) have been isolated and analysed according to current methodologies (airdrying, e.g. Vitturi, 1992) and do not demonstrated differences in the morphology of the head and flagellum compared to literature data (Barlett and Rananavare, 1983; Alzahrani *et al*., 2013).

Spermatogonial metaphases (figure 2a) and mitotic metaphases (figure 2b) after Giemsa staining indicate a diploid number of chromosomes 2n = 22 in 10 males examined. The homologous chromosomes identified on the basis of their length and position of the centromere, were arranged in 10 pairs of autosomes plus a couple of sex chromosomes (figure 3).

Autosomes (pairs 1-10) were found to be all metacentric while the X chromosome appears submetacentric and the estimated Y chromosome (figure 3a-b and arrow in figure 4a) appeared as a micro acrocentric chromosome with a large area weakly coloured, whose morphology appeared difficult to interpret because of the minute size.

The plates at the metaphase-II, based on the number, were of two types. Figure 3a shows the first type in meiosis II, including X chromosome, with 11, elements between which was always present the X, while the second type (figure 4b) contained the small Y.

Analysis with banding techniques, and DAPI/CMA_3

The C-banding on the spermatogonial plate indicate that heterochromatin spreads evenly in all chromosomes. Interesting to note also the profase I, with a more

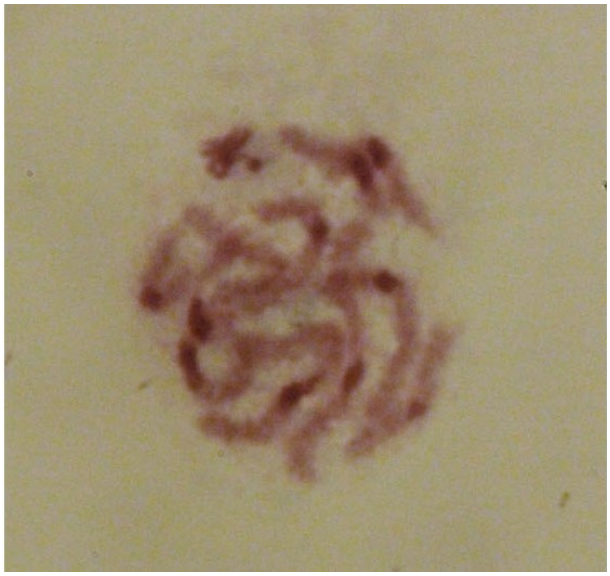

Figure 5. Prophase-I of males of RPW. Chromosomes show C-banding.

intense pericentromeric C-banding (figure 5), and the concentrated form assumed by the bivalent. After silver staining, Ag-Nor, the whole heterochromatic blocks react positively (figure 6). Heterochromatin could be distinguished from euchromatin after staining with fluorochrome base-specific as CMA_3 (GC-specific) (figures 3b and 7a) and DAPI (AT-specific) (figure 7b). It appears more fluorescent than euchromatin after staining with CMA_3 and less fluorescent, instead, after staining with DAPI. The same type of reaction could be observed on the chromosomes to the metaphase-I meiotic (figure 8). The sex chromosome forming a "parachute" was clearly distinguished in metaphase I (figure 9).

Chromosome and morphotype

No remarkable differences were observed on the karyotype according in *R. ferrugineus* morphotypes.

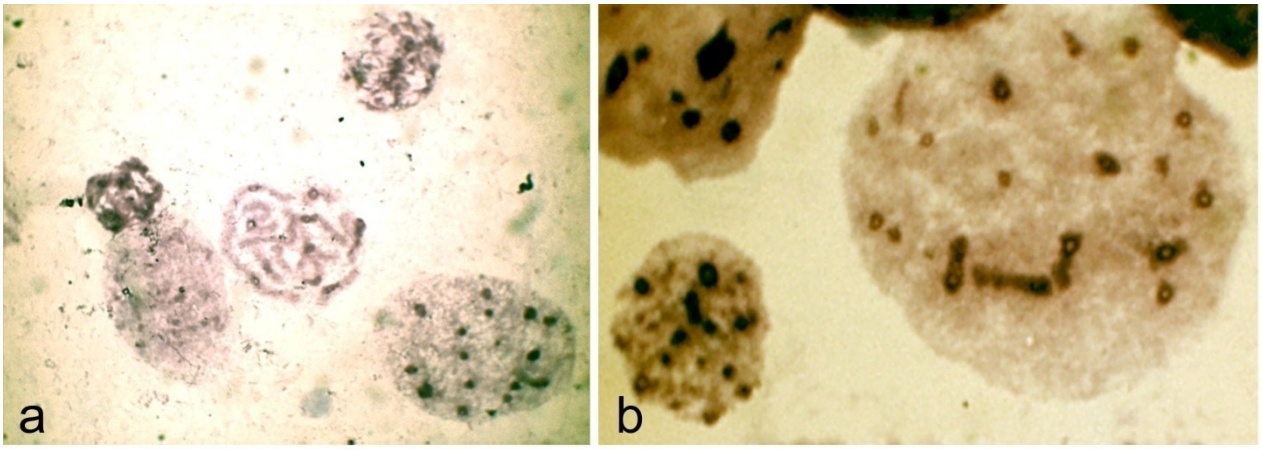

Figure 6. NOR pattern in mitotic cells of RPW submitted to silver nitrate impregnation (a: nucleus, b: prophase).

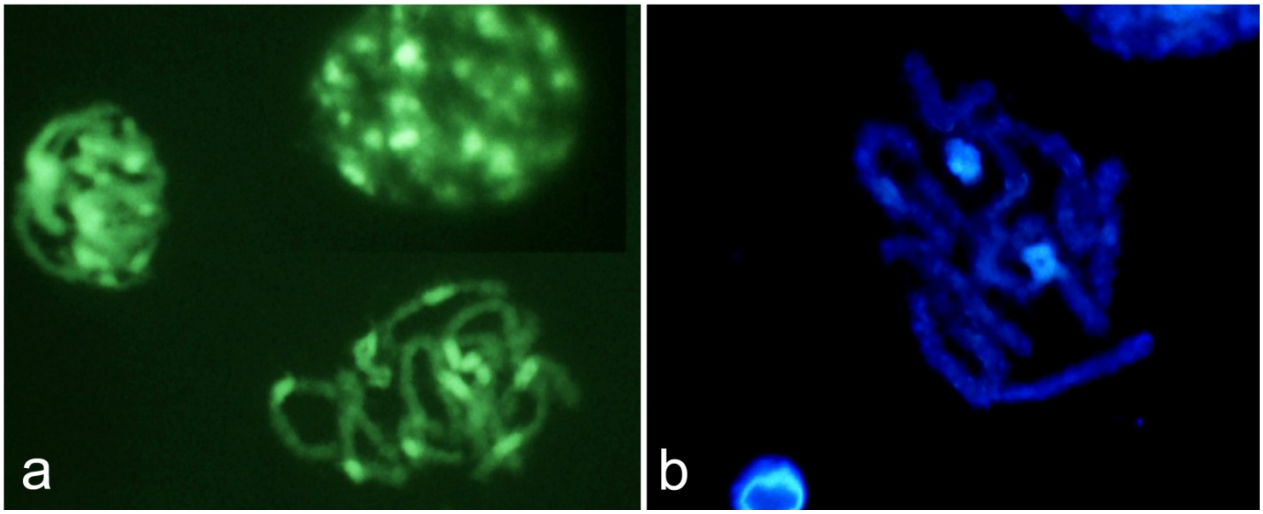

Figure 7. Prophase I in RPW males stained with CMA_3 (a) and Metaphase I (spermatocyte) stained with DAPI (b).

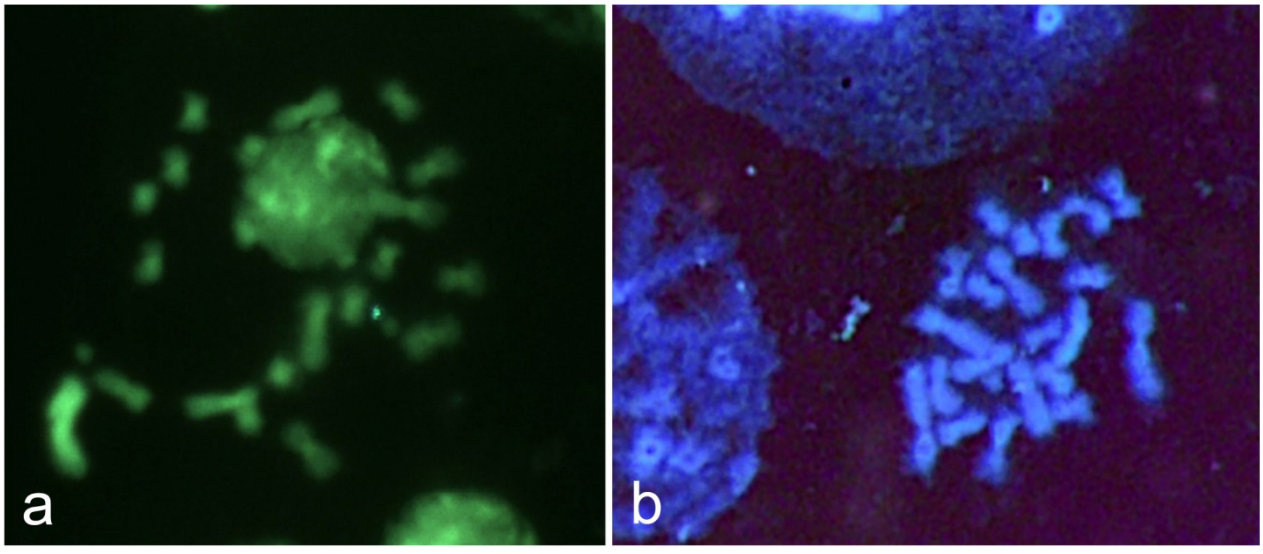

Figure 8. Meiotic metaphase-I of RPW males stained with CMA_3 (a) and DAPI (b).

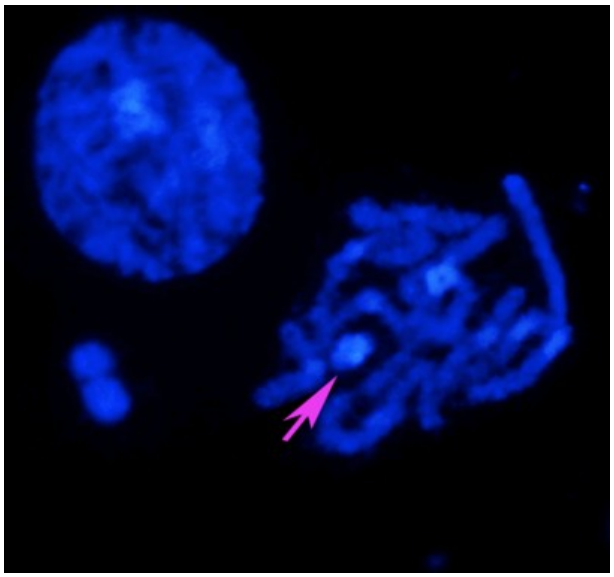

Figure 9. Metaphase I (spermatocyte) in RPW in DAPI staining. Arrow: parachute sex bivalent.

Discussion

Among Curculionidae many species are considered invasive species and able to shift on different host plants and in different geographical areas and climates, demonstrating a great adaptive plasticity at different biological and ecological level. Phenotypic plasticity is a phenomenon that covers all types of environmentally-induced phenotypic variation (Stearns, 1989). It is the ability of a single genotype to produce more than one phenotype, be it morphological, physiological or behavioural in response to environmental conditions (West-Eberhard, 1989). Molecular analysis of living species faces now with the extraordinary level of variability and polymorphisms in genomes. The specific status and phenotypic differences are not easily conducible to genomic differences. A more definite relationship in between genes and phenes can be achieved in the moment species are karyologically distinguishable. Karyotype differences can be found among populations and they may be potentially important for their specific divergence (Da Silva *et al*., 2015). Chromosomal differences are involved in reproductive isolation of animal species and may arose in animal and plant population for a founder effect related to migration and genetic drift.

RPW shows different morphotypes, and the ability to spread over geographical areas adapting quickly to the new plant hosts available and to the new environment. A similar situation was recorded also for other Curculionidae and/or related species of RPW. These organisms, as reported by many scientific articles, have been studied on bio-geographical, behavioural and reproductive point of view (Oberprieler *et al*., 2014). On the contrary, these taxa have been less studied from the genomic and cytogenetic aspect. In particular, the karyological data available on Curculionidae, although referring to nine species, have been initially limited to the determination of the haploid (n) and/or diploid (2n) number of chromosomes and the description of the karyotype of some of them, or to the description of the reproductive system (Bartlett and Rananavare, 1983; Dutrillaux *et al*., 2008). For the Curculionoidea, a diploid chromosome number 2n = 22 is present in 7 of the 9 species analyzed, representatives of the two major groups in which these insects are divided (Holecová *et al*., 2008). Previous results authorize both to propose a high karyological stability in these animals and confirmed that sex determination within the Curculionoidea is driven by Xy_p heterochromosomes as reported by Smith and Virkki (1978). On the contrary some Curculionidae species have shown change in the karyotype according to the geographical distribution and host plants (Dutrillaux *et al*., 2008; Da Silva *et al*., 2015).

Our results on meiotic chromosomes of Sicilian RPW morphotypes suggest that, in spite of phenotypical differences, the individual studied are characterised by a strict homogeneity in chromosome number and morphology, data in accordance to the findings of other authors on other weevil species (Bartlett and Rananavare, 1983; Al-Qahtani *et al*., 2014; Abad *et al*., 2014). These results confirm such an alleged homogeneity in chromosomal organization level. The air-drying technique, associated to the analysis of the chromosomes to mitotic metaphase, in addition to meiotic metaphase-I, allowed us to confirm that RPW karyotype is characterized by metacentrics and submetacentrics autosomes. The karyological uniformity within this family is further confirmed when the analysis is extended to the NORs and to the base composition of heterochromatins (constitutive heterochromatin and NORs associated heterochromatin).

After fluorochrome staining (CMA_3), all the heterochromatic DNA appear compartmentalized in GC rich areas. The affinity with silver nitrate ($AgNO_3$) around the heterochromatic DNA instead of only NORs, is a feature observed in all species belonging to Curculionoidea, having been described in other species belonging to different families of beetles. However, we want to reiterate that this feature is somewhat unusual in other invertebrates (Vitturi *et al.*, 2000).

On the basis of these results we derive some observations:

1- The silver staining, commonly specific to locate the NORs transcriptionally active in almost all organisms, cannot perform this task when applied to the chromosomes of Curculionoidea.
2- In so far examined species of Curculionida, Dryophthoridae, the constitutive heterochromatin is a particular class of heterochromatin when compared with other types of heterochromatin present in invertebrates.
3- Since these highly reactive silver heterochromatic areas may be rich in GC bases as noted in RPW, we can conclude that this reactivity is not correlated with the base composition of genome, but may depend on other factors. One of these could be represented by the presence of silver-congenial proteins throughout the heterochromatic areas of these animals, of which only a portion would be associated with the ribosomal clusters.

4- RPW presents karyotype with intermediate characteristics between the curculionid subfamilies Dryophthorinae and Curculioninae such as micro Y chromosome and the typical "sphere-shape" ("parachute": Dutrillaux *et al*., 2008) of the sexual bivalent in prophase -I or after C-banding.

In addition to the extended chromaffinity of heterochromatin with silver nitrate, as mentioned above, RPW did not differ from other species of beetles. In other invertebrate like *Ommatoiulus oxypygus* (Brandt) and in *Pentodon bidens punctatum* (Villers) the Ag-Nor is clustered on NOR areas (Vitturi *et al*., 2000; 2003). However, until it will be prepared a safe method for observing the activity of NORs in beetles, we should not discard the possibility that, as already observed in some grasshoppers (Lopez-Leon *et al*., 1999; Vitturi *et al*., 2008) and in beetles like *Bubas bison* (L.) (Colomba *et al*., 2006), part of the ribosomal DNA can be present as silent clusters. The karyotype of *R. ferrugineus* reacts to the simple Giemsa staining in the same way in which it reacts after C banding.

The results obtained after treatment with DAPI and CMA_3 base-specific fluorochrome, deserve to be commented for two reasons. The first is that the DAPI/NORs heterochromatin negativity in RPW, and subsequent CMA_3 positivity do not deviates from the results up to now obtained for the other species of beetles analyzed and for other taxa (Torreiro *et al*., 1999), millipede (Vitturi *et al*., 2000), fish (Mandrioli *et al*., 2000; Castro *et al*., 2001) and amphibians (Schmid and Guttenbach, 1988), taxa in which the CMA_3 specifically colours the NORs. It is well documented the data that in higher organisms, the ribosomal transcription units are repeated in tandem, separated by regions NTS (non-transcribed spacers). In general in different animal groups NTS are rich in GC (Dutta and Verma, 1990).

In conclusion, the karyotype of RPW possesses similar features to most species of Curculionidae, and our analyses indicated that the methodologies employed were effective for the characterization of the RPW karyotype and could be further used for comparing karyotypes of other populations of this species. Actually the genomic differences that lead to population morphotypes and to their efficacy in host choice and survival after migration and genetic isolation, in the case of RPW, are not related to different chromosomal phenotypes but presumably, to more fine and hidden genetic features.

Acknowledgements

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Authors' addresses: Barbara MANACHINI (corresponding author: barbara.manachini@unipa.it), Luca SINEO, Stefania LO BIANCO, Vincenzo ARIZZA, Dipartimento Scienze e Tecnologie Biologiche Chimiche e Farmaceutiche "STEBICEF", University of Palermo, via Archirafi 18, 90123 Palermo, Italy; Antonella LANNINO, Dipartimento di Fisica e Chimica, University of Palermo, Ed. 17, viale delle Scienze, 90123 Palermo, Italy.

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