

Grasshoppers (Orthoptera)

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LIST OF ABBREVIATIONS

AFLP, amplified fragment length polymorphism
 BSA, bovine serum albumin
 CMA₃, chromomycin A₃
 Cot, concentration of DNA and time
 DA, distamycin A
 DAPI, 4',6 -diamidino-2-phenylindole
 dATP, deoxyadenosine triphosphate
 dCTP, deoxycytidine triphosphate
 dGTP, deoxyguanosine triphosphate
 DNA, deoxyribonucleic acid
 DNase, deoxyribonuclease
 dNTPs, deoxynucleotide triphosphates

DOP, degenerate oligonucleotide-primed
 DPX, distrene 80, plasticizer, xylene
 DTT, dithiothreitol
 dTTP, deoxythymidine triphosphate
 dUTP, 2'-deoxyuridine 5'-triphosphate
 EDTA, ethylenediaminetetraacetic acid
 FIAD, Feulgen image analysis densitometry
 FISH, fluorescence in situ hybridization
 IOD, integrated optical density
 ITS, internal transcribed spacers
 JPEG, joint photographic experts group
 LTRs, long terminal repeats
 NOR, nucleolus organizer region
 PBS, phosphate buffered saline
 PBT, phosphate buffered Tween 20
 PCR, polymerase chain reaction
 RAPD, random amplified polymorphism DNA
 rDNA, ribosomal DNA
 RNase, ribonuclease
 RPM, revolutions per minute
 SDS, sodium dodecyl sulfate
 SSC, saline sodium citrate
 TAE, Tris-acetate
Taq, *Thermus aquaticus*
 TE, Tris-EDTA
 TIFF, tagged image file format
 WGA, whole genome amplification

11.1 INTRODUCTION

11.1.1 Taxonomy

Grasshoppers belong to the insect order Orthoptera, which also includes locusts and crickets. This order comprises more than 26,000 species (<http://orthoptera.speciesfile.org>) with global distribution and higher diversity in the tropics. The name of the order is derived from “orthos,” meaning “straight,” and “pteron,” meaning “wing.” Most orthopteran species are included in two main suborders: Ensifera, including the long-horned grasshoppers (superfamily Tettigonioidea) and several types of crickets (Grylloidea and Gryllacridoidea), and Caelifera, including the short-horned grasshoppers (Acridoidea), the grouse locusts (Tetragoidea) and the pigmy mole crickets (Tridactyloidea).

Although most protocols described here were developed for caeliferan insects, many of them are also useful for orthopterans and other insects. Approximately half of the known orthopteran

species belong to the superfamily Acridoidea (more than 7600 species), with the Acrididae being the most diverse family. Although Acridoidea is considered monophyletic, the internal relationships between families are not well understood (Song 2010), but recently, Leavitt et al. (2013) have contributed to elucidate this topic by using the entire mitogenome for phylogenetic analysis. Acridid grasshoppers are phytophagous, although many species can be omnivores (even cannibalistic) when cultured in the laboratory. Females lay several clutches of eggs (egg pods) in the ground during their life (approximately one pod per week, depending on the species). Grasshoppers usually show cryptic coloration (Rentz 1991), and their most noticeable characteristic is the ability to jump. As part of the courtship ritual, males in many species “sing” by via stridulation of their rear legs and forewings.

11.1.2 Importance of Grasshopper Species

Approximately 20 acridid species in several different subfamilies exhibit gregarious behavior and migrate in dense swarms; these severe pests generate massive damage to crops. The most well known of these species are the desert locust (*Schistocerca gregaria*) and the migratory locust (*Locusta migratoria*) in Africa and the Middle East; *Schistocerca piceifrons* in tropical Mexico and Central America; *Melanoplus bivittatus*, *M. femurrubrum*, *M. differentialis*, and *Camnula pellucida* in North America; *Romalea guttata*, *Brachystola magna*, and *Sphenarium purpurascens* in northern and central Mexico; some species of *Rhammatocerus* in South America; and *Oedaleus senegalensis* and *Zonocerus variegatus* in Africa.

Grasshoppers constitute part of the diet in some African, American, and Asian countries, as they are a source of protein and fat. A recent FAO Forestry paper (Van Huis et al. 2013) indicates that approximately 80 grasshopper species are consumed worldwide. Most grasshopper species are edible, so they, and other insects, can be used for food. Locusts are particularly easy to harvest when they swarm. In Africa, the desert locust, the migratory locust, the red locust (*Nomadacris septemfasciata*), and the brown locust (*Locustana pardalina*) are eaten, although the insecticide treatment of these pests calls for caution in their consumption. This problem does not apply to nonswarming grasshoppers that are easily captured or cultured. Some countries with long traditions of using grasshoppers and/or crickets for food are Mexico, Niger, Thailand, the Lao People's Democratic Republic, and Cambodia, where these insects are farmed.

11.1.3 Karyotype

The large size and low number of grasshopper chromosomes have significantly contributed to the general understanding of chromosome structure and function during mitosis and meiosis. No other organism provides such a convenient and complete collection of stages showing the complete course of meiosis, that is, the most complex type of cell division. The pioneer work of McClung (1902) describing male grasshopper meiosis opened a long series of meiotic studies using grasshoppers as the preferred material. Even today, male grasshopper meiosis is visualized in student practical activities in many universities. Key topics of chromosome biology, such as chromosome structure, condensation, pairing, movement, chiasma formation, and chromosome rearrangements, have been elucidated using grasshopper cytogenetic materials. Grasshopper meiosis can also be analyzed in vivo in short-term cultures of spermatocytes (Nicklas 1961; Rebollo and Arana 1995; Rebollo et al. 1998), and grasshoppers are one of the few animals in which female meiosis has been analyzed in detail (Hewitt 1976; Henriques-Gil et al. 1987; Cano and Santos 1989).

Most grasshopper species, especially those in the family Acrididae, have 23 chromosomes in males and 24 in females. This difference is due to their X0/XX sex chromosome determinism. The karyotype composed from $2n = 23, X0\delta/24, XX\phi$ is considered atavistic, at least for Caelifera representatives (White 1973; Hewitt 1979). Between species, variation in chromosome number and derived sex systems, such as neo-XY and neo- X_1X_2Y , occurs in some Acridid groups (Hewitt 1979, Castillo et al. 2010), and there are also cases of extensive intraspecific variation caused by polymorphic chromosome rearrangements, supernumerary segments, and supernumerary (B) chromosomes (Hewitt 1979; Jones and Rees 1982; Camacho et al. 2000; Camacho 2004, 2005).

Many grasshopper species have an acro/telocentric chromosome morphology, with most chromosomes appearing to have a single arm with the centromere placed close to one end. The karyotypes are composed of a continuous series of chromosomes gradually decreasing in size, which frequently complicates their identification. However, grasshopper autosomes are classified into three size groups, that is, long (L), medium (M), and short (S). In many cases, the frontier between the L and M autosomes is marked by the size of the X chromosome, whereas between the M and S autosomes is marked by the so-called "megameric bivalent." This autosomal bivalent shows positive heteropyknosis (high condensation) during meiotic prophase (Corey 1938). It is usually the ninth autosomal bivalent in size in species with $2n\delta = 23$ ($n = 11 + X0$) and the sixth one in species with $2n\delta = 17$ ($n = 8 + X0$). As a

borderline bivalent, the megameric bivalent is named M9 (or M6) in some species but S9 (or S6) in others. Similarly, the X chromosome is similar in size to the L chromosomes in some species but similar to the M chromosomes in others (Camacho 1980).

Chiasma frequency in grasshoppers has profusely been used in comparisons between sexes (Fletcher and Hewitt 1980; Cano et al. 1987; Cano and Santos 1990), B chromosome effects (John and Hewitt 1965; Cano and Santos 1988; Camacho et al. 2002), temperature or x-ray effects (Church and Wimber 1969), and changes associated with locust phase transformation (Dearn 1974).

The C-banding technique (Sumner 1972) allowed the characterization of heterochromatin distribution in many species of grasshoppers (King and John 1980; Santos et al. 1983; Cabrero and Camacho 1986a), and the silver impregnation technique (Goodpasture and Bloom 1975; Rufas et al. 1982) revealed the localization of the active nucleolus organizer regions (Cabrero and Camacho 1986b). Similarly, triple CMA₃-DA-DAPI staining (Schweizer 1980) provided information on the chromosome location of chromatin regions that are rich in A+T or G+C, as these regions preferentially bind to the DAPI or CMA₃ fluorochromes, respectively (Schweizer et al. 1983; John et al. 1985; Camacho et al. 1991).

More recently, fluorescence in situ hybridization (FISH) has opened the door to the physical mapping of several repetitive DNA families, such as 45S ribosomal DNA (rDNA) (Cabrero and Camacho 2008), histone genes (Cabrero et al. 2009), and 5S rDNA (Cabral de Mello et al. 2011a,b). In addition, the mapping of satellite DNAs and transposable elements has elucidated B chromosome evolution in the species *Eyprepocnemis plorans* (Cabrero et al. 2003; Montiel et al. 2012).

11.1.4 Genome Size

Grasshopper genomes are among the largest genomes in insects. For instance, the migratory locust has a C value (6 pg) that is double that of human beings, and it is even higher in the grasshopper *Podisma pedestris* (16.93 pg) (Westerman et al. 1987). The mass of total genomic DNA is known in only 39 grasshopper species (Animal Genome Size Database: <http://www.genomesize.com>) (Hanrahan and Johnston 2011), and most of these estimations were evaluated by microdensitometry. Currently, flow cytometry (Geraci et al. 2007), Feulgen Image Analysis Densitometry (FIAD) (Hardie et al. 2002), and real-time PCR (Wilhelm et al. 2003) are the most common techniques for measuring genomic DNA. Given the excellent correlation between genome size measurements performed by flow cytometry and FIAD (Dolezel et al. 1998), the simplicity, ease, accuracy, and cost-effectiveness of the latter (Hardie et al. 2002) make it the best choice for most cytogenetic laboratories.

11.1.5 Genome Sequencing Projects

Because of their huge size, grasshopper genomes constitute a challenge for full sequencing projects. In spite of that, the decreasing costs of high-throughput sequencing methods have recently allowed the publication of the first complete draft sequence in the migratory locust (*L. migratoria*), which is the largest animal genome hitherto sequenced (Wang et al. 2014). It is thus presumable that the genomes of other species nominated for sequencing in the i5k initiative, including some *Schistocerca* and *Chorthippus* species (accessed on July 31, 2013, at http://arthropodgenomes.org/wiki/i5K_nominations), will be promptly sequenced.

The 6.3 Gb *L. migratoria* draft genome sequence published has uncovered some interesting characteristics of this genome, compared to the genomes of other insects. For instance, whereas there is no difference in the length of coding regions, compared with *Drosophila melanogaster*, the *L. migratoria* genome shows much longer introns and intergenic regions, presumably because of the proliferation of mobile elements combined with slow rates of loss for these elements (Wang et al. 2014). These authors have performed the most complete genomic analysis ever published in a single paper, by also performing methylome and transcriptome analyses. This has revealed complex regulatory mechanisms involved in microtubule dynamic-mediated synapse plasticity during phase change, and expansion of gene families associated with energy consumption and detoxification, the latter being consistent with long-distance flight and phytophagy characteristics of gregarious locusts. Remarkably, these authors have found in this genome hundreds of potential insecticide target genes, thus offering new insights into the biology and sustainable management of this pest species.

11.2 PROTOCOLS

11.2.1 Biological Materials and Grasshopper Culture

The easiest way to visualize grasshopper chromosomes is to analyze male meiosis because no colchicine pretreatment is necessary. Female meiosis is also amenable to analysis, but the technique is rather complex, and the resulting cells cannot be used for chromosome-banding techniques (Hewitt 1976; Henriques-Gil et al. 1987). The best mitotic chromosomes are obtained from embryos, especially from the neuroblast cells. In adult females, mitosis can be visualized in cells from ovariole walls or gastric caeca.

When collecting grasshoppers in the field, the first challenge for beginners is to distinguish males from females. In all grasshopper

species, females are larger than males, and the end of the abdomen is pointed in females but rounded in males (<http://keys.lucidcentral.org/keys/grasshopper/nonkey/html/Gender/Gender.htm>)

Field-collected males and females can be prepared for cytological analysis or maintained in the laboratory for controlled crosses, obtaining embryos for cytological analysis or next-generation adults. Culture conditions are simple, as grasshoppers have scarce requirements: 27°C–30°C with 30% humidity and a 12:12 photoperiod (Figure 11.1a and b), although these conditions can vary among species. They can be fed almost anything, but lettuce, cabbage, and bran work well for most species.

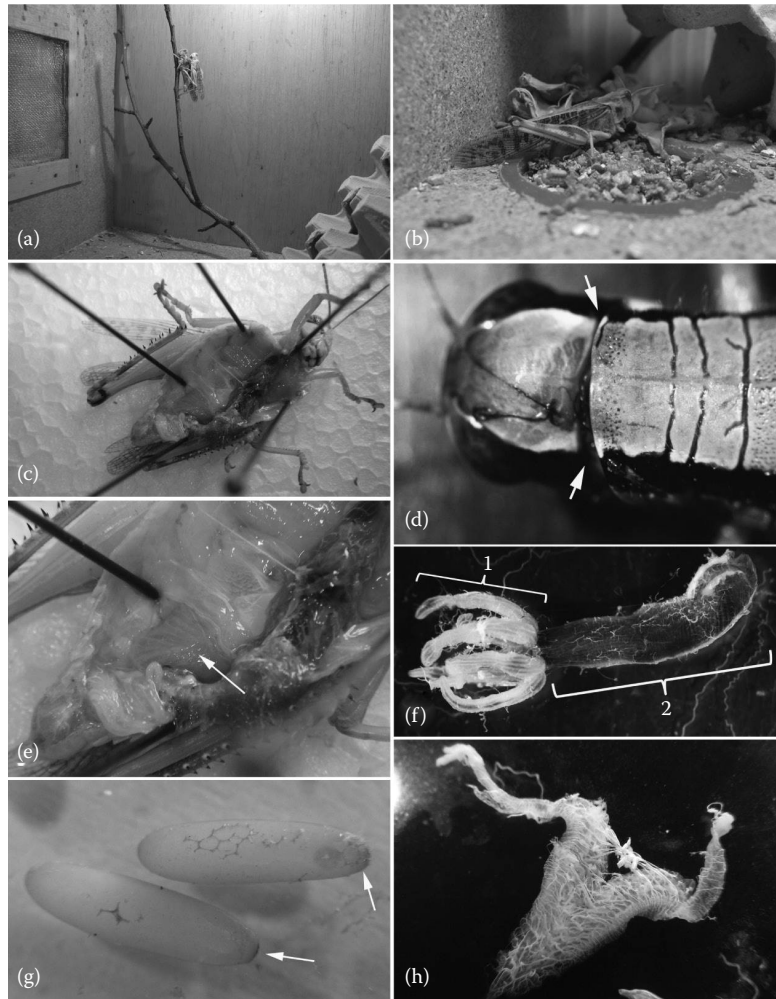


FIGURE 11.1 (See color insert.) Grasshopper culture and dissection of appropriate tissues for chromosome analysis. (a) Mating pair of *Locusta migratoria*; (b) laying female of *L. migratoria*; (c) dissected male of *Eyprepocnemis plorans* showing a yellowish mass (indicated by an arrow in e) corresponding to the testes; (d) dorsal view of the head and pronotum of the grasshopper *Parascopas sanguineus* showing where to cut (arrows) for a rapid dissection of gastric caeca (1 in f) and gizzard (2 in f); (g) eggs showing the micropyle end (arrows); (h) ovaries.

11.2.2 Equipment, Materials, and Reagents

A laboratory used to perform the protocols included in this chapter should be equipped with the following:

Equipment

- Fluorescence microscope (Cat. No. 909, Olympus BX41, Olympus, Tokyo, Japan) with a digital camera (Olympus DP70) and appropriate filter set
- Zoom Stereomicroscope (Cat. No. SMZ-1000, Nikon, Tokyo, Japan)
- Thermal cycler Mastercycler ep gradient S (Cat. No. 13038553, Eppendorf, Hamburg, Germany)
- Freezers (-20°C and -80°C) (Cat. No. 365GTL, AEG; Cat. No. 14230-102, VWR, Radnor, PA)
- Refrigerators Samsung no frost (Cat. No. RL58GEGSW1, Samsung, Seoul, South Korea)
- SW22 Shaking Water bath (Cat. No. 9550322, Julabo, Seelbach, Germany)
- Biological safety cabinet ESCO Class II (Cat. No. SC2-4A1, ESCO, Singapore)
- Mini-sub cell GT, Electrophoresis apparatus; PowerPac 3000 (Cat. No. 166-4288EDU, Cat. No. 165-5056, Bio-rad, CA)
- UVP Visi-Blue transilluminator (Cat. No. UV95-0461-01, Fischer Scientific, Hampton, NH)
- BioPhotometer plus (Cat. No. 6132 000.008, Eppendorf)
- Universal Precision Ovens (Cat. No. 2005151, Selecta, Barcelona, Spain)
- TransferMan NK 2, Micromanipulator (Cat. No. 920000011, Eppendorf) coupled to an inverted microscope (Axiovert 200, Zeiss, Jena, Germany) for chromosome microdissection
- Hot plate X5 (Cat. No. 23-PC800, Bio-Optica, Milan, Italy)
- Microcentrifuge 5415D (Cat. No. 022621408, Eppendorf)
- Autoclave (Cat. No. AHS-75 N, Raypa, Barcelona, Spain)
- Shaking platform MVH-40 (Cat. No. 2063MVH40, ICT, SL, La Rioja, Spain, Lardero, La Rioja, Spain)

Materials

- Coplin jar (Cat. No. 12954000, Endo glassware, Beijing, China)
- Coverslips (Cat. No. BB018018A1, Menzel-Gläser, Braunschweig, Germany)
- Dissecting scissors (Cat. No. 72940, Dumont, Montignez, Switzerland)
- Dissecting tweezers (Cat. No. 72873D, Dumont)

- Eppendorf micropestle for 1.2- to 2-mL tubes (Cat. No. Z317314, Sigma-Aldrich, St. Louis, MO)
- Filter paper (Cat. No. 1305, Filtros Anovia, Barcelona, Spain)
- Homogenizer (Cat. No. 6102, Kartell, Melbourne, Australia)
- Laboratory film (Cat. No. PM996, Parafilm, Pechiney Plastic Packaging Company, Chicago, IL)
- Micropipette different volumes Nichipet (Cat. No. NPX-2, NPX-20, NPX-200, NPX-1000, Nichiryō, Tokyo, Japan)
- Microcentrifuge tube Eppendorf (Cat. No. 175508N, Daslab, Barcelona, Spain)
- Petri dish (Cat. No. P 9.0-720, Soria Genlab, Madrid, Spain)
- Razor blade (Cat. No. 61204100, Nahita, Auxilab SL, Beriáin, Navarra, Spain)
- Microscope Slides (Cat. No. AB00000112E, Menzel-Gläser)

Reagents

- Acetic acid glacial (Cat. No.131008.1211, Panreac, Barcelona, Spain)
- Acridine orange solution (Cat. No. A9231, Sigma-Aldrich)
- Agarose (Cat. No. A9539, Sigma)
- Anti-digoxigenin-rhodamine (Cat. No. 11207750910, Roche, Basilea, Switzerland)
- Barium hydroxide octahydrate (Cat. No. 101737, Merck, Hunterdon County, NJ)
- BioNick DNA Labeling System (Cat. No. 18247-015, Invitrogen, Life Technologies, Carlsbad, CA)
- BSA (bovine serum albumin) (Cat. No. A3294, Sigma-Aldrich)
- Calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) (Cat. No. 131232, Panreac)
- Chloroform (Cat. No. EC 200-663-8, Amresco, Solon, OH)
- Citric acid (Cat. No. 131808, Panreac)
- Colchicine (Cat. No. C9754, Sigma-Aldrich)
- Chromomycin A (Cat. No. C2659, Sigma-Aldrich)
- DAPI (4', 6'-diamidino-2-phenylindole) (Cat. No. D9542, Sigma-Aldrich)
- Dextran sulfate (Cat. No. D8906, Sigma-Aldrich)
- DIG-nick translation mix (Cat. No. 11 745 816 910, Roche)
- Disodium phosphate (Na_2HPO_4) (Cat. No. 141655.1210, Panreac)

- Distamycin A (Cat. No. D6135, Sigma-Aldrich)
- Dithiothreitol (Cat. No. 43815, Sigma-Aldrich)
- DNA polymerase I/DNase I (Cat. No. 18162-016, Invitrogen)
- dNTPs set (Cat. No. DNTP10, Sigma-Aldrich)
- DPX (mountant for microscopy) (Cat. No. 36029, BDH, VWR)
- Ethanol (Cat. No. 121086.1211, Panreac)
- Formamide (Cat. No. F7503, Sigma-Aldrich)
- Formaldehyde (Cat. No. F8775, Sigma-Aldrich)
- Formic acid (Cat. No. 131030, Panreac)
- GenElute PCR Clean-Up Kit (Cat. No. NA1020, Sigma-Aldrich)
- GenomePlex WGA Reamplification Kit (Cat. No. WGA3, Sigma-Aldrich)
- GenomePlex Single Cell Whole Genome Amplification Kit (Cat. No. WGA4, Sigma-Aldrich)
- Giemsa (Cat. No. 1.09204, Merck)
- Glycogen (Cat. No. 10 901 393 001, Roche)
- Hydrochloric acid (HCl) (Cat. No. 20 252 290, BDH, VWR)
- Hyperladder DNA marker (Cat. No. BIO-33039, Bioline, London, UK)
- Illustra GenomiPhi V2 DNA Amplification Kit (Cat. No. 25 6600 30, GE Healthcare Life Sciences, Little Chalfont, UK)
- Isoamyl alcohol (Cat. No. 121372.1611, Panreac)
- Labeled nucleotide, Fluorescein 12-dUTP (Cat. No. 11 373 242 910) and Tetramethylrhodamine-5-dUTP (Cat. No. 11 534 378 910, Roche)
- Lactic acid (Cat. No. 141034.1211, Panreac)
- Magnesium chloride ($MgCl_2$) (Cat. No. M1028, Sigma-Aldrich)
- Monopotassium phosphate (KH_2PO_4) (Cat. No. 131509, Panreac)
- Orcein (Cat. No. 251324.1604, Panreac)
- Paraformaldehyde (Cat. No. P6148, Sigma-Aldrich)
- Pepsin (Cat. No. P6887, Sigma-Aldrich)
- Phenol (Cat. No. P4682, Sigma-Aldrich)
- Potassium chloride (KCl) (Cat. No. 131494, Panreac)
- Potassium ferricyanide ($K_3[Fe(CN)_6]$) (Cat. No. P4066, Sigma-Aldrich)
- Propionic acid (Cat. No. P1386, Sigma-Aldrich)
- Proteinase K (Cat. No. P2308, Sigma-Aldrich)
- RNase A (Cat. No. R6513, Sigma-Aldrich)
- Salmon testes DNA (Cat. No. D7656, Sigma-Aldrich)
- S1 nuclease (Cat. No. 18001-016, Invitrogen)

- Schiff's reagent (Cat. No. 3952016, Sigma-Aldrich)
- Silver nitrate (Cat. No. 101512, Merck)
- Sodium acetate (Cat. No. 131633.1210, Panreac)
- Sodium bicarbonate (NaHCO_3) (Cat. No. S5761, Sigma-Aldrich)
- Sodium chloride (NaCl) (Cat. No. 121659.1211, Panreac)
- Tri-sodium citrate ($\text{C}_6\text{H}_5\text{Na}_3\text{O}_7$) (Cat. No. 141655.1210, Panreac)
- SDS (sodium dodecyl sulfate) (Cat. No. L5750, Sigma-Aldrich)
- Sodium hydroxide (NaOH) (Cat. No. 131687.1210, Panreac)
- Sodium metabisulfite (Cat. No. 13459, Sigma-Aldrich)
- Sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) (Cat. No. S7026, Sigma-Aldrich)
- SpectrumOrange dUTP (Cat. No. 02N33 050, Vysis, Abbott Molecular, Abbott Park, IL)
- Streptavidin-Alexa Fluor 488 Conjugate (Cat. No. S11223, Life Technologies, ThermoFisher Scientific)
- SYBR safe DNA gel stain (Cat. No. S33102, Invitrogen)
- *Taq* DNA polymerase (Cat. No. P0023, Canvax, Córdoba, Spain)
- Tris (Cat. No. A7455, AppliChem, Darmstadt, Germany)
- Tween 20 (Cat. No. P5927, Sigma-Aldrich)
- Ultrapure water (Cat. No. W4502, Sigma-Aldrich)
- VECTASHIELD mounting medium (Cat. No. H-1000, Vector Laboratories, Burlingame, CA)

11.2.3 Sources of Chromosomes

In adults, the best mitotic cells are obtained from gastric caeca in males and females and from female ovarioles. Testes do not require colchicine pretreatment and provide convenient mitotic metaphases from pre-meiotic spermatogonial cell divisions. In the remaining cases, colchicine treatment is necessary to increase the proportion of cells in mitotic metaphase, which is the best stage to visualize chromosomes for karyotyping and physical mapping. Undoubtedly, the best source of mitotic chromosomes is embryos obtained from eggs incubated in the laboratory.

11.2.4 Tissue Extraction and Fixation

11.2.4.1 Mitotic Chromosomes in Adults

The following protocol is quite simple and useful for obtaining mitotic plates from ovarioles and gastric caeca, which avoids the requirement of laboratory strain maintenance. Although it is

possible to obtain good metaphase plates using this approach, the number of cells could be reduced, depending on the animal.

1. Dissolve colchicine in insect saline solution to a final concentration of 0.05% and inject it into the abdomen in amounts corresponding to body size. For an average grasshopper, 0.1 mL may be enough.
2. After 6–8 hours, anesthetize the animal in ethyl acetate vapors and remove the desired organ.
3. With scissors, make a longitudinal and ventral cut of the abdomen (Figure 11.1c), open it with entomological pins, and, with forceps, extract the testes (Figure 11.1e) or the ovarioles (Figure 11.1h), which are located under the digestive tube. Gastric caeca can also be obtained this way or via a dorsal cut between the head and the thorax (Figure 11.1d and f). Immediately fix all the materials in freshly prepared 3:1 solution of absolute ethanol–acetic acid.
4. Alternatively, place the material in potassium chloride (KCl) hypotonic solution (0.75%) for 45–60 minutes before fixation to improve chromosome spreading.
5. Then, immerse the materials in fresh 3:1 absolute ethanol–acetic acid for 1 hour at room temperature (RT) and store them at 4°C or –20°C.
6. In ovarioles, the interesting part is the terminal filament. Under a stereomicroscope, dissect the ovarioles and eliminate the developing egg (if it is large). Similarly, clean gastric caeca (in glacial acetic acid) to remove digestive remains.

In some cases, we want to obtain colchicine-treated mitotic metaphase cells and use body remains without the effect of colchicine, for example, to extract RNA. In this case, we dissect the animal, extract the desired organ for chromosome analysis (e.g., ovarioles and/or gastric caeca) and immerse it in 2% colchicine in insect saline for 2–6 hours. They are then fixed in 3:1 ethanol/acetic acid and, after a 1-hour fixation at RT, are stored at 4°C until study. Body remains are immediately frozen in liquid nitrogen and stored at –80°C.

11.2.4.2 Mitotic Chromosomes in Embryos

Embryos are excellent for chromosome studies because many mitotic metaphases are obtained from a single embryo, and the chromosomes from these cells are easy to spread, providing high-quality material for chromosome banding and physical mapping. In addition, embryo neuroblasts are the very best cells for karyotyping, banding, and mapping. These cells have very large chromosomes because of a special low-condensation state, allowing

the detection of very thin bands that are difficult to detect in other types of cells. To obtain embryos and fix them for cytological analysis, we proceed as follows:

1. Maintain gravid females in culture cages with humid vermiculite (or sand) to facilitate laying (Figure 11.1b). Monitor vermiculite every day for egg pods.
2. Place egg pods in a petri dish with 10% humid vermiculite and incubate at 25°C–28°C, with readjustments to humidity every 4–5 days. Fix mitosis-rich embryos before they enter diapause, typically before embryo eyes are pigmented. Depending on the species, the appropriate incubation period to obtain an optimum number of mitotic metaphase cells varies, for example, from 6 days in *L. migratoria* to 10 days in *E. plorans* or even 15 days in *Chorthippus jacobsi*.
3. At the end of the incubation period, immerse egg pods in insect saline solution and dissect them to separate the eggs and extract the embryo from each egg. For this purpose, perform a transversal cut close to the micropyle end, which has a crown of minute holes (Figure 11.1g).
4. Immerse embryos in 2 mL 0.05% colchicine in insect saline solution for 2 hours and in 2 mL distilled water (hypotonic treatment) for 10–15 minutes, depending on the species.
5. Immerse embryos in 3:1 absolute ethanol–acetic acid for 1 hour at RT and store them at 4°C.

11.2.4.3 Meiotic Chromosomes

Meiosis is easy to observe in adult males. No colchicine treatment is necessary, and it is preferable not using it. Female meiosis is more difficult to observe, but Henriques-Gil et al. (1987) developed a protocol for observing primary and secondary oocytes from metaphase I onward.

Male meiosis is analyzed in testis tubules of adult individuals previously anaesthetized with ethyl acetate vapors to extract the testis mass (a yellowish mass dorsally placed in the abdomen and including both testes together) (Figure 11.1e).

Testes are immersed in freshly prepared 3:1 absolute ethanol–acetic acid in a tube, which is vigorously shaken to separate the testis tubules (if necessary, the tubules in the fixative could be separated with two needles under a stereomicroscope). The fixative should be exchanged with fresh solution several times. After approximately 1 hour at RT, the fixed material should be stored at 4°C or at –20°C.

Alternatively, a small amount of 0.05% colchicine dissolved in insect saline solution could be injected in the abdomen of the

animal 5–8 hours before fixation and follicle dissection. In addition, the testis follicles could be placed in potassium chloride (KCl) hypotonic solution (0.75%) for 45–60 minutes.

11.2.5 Chromosome Preparations

11.2.5.1 Squashing

In all types of adult tissues (testis tubules, ovarioles, and gastric caeca), the cytological preparations can be performed similarly, except that gastric caeca should be immersed for 1–2 minutes in glacial acetic acid to eliminate digestive remains. The preparations are performed by squashing as follows:

1. On a clean slide, immerse two testis tubules (or ovarioles) in a drop of 45%–50% acetic acid.
2. Crush the material with the flat end of an appropriate macerator to separate the tissue into individual cells. Place a coverslip over the material, and holding the coverslip in one corner with filter paper, gently push with a needle to eliminate air drops and spread the cells between the slide and the coverslip. Finally, remove excess fixative and squash by placing new filter paper on the preparation and pressing strongly with the thumb.
3. After 10 minutes, immerse the preparation in liquid nitrogen for 1 minute. Separate the coverslip with a razor blade and dry the slide for 15 minutes at RT.

11.2.5.2 Spreading

Alternatively, preparations can be made with the following protocol:

1. Place 2–3 testis follicles (or ovarioles) on a slide, add a drop of 50% acetic acid and macerate the tissue to separate cells.
2. Add another drop of 50% acetic acid and spread the solution over the slide.
3. Place the slide onto a hot plate at 45°C–50°C to dry the solution. Tilt the slide to facilitate the spreading of the cell solution. Avoid higher temperatures that may degrade DNA or chromosomes.

Spreading preparations can also be made as follows (modified from Castillo et al. 2011):

1. Cut a small piece of gastric caecum and place it in a small petri dish containing 700 μL 50% acetic acid. Disperse the caecum by pipetting with a Pasteur pipette.

2. To improve the quality of the preparation, use a hot plate at 45°C–50°C and spread cells with a pipette.
3. Place a dry and clean slide onto the hot plate. Transfer 100 μL of disaggregated caecum to the slide. Aspirate the solution and place it again on another region of the slide. Repeat this step as necessary until several preparations are obtained, with several drops of cell suspension on each slide (as explained in more detail for embryo preparations).
4. Air-dry the slides at RT.

Although embryo preparations can be made by squashing (see Section 11.2.5.1), the best results are obtained with Meredith's technique for mammal meiosis (Meredith 1969), with slight modifications as follows:

1. Place the embryo in an Eppendorf tube with 20 μL of 75% acetic acid. Wait for 3 minutes to soften the material and then perform repeated pipetting with a micropipette to separate embryo cells.
2. Pipette 20 μL with the micropipette and slowly place a drop of the cell suspension on a slide previously warmed on a hot plate at 60°C. Repeat this process with the same micropipette to transfer 6–8 nonoverlapping drops per slide. Dry the slide on the warm plate.

Embryo preparations can also be produced by spreading, as described by Crozier (1968). An embryo is crushed in a drop of 60% acetic acid and one or two drops of 3:1 methanol/acetic acid is added to facilitate cell spreading. The preparations are air-dried. Preparations from embryo or adult materials can be stored at -20°C for 2 days or dehydrated in an alcohol series (3 minutes in 70%, 5 minutes in 90%, and 8 minutes in absolute ethanol) and frozen at -80°C .

11.2.5.3 Chromosome Microdissection

Few studies of chromosome painting have been performed in grasshopper species, with the only exceptions being those performed on the X and B chromosomes of *L. migratoria* (Teruel et al. 2009a) and *E. plorans* (Teruel et al. 2009b) and the B chromosomes of *Podisma kanoi* (Bugrov et al. 2007). However, this interesting assay elucidates chromosome evolution, and it provides precious information about the molecular content of specific chromosomes or chromosome regions.

Chromosome microdissection can be performed from testis tubules, ovarioles, or embryo cells. To minimize DNA damage, the

material needs to be fixed in 3:1 absolute ethanol–acetic acid for 10 minutes and stored in 70% ethanol at -20°C until use (for months).

Before making the preparation, the coverslips can be subjected to a salt treatment, which facilitates separation of the chromosomes with the microdissection needle, but the treatment is not strictly necessary. This treatment is performed by incubating them overnight in $10\times$ SSC (saline sodium citrate). Excess salt is removed by washing with hot running water and distilled water. The coverslips are then air-dried and stored until use. The salts form a film on the coverslip, which subsequently facilitates the separation of chromosomes from the glass.

Immediately before microdissection, preparations are made in 50% acetic acid on a 24×60 -mm coverslip at 27°C on a warm plate, following Meredith's method (see Section 11.2.5.2). A single drop is placed in the center of the coverslip because the interesting cells usually occupy the periphery of the drop, and they can be repeatedly localized by their position in "hours," as on a round clock face. Two or more drops can also be placed, but if different drops overlap, it is difficult to establish cell localization.

Chromosome microdissection is carried out with glass needles in an inverted microscope coupled to an electronic micromanipulator. These needles are manually made from 2-mm-diameter glass capillaries with a horizontal pipette puller and are steps of UV sterilized twice. Appropriate chromosomes for microdissection can be obtained from pachytene, diplotene, diakinesis or metaphase I cells, where the desired chromosome or bivalent is completely separated from the remaining chromosomes to avoid contamination. The microdissected chromosomes are collected in 0.2-mL tubes with 20 μL of $1\times$ PCR buffer (Roche) (10 mM Tris-HCl, 1.5 mM MgCl_2 , 50 mM KCl, pH 8.3) when they are going to be amplified by degenerate oligonucleotide-primed PCR (DOP-PCR) or in 9 μL DNase-free ultrapure water for the whole genome amplification (WGA) method (GenomePlex, Sigma).

11.2.5.4 Chromosomes for Fiber-FISH

The material analyzed is the cerebral ganglion of adult individuals. Insect ganglia are dissected under a stereomicroscope and immersed in saline solution, and proceeded as described in the following protocol:

1. Immerse the cerebral ganglion in 250 μL of 60% acetic acid for 1 minute.
2. Set the ganglion in a homogenizer in 500 μL of 60% acetic acid and homogenize 10–15 times.
3. Pick up the homogenate with a micropipette and transfer it to an Eppendorf tube.

4. For very soft materials, it is sufficient to homogenize the tissue in an Eppendorf tube with 750 μL of 60% acetic acid and pipetting until complete disintegration.
5. Centrifuge for 10 minutes at 1000 rpm and discard the supernatant.
6. Resuspend in 500–750 μL fixative (3:1 absolute ethanol–acetic acid).

To make preparations:

1. Set a clean slide on a warm plate at 50°C.
2. With a 200- μL micropipette, place several drops of the material on the slide.
3. Wait for the fixative to evaporate and immediately immerse the slide in 1 \times PBS (phosphate buffered saline) for 1 minute.
4. Pour 200 μL of 0.05 M NaOH (in 30% ethanol) on one end of the slide. Move the end of another slide, slightly inclined, along the entire slide. Discard the latter slide.
5. Add a few drops of absolute ethanol (~500 μL) on the slide, keeping it inclined.
6. Air-dry the slides. Select the best preparations under a microscope.
7. Dehydrate in an ethanol series for 3 minutes (70%), 5 minutes (90%), and 8 minutes (absolute).
8. Freeze at -20°C or at -80°C .

11.2.6 Staining Protocols

11.2.6.1 Conventional Staining

Pour a small drop of 2% acetic (or lactopropionic) orcein on the center of a clean slide and immerse one or two testis tubules or ovarioles. The material is crushed with the flat end of a macerator to disintegrate the tissue and separate cells. Place a coverslip on the orcein. Using a needle or lancet and filter paper, remove air bubbles and excess orcein. Place several pieces of filter paper on the coverslip and firmly press the preparation (see examples in Figures 11.2 and 11.3a and b).

11.2.6.2 C-Banding

The purpose of this technique is to visualize constitutive heterochromatin (C-positive blocks) (Figure 11.3c). Preparations obtained by the methods mentioned above are treated as follows:

1. Hydrolyze in 0.2 N HCl at 28°C for 30 minutes.
2. Wash preparations in tap water for 2 minutes.

3. Immerse the preparations in a saturated and filtered solution of 5% barium hydroxide at 28°C for 3–10 minutes, depending on the type of material and fixation.
4. Wash vigorously in tap water for 2 minutes.
5. Wash briefly in 0.2 N HCl to eliminate barium hydroxide remains.
6. Wash vigorously in tap water for 2 minutes.
7. Immerse the preparations in 2× SSC at 60°C for 1 hour.
8. Wash vigorously in tap water for 2 minutes.
9. Stain with 5% Giemsa in phosphate buffer for 1–5 minutes, depending on the material.
10. Mount the air-dried preparations in DPX (distrene 80, plasticizer, xylene).

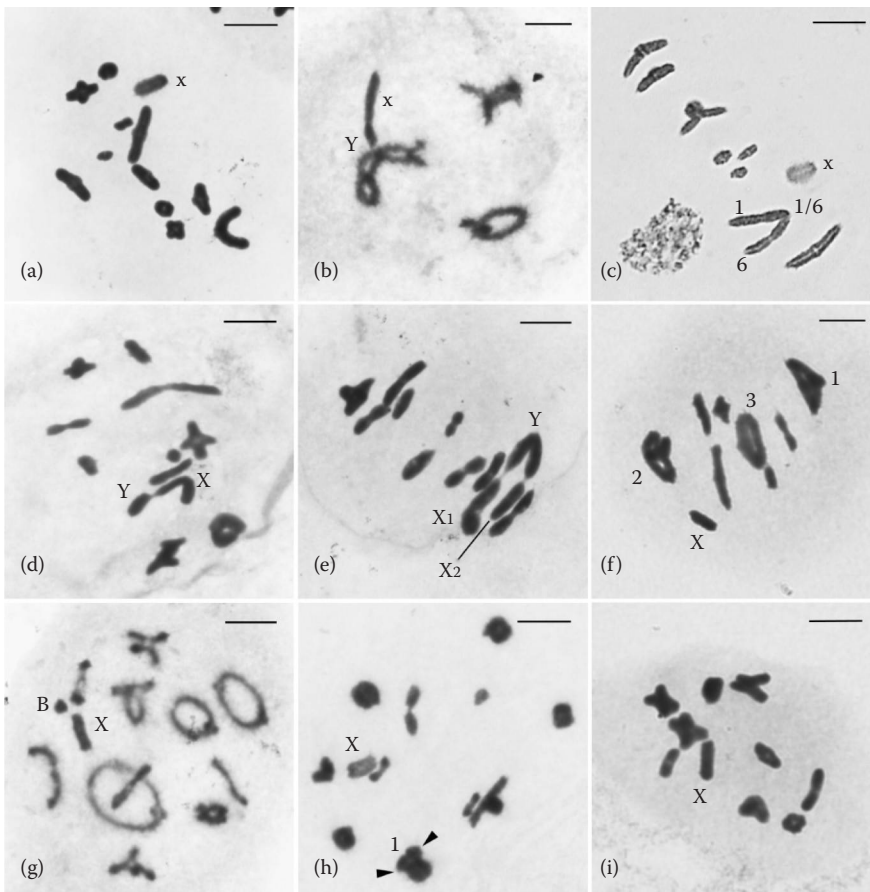


FIGURE 11.2 Conventional staining of meiotic cells at metaphase I in nine species of grasshoppers from three distinct families showing the chromosomal diversity observed in the group. (a) *Abracris flavomileata*, $2n = 23$, X0; (b) *Dichroplus silveiraguidoi*, $2n = 8$, neo-XY; (c) *Dichroplus pratensis*, $2n = 18$, X0 with heterozygote fusion between chromosomes 1 and 6; (d) *Ronderosia bergi*, $2n = 22$, neo-XY; (e) *Dichromatos lilloanus*, $2n = 21$, neo- X_1X_2Y ; (f) *Chorthippus nevadensis*, $2n = 17$; (g) *Eyprepocnemis plorans*, $2n = 23$, X0 plus one B chromosome; (h) *Ommexecha virens*, $2n = 23$, X0; (i) *Stiphra robusta*, $2n = 19$, X0. Arrowheads in (h) show centromere position in the largest bivalent with metacentric morphology. Bar = 5 μ m.

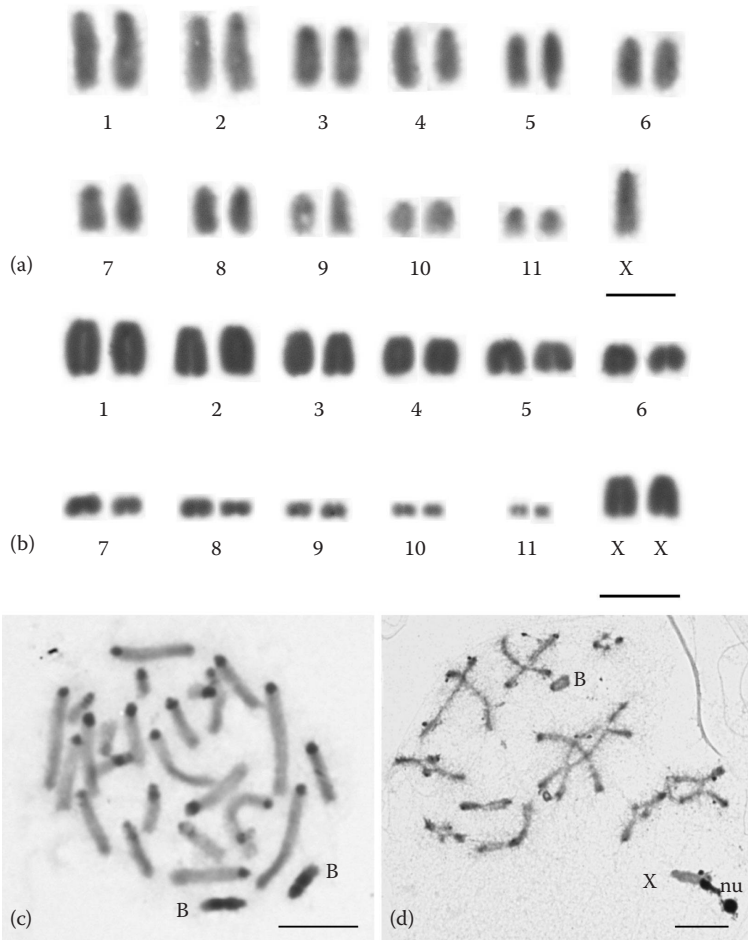


FIGURE 11.3 (See color insert.) Typical grasshopper karyotypes (a and b) and C-banded (c) and silver stained (d) chromosomes. (a) Male $2n = 23$, X0 karyotype obtained from an embryo cell of *Eyprepocnemis plorans*; (b) female $2n = 24$, XX karyotype obtained from a gastric caecum cell of *Adimantus ornatus*; (c) C-banded embryo mitotic metaphase cell from *E. plorans*; (d) silver-stained diplotene cell from an *E. plorans* male. nu = nucleolus, Bar = 5 μm .

11.2.6.3 Silver Impregnation

In grasshoppers, silver impregnation stains the nucleolus but not the nucleolar organizer regions (NORs) (Figure 11.3d). However, we can infer the chromosome localization of NORs from the chromosome regions that are closely associated with nucleoli during the first meiotic prophase (especially pachytene and diplotene). The best material for this technique is the testis, and a very simple version of this technique was developed by Rufas et al. (1982).

1. Wash the preparations with a formic acid solution prepared by adding a few drops of formic acid (pH 3–3.5) to 200 mL deionized water and dry the preparations with warm air.
2. Prepare a solution of 0.5 g silver nitrate and 0.5 mL above-mentioned formic acid solution.
3. Place a drop of this solution on the slide and place a coverslip.
4. Incubate the preparations in a humid chamber, in the dark, at 60°C.
5. Wash with distilled water.
6. Dry and mount in DPX.

11.2.6.4 Double Silver Impregnation and Fluorescence In Situ Hybridization

This technique reveals the physical location of NORs (by FISH) and nucleoli attached to the chromosomes (by silver impregnation), thus allowing to ascertain which NOR was active in every cell. The following sequential staining technique is based on the one described by Zurita et al. (1998):

1. Perform the conventional silver staining technique for testis preparations (Section 11.2.6.3).
2. Place a drop of distilled water and a coverslip on the slide and observe it under the microscope. Select and photograph interesting cells and write cell coordinates in the preparation.
3. To eliminate silver nitrate, immerse the preparations in 7.5% potassium ferricyanide for 4 minutes and then immediately immerse them in 20% sodium thiosulfate for 5 minutes.
4. Wash with distilled water.
5. Dry the preparations and perform FISH, as described in Section 11.2.8.
6. It is important to permeabilize the material before performing FISH (see Section 11.2.8).
7. Search for the same cells previously photographed after silver impregnation, photograph them with the appropriate fluorescence filters and then merge the images with the appropriate software.

11.2.6.5 Triple Fluorescent CMA₃-DA-DAPI Staining

Triple fluorescent staining with CMA₃, DA, and DAPI is based on the procedure described by Schweizer (1980, 1981). This technique reveals two types of chromosome bands: those containing G+C-rich chromatin (which are CMA₃+) and those containing A+T-rich chromatin (DAPI+). The procedure is performed in the dark as follows:

1. Pour approximately 100 μL CMA_3 (0.5 mg/mL) in McIlvaine's buffer on the slide and add a paraffin coverslip. Incubate for 1 hour at 37°C in the dark.
2. Wash with tap water removing the coverslip.
3. Wash with distilled water.
4. Dry the preparation with warm air.
5. Pour 100 μL DA (0.05–0.1 mg/mL) in McIlvaine's buffer on the preparation, place a paraffin coverslip and incubate in the dark at 37°C for 15 minutes.
6. Immerse the preparation in a mixture containing 1:1 McIlvaine's buffer and distilled water to separate the coverslip.
7. Pour 100 μL DAPI (1 μL /mL) in McIlvaine's buffer on the slide and incubate it for 40 minutes at RT in the dark.
8. Wash with distilled water or PBS.
9. Mount in VECTASHIELD.
10. Store the preparations at 37°C in the dark for a minimum of 48 hours before microscopy.

11.2.6.6 Acridine Orange Staining

This technique is used for N banding in grasshoppers (Fox and Santos 1985). After comparing it with FISH for several repetitive DNA probes, it was observed that the N bands include repetitive G+C-rich DNAs, such as rDNA and histone genes. The best material for this technique is mitosis in embryos. It is performed with the following steps:

1. Immerse the slides in absolute ethanol for 2–5 minutes.
2. Dry them with warm air.
3. Immerse the slides in a 1:1 mixture of formamide and $2\times$ SSC at 60°C for 1 hour.
4. Wash the slides vigorously with tap water.
5. Without drying them, immerse the slides in a 0.1% acridine orange solution for 1 minute.
6. Immerse the slides in a decoloring buffer (pH = 7.0) for 15 minutes. Repeat three times.
7. Wash in abundant water.
8. Mount the preparation with a drop of buffer and observe under the fluorescence microscope.

The chromosomes will appear light green, whereas the N bands will be dark green. Alternatively, staining in 5–10% Giemsa for 2–5 minutes can improve contrast. In all cases, dry the preparations and mount them in DPX.

To make the decoloring buffer, prepare solution 1 (28.392 g/L Na_2HPO_4) and solution 2 (21.01 g/L citric acid) and mix 82.35 mL solution 1 and 17.65 mL solution 2.

11.2.7 Molecular Techniques

11.2.7.1 Genomic DNA Isolation

The manual extraction using phenol/chloroform/isoamyl alcohol (25:24:1) works well for DNA extraction in grasshoppers, although some commercial kits are also useful. DNA can be extracted from all body parts, but the muscle from the posterior leg provides high amount of good-quality DNA for subsequent procedures, such as probe generation. For each sample, follow this procedure:

1. Prepare 500 μL of the following solution in a 1.5 mL microtube
 1. 5 M Sodium chloride (NaCl) 10 μL
 2. 1 M Tris-HCl, pH 8.00 5 μL
 3. 0.5 M EDTA, pH 8.00 25 μL
 4. 10% SDS 25 μL
 5. 10 mg/mL Proteinase K 10 μL
 6. Distilled H_2O 425 μL
2. Drain the ethanol from the tissue and macerate the material in the microtube with the solution described above using scissors or a pestle.
3. Incubate the microtube in a water bath at 45°C for 90–120 minutes or until the tissue dissolves. Homogenize periodically during this period.
4. Add 500 μL phenol/chloroform/isoamyl alcohol (25:24:1) and homogenize with circular rotation for 15 minutes.
5. Centrifuge at 15,000 rpm for 15 minutes at 4°C.
6. Transfer the upper aqueous phase to a clean 1.5 mL microtube.
7. To precipitate DNA, add 0.2 \times volumes 1 M NaCl and 2 \times volumes absolute cold ethanol and mix by inversion.
8. Centrifuge at 15,000 rpm for 15 minutes at 4°C.
9. Discard the supernatant and add 375 μL of 70% cold ethanol, without agitation.
10. Centrifuge at 15,000 rpm for 15 minutes at 4°C.
11. Discard the supernatant and incubate at 37°C or at RT to dry the pellet.
12. Rehydrate the DNA in 100 μL ultrapure water for 1 hour.
13. To check DNA quality, load 5 μL sample on a 0.8% agarose gel and measure the concentration using a spectrophotometer.
14. If necessary, treat each 100 μL extracted DNA with 1 μL RNase (1 mg/mL) and incubate for 60 minutes at 37°C.

11.2.7.2 DNA Amplification from Microdissected Chromosomes

There are several methods to amplify the DNA obtained from microdissected chromosomes. We will describe three of them, namely DOP-PCR, GenomePlex, and Genomiphi methods.

The DOP-PCR technique was developed by Telenius et al. (1992), and it is commonly used to amplify DNA in chromosomes obtained by microdissection or flow sorting. The primers are partially degenerate oligonucleotides, which are used in two rounds of amplification. The first primer has a low hybridization temperature to facilitate primer binding to many genomic regions, whereas the second primer is more specific and has a higher hybridization temperature. The second round may also serve to mark probes for chromosome painting.

The microdissected chromosomes are placed in 0.2-mL tubes containing 20 μL of 1 \times Roche buffer (10 mM Tris-HCl, 1.5 mM MgCl_2 , 50 mM KCl, pH 8.3).

The first round PCR is performed as follows:

1. Add 1 μM oligo-DOP, 200 μM dNTPs, 100 $\text{pg}/\mu\text{L}$ BSA, 15 mM MgCl_2 , 2 U DNA polymerase and perform the following PCR program: initial denaturation at 94°C for 5 minutes, 8 cycles of denaturation at 94°C for 1 minute, hybridization at 45°C for 1 minute and extension at 72°C for 3 minutes, 28 cycles of denaturation at 94°C for 1 minute, hybridization at 56°C for 1 minute and extension at 72°C for 3 minutes and a final extension at 72°C for 5 minutes. The product should be maintained at 4°C.
2. Visualize the amplification product (5 μL) on a 2% agarose gel with 5 μL of 1 \times SYBR safe or an adequate quantity of another DNA stain.

The second PCR is done as follows:

1. Add 1 \times PCR buffer (100 mM Tris-HCl, 15 mM MgCl_2 , 500 mM HCl pH 8.3), 4 μM oligo-DOP, 200 μM dNTPs, 20 μM SpectrumOrange-dUTP (Vysis), 2 U DNA polymerase to 5 μL of the first PCR product.
2. Perform a PCR program consisting of an initial denaturation at 94°C for 5 minutes, 5 cycles of denaturation at 94°C for 30 seconds, hybridization at 45°C for 30 seconds and extension at 72°C for 90 seconds, 28 cycles of denaturation at 94°C for 30 seconds, hybridization at 56°C for 30 seconds and extension at 72°C for 90 seconds and a final extension at 72°C for 7 minutes.

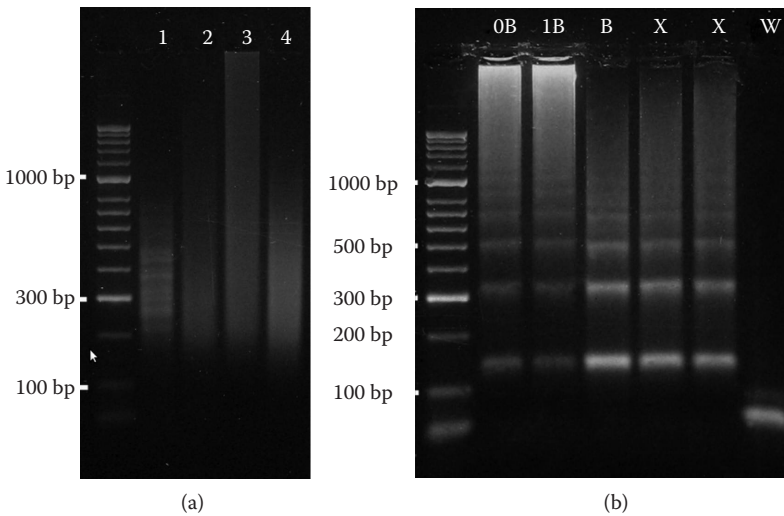


FIGURE 11.4 PCR amplification of (a) microdissected B and X chromosomes or (b) satellite DNA in *E. plorans*. (a) Agarose gel (1.5%) showing the fragments obtained after DOP-PCR (lane 1) or GenomePlex (lanes 2–4) amplification. Lanes 1 and 2 correspond to the microdissected B₂₄ and lanes 3 and 4 correspond to the X chromosome; (b) PCR amplification of the satellite DNA in 0B and 1B individuals as well as in microdissected DNA from B and X chromosomes. w = white.

3. To verify the result, run 5 μL of the reaction on a 2% agarose gel with 5 μL of 1 \times SYBR safe or an adequate quantity of another DNA stain (Figure 11.4a).

The GenomePlex method performs WGA by LA-PCR (linker-adapted PCR). Random genome fragmentation is followed by ligation of the OmniPlex Library adaptors and PCR amplification with primers specific for the adaptors.

The amplification of chromosome DNA with the GenomePlex Single Cell kit is performed following the manufacturer's (Sigma) recommendations with no modifications.

1. Place the microdissected chromosomes in 9 μL DNase-free ultrapure water and fragment DNA by adding 1 μL Lysis and Fragmentation-K solution (32 μL of 10 \times Lysis and Fragmentation plus 1 μL proteinase K). Incubate the mixture for 1 hour at 55°C, heat shock it at 99°C for 4 minutes to inactivate the enzyme and place it on ice.
2. Ligate the OmniPlex Library primers by adding 2 μL 1 \times Library Preparation and 1 μL Stabilization Solution to the product of the former reaction. Incubate the mixture for 2 minutes at 95°C and place it on ice.

3. Add 1 μL Enzyme Library to generate fragments with the following program: 20 minutes at 16°C, 20 minutes at 24°C, 20 minutes at 37°C, and 20 minutes at 75°C. Maintain the product at 4°C.
4. Finally, amplify the generated fragments in a final volume of 70 μL by adding the following reagents and to 14 μL of the previous product: 48.5 μL DNase-free ultrapure water, 7.5 μL of 10 \times Amplification Master Mix, and 5 μL WGA DNA polymerase.
5. Apply a PCR program consisting of an initial denaturation at 95°C for 3 minutes, 25 cycles of denaturation at 94°C for 30 seconds and hybridization/extension at 65°C for 5 minutes and a final step maintaining the product at 4°C.
6. Visualize the product (5 μL) on a 2% agarose gel with 5 μL of 1 \times SYBR safe or an adequate quantity of another DNA stain (Figure 11.4a).
7. Purify the amplified DNA with the GenElute PCR Clean-Up Kit (Sigma). Quantify the DNA visualization on a 2% agarose gel, as described in step 6 and store it at -20°C.

The chromosome DNA obtained as described in Section 11.2.7.1 should be reamplified to obtain several working aliquots. Reamplification is performed following the protocol of the GenomePlex WGA Reamplification Kit (Sigma) with some modifications.

1. Perform the amplification reaction in a final volume of 70 μL by adding the following reagents to 5 μL of the previously amplified DNA: 52.5 μL DNase-free ultrapure water, 7.5 μL of 10 \times Amplification Master Mix and 5 μL WGA DNA polymerase.
2. Use a PCR program consisting of an initial denaturation at 95°C for 3 minutes, 15 cycles of denaturation at 94°C for 25 seconds and hybridization/extension at 65°C for 5 minutes and maintenance at 4°C.
3. Purify the amplified DNA with the GenElute PCR Clean-Up Kit (Sigma), quantify it by running 5 μL on a 2% agarose gel (described earlier) and store it at -20°C.

The GenomiPhi (Φ 29) method uses the Φ 29 DNA polymerase and random hexamer primers for unbiased WGA. We used the illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare Life Sciences). It contains all components necessary for miniscale WGA by isothermal strand displacement. This kit yields 4–7 μg

of representative genomic DNA (gDNA) in 1.5 hours from 1–10 ng input gDNA, with no amplification in nontemplate negative controls. The high processivity and fidelity of the Φ 29 DNA polymerase allows highly uniform amplification across the genome. The average amplification is 10,000-fold with an average product length >10 kb.

We have successfully used two different amplification programs:

Program 1: 95°C treatment for 3 minutes and keep at 4°C.

Program 2: 30°C for 2 hours (it can be extended to 3 hours if the starting amount of DNA is low), 65°C for 10 minutes, and keep at 4°C.

To perform this type of DNA amplification, do the following:

1. In a 0.2-mL Eppendorf tube, add 9 μ L sample buffer to the microdissected chromosome. Pulse spin. Run Program 1 to denature DNA. Pulse spin and store the Eppendorf tubes on ice to anneal random hexamers.
2. Add 9 μ L reaction buffer and 1 μ L Φ 29 enzyme to the tubes on ice. Run Program 2. The enzyme will be inactivated during the final step at 65°C. Store the samples at -20°C .
3. Purify the product with a purification kit (this step can be omitted if the initial amount of DNA is low).
4. Test 1 μ L product on a 1% agarose gel.

To obtain several working aliquots, we can reamplify the chromosomal DNA through the following procedure:

1. Amplify 1 μ L chromosomal DNA with the Φ 29 DNA polymerase and dilute 1:10 in DNase-free ultrapure water.
2. To quantify the resulting DNA, run 1 μ L DNA on a 1% agarose gel. Store the DNA at -20°C until use.

11.2.8 Fluorescence In Situ Hybridization

Since its first description by Gall and Pardue (1969), in situ hybridization is the technique of choice for the physical mapping of chromosomes in plant and animal species. Currently, FISH, using one or a pool of fluorescent probes, is widely used in cytogenetic laboratories because of its quickness, high resolution, and versatility. These qualities are especially true with direct fluorophore probe labeling because no further detection steps are needed and nonspecific background is avoided. Probes made of biotin- or digoxigenin-conjugated oligonucleotides need additional antibody-based detection procedures but provide greater sensitivity. Typical FISH experiments use DNA or RNA

as probes, which may be composed of a specific sequence, a fraction of genomic DNA or amplified DNA from either an entire microdissected chromosome or a specific chromosomal region. The most common strategies for probe labeling are PCR and nick translation. Here, we describe three FISH protocols—specially focused on grasshopper chromosomes—that routinely give the best results in our labs.

11.2.8.1 Probe Generation

Probe sequences can be isolated directly from genomic DNA, or they can be cloned into plasmids to amplify DNA before labeling. If the sequence of interest is known, it can be amplified by PCR, or synthetic oligonucleotides can be purchased commercially. Some examples of commonly used probes in grasshoppers and the method for obtaining these probes are presented.

11.2.8.1.1 *C₀t-1 Repetitive DNA*

The isolation of highly and moderately repetitive DNA is based on the reassociation kinetics of genomic DNA, as described by Zwick et al. (1997), and the process is listed as follows:

1. Dilute the genomic DNA to 100–500 ng/μL in 0.3 M NaCl in a 1.5-mL microtube. It is important to use nondegraded genomic DNA.
2. Fragment the DNA by autoclaving at 1.4 atm/120°C or using DNase I. The time used for DNA fragmentation is variable, and it is useful to test a range of times to obtain an optimal result.
3. Run 3 μL autoclaved or digested DNA on a 1% agarose gel to check the size of the fragments. The recommended size of DNA fragments ranges from 100 to 1000 bp.
4. Denature at least three samples (tubes 1, 2, and 3) of 50 μL fragmented DNA using a thermocycler or water bath at 95°C for 10 minutes.
5. Place the tubes on ice for 10 seconds. Add S1 nuclease enzyme to tube 1 and incubate at 37°C for 8 minutes. After 10 seconds on ice, immediately transfer tubes 2 and 3 to a water bath/thermocycler at 65°C to renature the DNA.
6. After 1 minute, add S1 nuclease enzyme to tube 2, and after 5 minutes, add S1 nuclease enzyme to tube 3. Incubate at 37°C for 8 minutes. Other times could be tested to obtain a large amount of repetitive DNA. Use 1 U of S1 nuclease enzyme and 5.5 μL of 10× nuclease buffer for each 1 μg DNA.
7. Add an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and rotate the tubes.
8. Centrifuge for 5 minutes at 13,000 rpm and transfer the supernatant (aqueous phase) to a clean 1.5-mL microtube.

9. Add 2.5 volumes of cold absolute ethanol to precipitate the DNA and place the tube in a -70°C deep freezer for 30 minutes.
10. Centrifuge for 15 minutes at 15,000 rpm at 4°C .
11. Dry the pellet at RT and add 30–50 μL ultrapure water.
12. Check the fragment sizes on a 0.8% agarose gel. The fragments should be 50–500 bp.
13. Quantify the DNA.

To use the C_{ot} -1 repetitive DNA in chromosomal mapping, label the probe by a nick translation reaction with the appropriate amount of DNA (see Section 11.2.8.2).

11.2.8.1.2 *Telomere Repeat*

The telomere motif in grasshoppers is TTAGG and the self-annealing primers F (TTAGG)₅ and R (CCTAA)₅ should be used to obtain this type of probe, as suggested by Ijdo et al. (1991).

The reaction is done with the following products:

1. 5 μL *Taq* DNA polymerase enzyme buffer (10 \times)
2. 0.5 μL MgCl_2 (50 mM)
3. 2 μL F primer (10 mM)
4. 2 μL R primer (10 mM)
5. 1 μL dATP (2 mM)
6. 1 μL dCTP (2 mM)
7. 1 μL dGTP (2 mM)
8. 0.7 μL dTTP (2 mM)
9. 0.6 μL labeled dUTP (1 mM)
10. 0.4 μL *Taq* DNA polymerase (5 U/ μL)
11. Sterile ultrapure water (up to 50 μL)

And PCR cycles are performed as indicated in [Table 11.1](#).

Check the amplification on a 1% agarose gel. The fragments should be a smear between 100 and 1000 bp, but if they are higher, use DNase I to cut the fragments. Alternatively, the reaction can be adjusted with higher primer concentrations. Note that for telomeric probe generation it is not necessary to use a DNA template.

Telomeric probes can also be obtained from commercial suppliers as 3'-, 5'-, or both end-labeled synthetic oligonucleotides ([TTAGG]₇, [CCTAA]₇). See examples of FISH with telomeric probes in Figure 11.5a and b.

11.2.8.1.3 *Multigene Families, Transposable Elements, and Satellite DNA*

Moderate and highly repetitive sequences, such as distinct families of satellite DNA (Figures 11.4b and 11.5c) and transposable

TABLE 11.1
PCR Program for Amplifying Telomeric DNA

Step	Temperature	Time
Initial denaturation	95°C	5 minutes
10 Cycles	95°C	1 minute
	55°C	30 seconds
	72°C	1 minute
	95°C	1 minute
35 Cycles	60°C	30 seconds
	72°C	1 minute and 30 seconds
	95°C	1 minute
	72°C	30 seconds
Final extension	72°C	5 minutes
Hold	4°C	

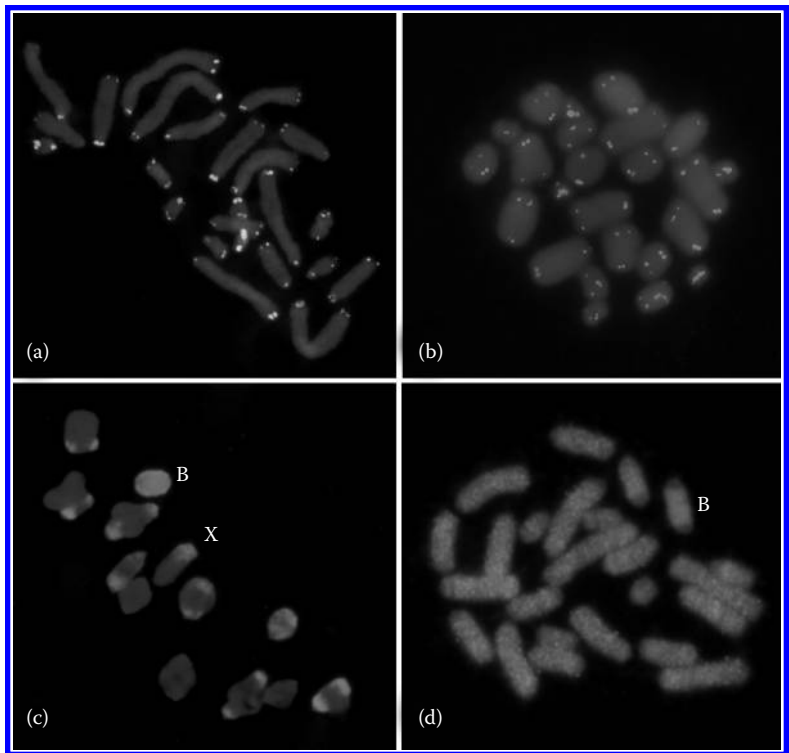


FIGURE 11.5 (See color insert.) FISH mapping of tandem (a, b, c) and scattered (d) repetitive DNA sequences in *L. migratoria* (a), *Adimantostissimus* (b) and *E. plorans* (c, d). (a, b) Telomeric DNA, (c) a 180-bp satDNA, (d) Gypsy transposable element. (a, d) Embryonic mitotic cells, (c) first meiotic metaphase cell, (b) mitotic cell obtained from gastric caeca. Note the presence of B chromosomes in c and d.

elements (Figure 11.5d), can also be identified and isolated by standard cloning protocols or next-generation sequencing (NGS) approaches. Amplification is performed by PCR using specific primers. Some examples of primers used for the amplification

TABLE 11.2

Primers Used for PCR Amplification of Some Repetitive DNAs in the Grasshopper *Eyprepocnemis plorans*, Such as a 180-bp Satellite DNA and Three Mobile Elements

Target	Primer Sequences	Estimated Fragment Size	Reference
180-bp satellite DNA	F 5' GCACTGCTTTCCAGATATCACACTAAAATG 3' R 5' CGCATTCTGCCGCCTGTGGCGCTACATT 3'	148 bp	Teruel (2009)
Gypsy, LTR retrotransposon	F 5' GTKTTIKTIGAYACIGGIKC 3' R 5' GCGTTIKTIAGICCRAAIGGCAT 3'	1107 bp	Miller et al. (1999)
RTE, non-LTR retrotransposon	F 5' CTGGACGCAGAGAARGCVTTYGAC 3' R 5' CGGGCTCAGNGGWCANCCYTGA 3'	1242 bp	Montiel et al. (2012)
Mariner transposon	F 5' CGCGCATGAATGGATTAACG 3' R 5' AAGAGCCGACATCGAAGGATC 3'	1112 bp	Burke et al. (1993)

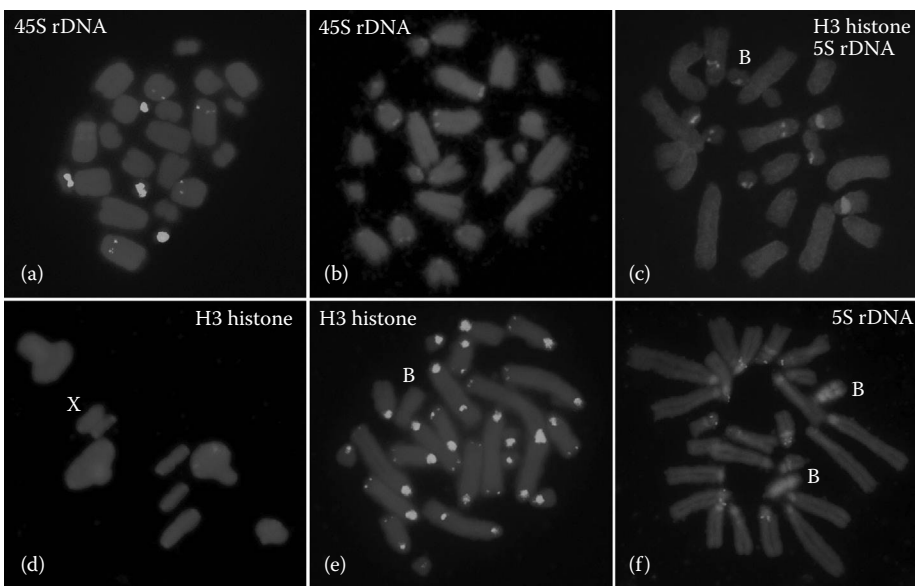


FIGURE 11.6 (See color insert.) FISH using DNA probes for distinct multigene families in five grasshopper species, (a) *Adimantos arnatissimus*, (b and c) *Locusta migratoria*, (d) *Chorthippus jacobsi*, (e) *Abracris flavolineata*, and (f) *Eyprepocnemis plorans*. (a and e) Mitotic cells obtained from gastric caeca; (b, c, f) embryo mitotic cells, (d) meiotic metaphase I cell. The probes used are indicated in the cells. Note the presence of B chromosomes in c, e, f.

of these repetitive sequences in the grasshopper, *E. plorans*, are listed in Table 11.2.

Multigene families of DNA sequences (Figure 11.6) are amplified through PCR using specific or universal primers. Some primers currently used to obtain these sequences in grasshoppers from distinct families are listed in Table 11.3.

The PCR reaction should be performed using the parameters shown in Table 11.4.

It is important to sequence the obtained fragment to perform the FISH technique, to be sure of fragment identity. Test different

TABLE 11.3**Primers Used for PCR Amplification of Some Multigene Families in Grasshoppers**

Target Gene	Primer Sequences	Estimated Fragment Size	Reference
18S rDNA	F 5' CCCCCTAATCGGAATGAGTA 3' R 5' GAGGTTTCCCCTGTTGAGTC 3'	822 bp	Cabral-de-Mello et al. (2010)
5S rDNA	F 5' AACGACCATAACCACGCTGAA 3' R 5' AAGCGGTCCCCCATCTAAGT 3'	92 bp	Loreto et al. (2008)
U1 snDNA	F 5' CTTACCTGGCGTRGRGGWY 3' R 5' CAKCCCRRCTACCAAAAATT 3'	127 bp	Cabral-de-Mello et al. (2012)
U2 snDNA	F 5' ATCGCTTCTCGGCCTTATG 3' R 5' TCCCGGCGGTACTGCAATA 3'	178 bp	Bueno et al. (2013)
H3 histone	F 5' ATGGCTCGTACCAAGCAGACVGC 3' R 5' ATATCCTTRGGCATRATRGTGAC 3'	370 bp	Colgan et al. (1998)
H4 histone	F 5' TSCGIGAYAACATYCACGGIATCAC 3' R 5' CKYTTIAGIGCRTAIACCACRTECCAT 3'	210 bp	Pineau et al. (2005)

TABLE 11.4**PCR Program for Amplifying Multigene Families**

Step	Temperature	Time
Initial denaturation	95°C	5 minutes
30 Cycles	95°C	1 minute
	(45°C–60°C)*	30 seconds
	72°C	1 minute
Final extension	72°C	5 minutes
Hold	4°C	

*Distinct temperatures should be tested in the indicated range

temperatures within the indicated range. The theoretical optimal temperature is provided by the primer manufacturer, but it should be empirically determined. Check the fragment sizes on a 1% agarose gel.

Double FISH can be performed by combining different probes and colors (Figure 11.6c), which is especially indicated for the fiber-FISH technique (Figure 11.7a).

11.2.8.2 Probe Labeling

PCR and nick translation are the most frequently used strategies for probe labeling in FISH experiments. The choice of labeling method depends on the type of DNA probe.

PCR labeling is useful for small DNA sequences. For DNA fragments larger than 600 bp, the PCR product should be fragmented with DNase I to allow the probe to access the target chromosomal DNA. This fragmentation prevents the occurrence of background after posthybridization washes. The probe-labeling PCR is identical to a regular PCR, except that the nucleotide concentrations are modified. The ratio of regular to modified nucleotides (e.g.,

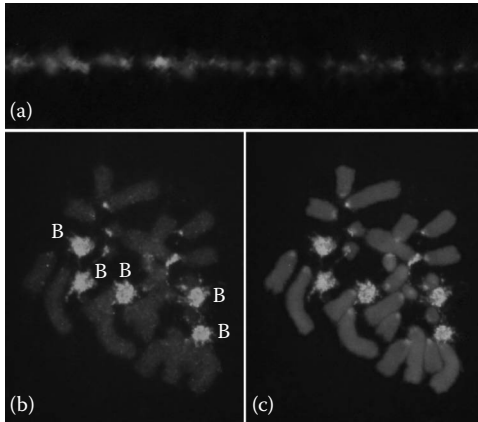


FIGURE 11.7 (See color insert.) (a) Double FISH for 45S rDNA (red) and a sequence-characterized amplified region (SCAR) marker specific to B chromosomes in *E. plorans* was performed on chromatin fiber. Note the alternating arrangement of the two sequences; chromosome painting on an embryonic mitotic cell of *Locusta migratoria* using a B-chromosome DNA probe obtained through microdissection, (b) paint probe signal, (c) DAPI pattern in gray scale merged with paint probe signal.

Dig-11-dUTP or Bio-16-dUTP) should be approximately 70% to 30%. A reaction example follows:

1. 2.5 μL *Taq* DNA polymerase enzyme buffer (10 \times)
2. 0.25 μL MgCl_2 (50 mM)
3. 1 μL F primer (10 mM)
4. 1 μL R primer (10 mM)
5. 0.5 μL dATP (2 mM)
6. 0.5 μL dCTP (2 mM)
7. 0.5 μL dGTP (2 mM)
8. 0.35 μL dTTP (2 mM)
9. 0.3 μL labeled dUTP (1 mM)
10. 0.1 μL *Taq* DNA polymerase (5 U/ μL)
11. 2 μL genomic DNA (50–100 ng/ μL)
12. Sterile ultrapure water (up to 25 μL)

Nick translation labeling is recommended for DNA fragments larger than 600 bp, but it is also suitable for shorter probes. Indirect probe labeling with biotin or digoxigenin can be performed with commercially available kits, such as the BioNick Labeling System (Cat. no. 18247-015, Invitrogen, Carlsbad, CA) and the DIG Nick Translation Mix (Roche). Nick translation can also be performed with a mix of DNA polymerase I and DNase I.

For direct probe fluorophore labeling using DNA polymerase I/ DNase I, follow this procedure:

1. Add the following to an Eppendorf tube on ice and mix:

10× nick translation buffer	5 μ L
0.2 mM unlabeled ACG nucleotide mixture	5 μ L
100 mM DTT	1 μ L
0.05 mM dTTP	1 μ L
DNA template	1 μ g
1 mM fluorophore-conjugated dUTP	1 μ L
Ultrapure water	up to 45 μ L
DNA polymerase I/DNase I (0.4 U/ μ L)	5 μ L
Total volume	50 μ L

2. Incubate for 2–3 hours at 15°C.

3. Stop reaction with 5 μ L 0.5 M EDTA (pH 8.0).

The reaction provides sufficient labeled probe for four to seven FISH reactions. For labeling of microdissected chromosomes, see Section 11.2.8.5.

For probe precipitation:

1. Add 5 μ L 3 M sodium acetate (pH 5.2) and 150 μ L chilled absolute ethanol for probe precipitation. Mix well.
2. Keep on ice for 15 minutes.
3. Store at -20°C overnight or at -80°C for 2 hours.
4. Centrifuge at 16,000 rpm for 30 minutes in a microcentrifuge.
5. Discard the supernatant.
6. Wash the labeled probe in chilled 70% ethanol.
7. Dry the probe.
8. Resuspend in 20 μ L distilled water or TE (pH 8.0).
9. Store at -20°C until use.

11.2.8.3 FISH with Direct Fluorophore Labeling and Detection

The following protocol is modified from the one by Schwarzacher and Heslop-Harrison (2000). It is a 3-day-long procedure that includes the first slide pretreatment and dehydration (day 1), additional slide pretreatments and hybridization reactions (day 2), and slide posthybridization washing (day 3). Detection steps are not needed after posthybridization washes.

Day 1

For optimum results, slides are pretreated with pepsin, which permeabilizes the cell membrane and eliminates cytoplasm, thus facilitating probe access to target chromosomes. RNA must also be removed to avoid background hybridization,

and a paraformaldehyde fixative is used to preserve the material during in situ hybridization.

Pepsin digestion is necessary for meiotic preparations where removing cytoplasm is essential for efficient probe binding. For this purpose:

1. Add 100 μL 50 $\mu\text{g}/\text{mL}$ pepsin solution in 0.01 N HCl to the preparation and cover with a parafilm coverslip to avoid drying.
2. Incubate the slides 2–5 minutes at 37°C in a humid chamber. Avoid chromosome degradation by stopping the reaction once the cytoplasm is removed.
3. Wash the preparation thoroughly in distilled water at RT and air-dry. This step can be omitted for embryonic mitotic preparations with hypotonic treatments.
4. Dehydrate chromosomes in a 70%, 90%, and absolute ethanol series for 3, 3, and 5 minutes, respectively, and incubate at 60°C overnight.

Day 2

1. Add 200 μL RNase (100 $\mu\text{g}/\text{mL}$ in 2 \times SSC) to each preparation, cover with a parafilm coverslip and incubate for 90–120 minutes at 37°C in a humid chamber.
2. Wash three times in 2 \times SSC at RT for 5 minutes.
3. Place the slides in a Coplin jar with 100 mL freshly prepared 4% paraformaldehyde and incubate them for 10 minutes in a fume hood.
4. Wash three times with shaking for 5 minutes each in 2 \times SSC at RT.
5. Dehydrate preparations in a 70% (3 minutes), 90% (3 minutes), and absolute (5 minutes) ethanol series.
6. Prepare a 30 μL hybridization reaction mix by adding the following reagents to an Eppendorf tube:

Formamide	12 μL
Dextran sulfate (50%)	6 μL
20 \times SSC	1.5 μL
10% SDS	0.5 μL
Salmon sperm DNA (5 $\mu\text{g}/\mu\text{L}$)	1 μL
Probe	100–250 ng per slide
Ultrapure water	up to 30 μL

7. Denature the probe in a water bath or thermocycler at 70°C for 10 minutes.
8. Place on ice for 5 minutes.

9. Apply the hybridization mixture to slides and cover with a parafilm coverslip. Avoid bubbles and cover the entire preparation.
10. Denature the chromosomal DNA by placing the slides with the hybridization mixture on a hot plate for 6 minutes at 80°C.
11. Incubate slides overnight at 37°C in a humid chamber.

Day 3

Slides must be protected from light during washing and detection procedures.

1. Carefully remove the coverslips from slides.
2. Put the slides in a Coplin jar and wash them twice with 2× SSC at 37°C, 5 minutes per wash, with gentle shaking in a water bath.
3. Wash slides with fresh 2× SSC at RT by vigorously shaking on a shaking platform.
4. Wash with 4× SSC/0.2% Tween 20 solution for 5 minutes.
5. Counterstain slides with 100 µL DAPI solution (2 µg/mL) in McIlvaine's buffer for 15 minutes with a parafilm coverslip. Protect the slides from light.
6. Wash briefly in 4× SSC/0.2% Tween 20 solution.
7. Add one drop of antifading solution (VECTASHIELD) and place glass coverslips on slides, avoiding bubbles.
8. Remove the excess antifading solution with filter paper. Repeat this step twice or until the filter paper remains clean after pressing.
9. Store slides horizontally in a box at 4°C in the dark. Visualize on an epifluorescence microscope coupled to appropriate filters.

11.2.8.4 FISH with Indirect Labeling and Antibody Detection

This protocol for chromosome in situ hybridization is divided into three main stages: (1) slide pretreatment (day 1), (2) DNA denaturation/hybridization (day 1) and (3) washing/probe detection (day 2), as modified from that of Cabral-de-Mello et al. (2010). Freshly made or stored (at -20°C) slides can be used for FISH experiments.

Day 1

1. Dehydrate slides in an ethanol series (70%, 85%, and absolute) for 5 minutes each at RT and air-dry at 37°C.
2. Incubate the preparation in 100 µg/mL RNase solution in 2× SSC under a parafilm coverslip for 1 hour at 37°C.
3. Wash three times in 2× SSC for 5 minutes each at RT.

4. Incubate the preparation in 10 µg/mL pepsin solution in 0.1 N HCl under a parafilm coverslip for 20 minutes at 37°C (optional).
5. Wash three times in 2× SSC for 5 minutes each at RT.
6. Place the slide in a Coplin jar with 3.7% formaldehyde diluted in wash-blocking buffer for 10 minutes.
7. Wash three times in 2× SSC for 5 minutes each at RT.
8. Dehydrate the slides in an ethanol series (70%, 85%, and absolute) for 5 minutes each and air-dry at 37°C.
9. Prepare the hybridization probe mixture. In a microtube, add at least 100 ng labeled DNA, formamide (final concentration 50%), SSC (final concentration 2×), and dextran sulfate (final concentration 10%); see an example below.

- 1 6 µL Labeled DNA (at least 100 ng)
- 2 15 µL of 100% Formamide
- 3 6 µL of 50% Dextran sulfate
- 4 3 µL of 20× SSC

10. Denature the hybridization probe mixture at 95°C for 10 minutes and immediately place the tube on ice for 5 minutes.
11. Place the hybridization probe mixture on the slide and cover with a glass coverslip, avoiding bubbles. The quantity of hybridization mixture will determine the coverslip size.
12. Incubate the slides with hybridization mixture at 75°C using a metal plate in a water bath or directly in a thermocycler for 5 minutes.
13. Incubate the slides overnight in a humid chamber at 37°C.

Day 2

1. Remove the coverslip and incubate the slides in a Coplin jar with 2× SSC for 5 minutes at RT.
2. Wash slides twice in 2× SSC at 42°C for 5 minutes each.
3. Wash slides twice in 0.1× SSC at 42°C for 5 minutes each.
4. Wash slides once in 2× SSC at 42°C for 5 minutes.
5. Place slides in 2× SSC at RT for 10 minutes.
6. Transfer the slides to a Coplin jar containing wash-blocking buffer.
7. Dilute the streptavidin-Alexa Fluor 488 conjugate (Life Technologies) for detecting probes labeled with biotin or the anti-digoxigenin-rhodamine (Roche, Basilea, Switzerland) for detecting probes labeled with digoxigenin in wash-blocking buffer, as follows:

1. 1:100 μL for streptavidin-Alexa Fluor 488 conjugate (initial concentration 2 mg/mL): wash-blocking buffer
2. 0.5:100 μL for anti-digoxigenin-rhodamine (initial concentration 200 g/mL): wash-blocking buffer.

Note: For two-color FISH experiments, dilute 0.5 μL anti-digoxigenin-rhodamine and 1 μL streptavidin-Alexa Fluor 488 conjugate in 100 μL wash-blocking buffer solution.

8. Add the solution on the slide and cover with a parafilm coverslip. Incubate at 37°C for 1 hour.
9. Wash the slide three times in wash-blocking buffer at 45°C for 5 minutes each.
10. Mount the slide with 0.5 μL DAPI (0.2 mg/mL) mixed in 15 μL VECTASHIELD antifade solution. Use an appropriately sized glass coverslip.
11. Store the slides in the dark at 4°C until the analysis.
12. Analyze the chromosome preparations under an epifluorescence microscope coupled to an adequate filter set.

11.2.8.5 Chromosome Painting

Chromosome painting is a FISH variation in which a specific chromosome probe homologous to part or the entire length of a particular chromosome is used on metaphase or interphase cells. Since it was developed several decades ago (Cremer et al. 1988; Lichter et al. 1988; Pinkel et al. 1988), the number of applications has increased over time, revealing that FISH procedure is useful for the identification of numerical and structural chromosome aberrations or for the establishment of evolutionary relationships between chromosomes from the same or different species (Figure 11.7b and c). The protocol described here is based on the work by Marchal et al. (2004).

The DNA probe is obtained by chromosome microdissection following the protocols described earlier. Amplified microdissected chromosome DNA is labeled using different approaches depending on the amplification methods used.

1. For chromosomal DNA amplified by DOP-PCR, use the second PCR described in Section 11.2.7.2.
2. For labeling chromosomal DNA amplified by GenomePlex (Sigma-Aldrich), use the standard nick translation procedure.

For probe precipitation, follow the steps:

1. Add the following to an Eppendorf tube and mix:

Distilled water	6.5 μL
-----------------	-------------------

3 M sodium acetate (pH 5.2)	4 μL
Salmon sperm (50 ng/ μL)	1 μL
Glycogen	0.5 μL
Labeled probe	8 μL
Absolute ethanol	20 μL
Total volume	40 μL

- Precipitate overnight at -20°C .
- Centrifuge at 16,000 rpm at 4°C for 30 minutes in a microcentrifuge.
- Discard the supernatant.
- Wash the probe with 100 μL chilled 70% ethanol.
- Centrifuge at 14,000 rpm at 4°C for 30 minutes.
- Discard the supernatant.
- Dry the probe.
- Resuspend the probe in the following solution:

Ultrapure water	9 μL
20 \times SSC	3 μL
Formamide	15 μL
Dextran sulfate (50%)	3 μL
Total volume	30 μL

- Mix and vortex for several seconds.
- Incubate for 3 h at 37°C .
- Store at -20°C until use.

The slides should be pretreated as follows:

- Incubate slides at 37°C for at least 24 hours.
- Dehydrate slides in a 70%, 90%, and absolute ethanol series for 5 minutes each.

Perform the hybridization the following way:

- Denature chromosomal DNA in 70% formamide in 2 \times SSC for 2.5 minutes.
- Put slides in 2 \times SSC at RT for 1 minute.
- Dehydrate slides in a 70%, 90%, and absolute ethanol series for 5 minutes each.
- Denature the probe for 6 minutes at 73°C in a water bath.
- Place the denatured probe on ice for 5 minutes.
- Add the probe mix to slides, cover with a parafilm coverslip and incubate at 37°C for 16 hours in a humidified chamber with formamide/2 \times SSC.

For posthybridization washing and chromosome painting detection, do the following:

1. Wash slides in 0.4× SSC/0.3% Tween 20 at 70°C for 2 minutes.
2. Wash in 2× SSC/0.1% Tween 20 at RT for 30 seconds.
3. Counterstain slides with 100 μL DAPI (20 μg/mL) in Mellvaine's buffer for 15 minutes with a parafilm coverslip in the dark.
4. Wash in PBT (phosphate buffered Tween 20), and mount slides with antifading VECTASHIELD (Vector) using a glass coverslip.
5. Remove the excess antifading solution by gently pressing with filter paper.
6. Store at 4°C several days before analysis under an epifluorescence microscope.

11.2.9 Measuring DNA by Feulgen Image Analysis Densitometry

The following protocol is used to measure the haploid DNA amount (C-value) or even the DNA content of single chromosomes. This method is useful for whole genome sequencing projects, coverage estimates, or simply for estimating the differences in chromosome size caused by chromosome polymorphisms. For instance, we recently estimated the size of three B chromosome variants in *E. plorans* to be 0.51, 0.54, and 0.64 pg, whereas the B chromosome in *L. migratoria* is only 0.15 pg (Ruiz-Ruano et al. 2011).

11.2.9.1 Sample Preparation

Testis tubules fixed in 3:1 absolute ethanol–acetic acid are a good choice for this technique, as they supply spermatids, for measuring the C-value, and spermatocytes, for measuring chromosome size. For C-value measurements, we need to include in the analysis an additional species with a known C-value to be used as a standard, which will allow to estimate the absolute DNA amount in picograms. It is convenient if both species' materials (the sample and the standard) were fixed at the same time in the same conditions (Hardie et al. 2002).

Preparations are made by squashing 2–3 testis tubules from the sample in 50% acetic acid on the left half of a slide and the same amount for the standard on the right half of the same slide. Thus, the Feulgen staining is performed in the same conditions for both the sample and the standard. Given that most grasshoppers show XX/X0 sex chromosome determinism, males are expected to contain two types of spermatids, which differ in DNA amount because

of the presence or absence of the X chromosome. The size of the X chromosome is inferred from the difference between +X and -X spermatids (Ruiz-Ruano et al. 2011). To measure chromosome size, we use the X chromosome of spermatocytes as an internal standard and measure the amount of DNA in the autosomes.

11.2.9.2 Feulgen Reaction

Feulgen reaction is a DNA-specific stoichiometric staining performed as follows:

1. Hydrolyze the preparations in 5 N HCl for 20 minutes at RT to depurinate DNA and generate free aldehyde groups. Then rinse the preparations in 0.1 N HCl. Adjust the hydrolysis time for each type of material. Perform the next steps in the dark.
2. Stain with Schiff's reagent (Sigma-Aldrich) for 90–120 minutes at RT. This reagent binds DNA and thus gives color to chromatin. Fresh Schiff's reagent is recommended, although it can be reused several times if stored at 4°C.
3. Remove the unbound stain with three 5-minute shaking washes in sulfurous water (300 mL containing 1.5 g sodium or potassium metabisulfite and 15 mL of 1 N HCl in distilled water). Wash in running tap water and rinse in distilled water.
4. Air-dry the slides, mount them in DPX, and store them at 4°C in the dark.

11.2.9.3 Image Capture

If image analysis is being performed for the first time, some tests are recommended to estimate the reliability of the microscope and camera. The linearity of the camera response can be tested with density filters, with uniformity testing so that the entire field is homogeneously captured by the camera, with no change in light intensity.

1. Turn on the microscope 10–20 minutes before image capture with the 100× objective to ensure stable measurements. During this time, test Köhler illumination and setup the camera.
2. Exposure time, resolution, and picture format must be the same for all captures. We use 1/120 seg, 1360 × 1024 pixels, and TIFF (tagged image file format), respectively. For higher sensitivity, 16-bit TIFF images are recommended. Although 8-bit images can be used, it is better to save them in the TIFF format. Do not use JPG format because information is lost during compression.
3. Select a region in the slide without materials and lacking dark spots. Open the condenser to an average value,

such that the green channel does not increase, and then adjust the light source until the maximum pixel value is approximately 200 to avoid overexposure of the camera cells. This point is critical for good quantification.

4. Find a region of the preparation with groups of nonoverlapping spermatids. The best results are obtained in spermatids that are starting to elongate because they show a more homogeneously stained nucleus (i.e., discard round spermatids). Assure that the spermatid compaction is similar in the sample and the standard, as inferred from a similar shape. Capture 50–100 spermatids. For measuring selected chromosomes, capture images of 10–20 complete diplotene or metaphases I cells where chromosomes do not overlap.

11.2.9.4 Image Analysis

1. Open the images in ImageJ software (Magelhaes et al. 2004). For 16-bit TIFF images, move the bottom bar to the middle to select the green channel. Trim each object, but include a small part of the background surrounding it. Paste it into a new file (File >> New >> Internal Clipboard), and save it as text with a txt extension. If you use 8-bit TIFF or JPG files, split the channels (Image >> Color >> Split channels), trim objects from the green channel, and save them in the same format (i.e., with the tif or jpg extension). Files from each sample and the standard need to be saved in different folders. Indicate identifiable chromosomes in the file name.
2. Integrated Optical Density (IOD) measures are calculated with a threshold set by Otsu's method and applying the Beer–Lambert's law with our open-source Python-written pyFIA software (Ruiz-Ruano et al. 2011), which can be downloaded from <http://code.google.com/p/pyfia/>. pyFIA has been tested in GNU/Linux Debian and Ubuntu distributions. Instructions for installing and running the program are found on the Web site.
3. In each folder, pyFIA creates a text file called “output” with the IOD values (for analysis) and the average of background values (which is subtracted from the optical density value for each stained pixel).

The format of the output file is optimized for the open-source spreadsheet Gnumeric, although it is compatible with other software of this type.

For spermatid analysis, build a histogram series with different interval amplitudes (Statistics >> Descriptive Statistics >> Frequency Tables >> Histogram), and

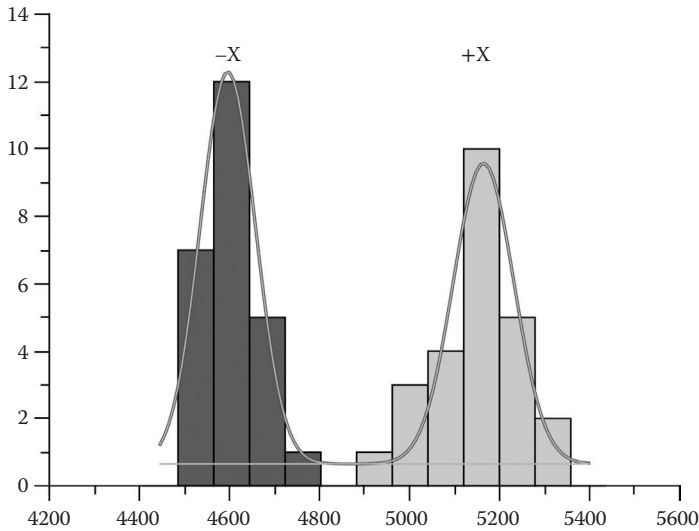


FIGURE 11.8 (See color insert.) Histogram of IOD values obtained from 50 spermatids of an *E. plorans* male. A bimodal distribution shows that one peak belongs to spermatids without (left) or with (right) the X chromosome. The latter peak corresponds to the C-value of the species, and the difference between the two peaks is equivalent to the DNA content of the X chromosome.

choose one that gives the best representation of the data. In the best conditions, a bimodal distribution is observed (Figure 11.8) where the peak with lower IOD corresponds to spermatids lacking the X chromosome. The peak with the higher IOD, however, corresponds to spermatids carrying the X chromosome and represents the C-value. Logically, the difference between the two peaks represents the DNA content of the X chromosome. We then copy the data on the histogram to the open-source software Qtiplot and make a graph (Plots >> Columns). With the histogram window selected, we select Analysis >> Fit Multi-peak >> Gaussian >> 2 peaks. Select the bar at the center of each distribution and calculate the mean of each peak by adjusting to a Gaussian curve. These values are the IOD for the analysis. To measure each object (i.e., the difference between -X and +X spermatids or the biggest chromosome) in the same individual, a coefficient of variation of up to 10% is permitted (Hardie et al. 2002).

4. Finally, the DNA amount of the haploid set (C-value) or the X chromosome can be calculated, in picograms, from the IOD values in the sample and the standard by the following equations:

$$C = \frac{IOD_c \times C_s}{IOD_s}$$

$$X = \frac{IOD_x \times C}{IOD_c}$$

where C = C-value for the sample (picogram); C_s = C-value for the standard (picogram); IOD_c = IOD for the +X peak in the sample; IOD_s = IOD for the +X peak in the standard; X = DNA amount of the X chromosome in the sample (picogram); IOD_x = Difference in IOD between the +X and -X peaks in the sample.

5. To measure selected chromosomes, it is better to measure only the autosomal bivalents because the X chromosome usually shows different condensation. Arrange the autosomal IODs from high to low, sum them, and calculate the relative amount for each autosome by dividing their sum. Calculate the size of each autosome in picograms by multiplying the obtained proportion by the C-value minus X chromosome size in picograms. Arrange all chromosome measurements in picograms, including the X chromosome, from larger to smaller values. This result will indicate the size order of the X chromosome, in respect to the autosomes, avoiding the problem of differential condensation. If the X chromosome size in picograms is unknown because the analysis of spermatids did not exhibit two peaks, the only option is to include the X chromosome in IOD measurements and double it. This method will solve the problem in primary spermatocytes of bivalent autosomes and univalent X chromosome.
6. As DNA amount is usually expressed in picograms, we can easily convert it to bp because 1 pg of DNA is equivalent to 0.978×10^9 bp (Dolezel et al. 2003).

11.2.10 Reagents and Solutions

1. BSA: Dilute in PBS at the required concentration. Make aliquot and store at -20°C .
2. CMA₃: Dissolve 5 mg chromomycin A₃ in 10 mL solution of 1:1 McIlvaine buffer pH 7.0 and distilled water. Add 10 μL of 5 M MgCl₂.
3. DAPI 20 $\mu\text{g}/\text{mL}$: Dilute in McIlvaine's buffer from a stock solution of 100 $\mu\text{g}/\text{mL}$ in water. Store at -20°C .

4. Dextran sulfate (50%): Mix the solution in distilled water by heating at 70°C until dissolved. Filter to sterilize.
5. 1 M DTT: Dissolve 30.9 g DTT in 20 mL 0.01 M sodium acetate (pH 5.2).
6. 100 mM DTT solution: Mix 100 μ L DTT 1 M, 3.3 μ L sodium acetate 3 M, pH 5.2 and 897 μ L ultrapure water.
7. EDTA (ethylenediaminetetraacetic acid): Dissolve 0.5 M EDTA solution in distilled water, pH 8.0.
8. Glycogen: 20 mg/mL in distilled water.
9. Insect saline solution: Dissolve 9 g NaCl, 0.42 g KCl, 0.33 g CaCl₂·2H₂O, 0.2 g NaHCO₃ in 1000 mL distilled water.
10. McIlvaine's buffer: Prepare two separate solutions of 200 mM PO₄HNa₂ and 100 mM citric acid. Add 100 mM citric acid solution (18 mL) to the 200 mM PO₄HNa₂ solution (82 mL) for a 100 mL solution, pH 7.0.
11. 10× Nick translation buffer: 0.5 M Tris-HCl pH 7.8, 50 mM MgCl₂, 5 mg/mL BSA.
12. Orcein (lactopropionic): Dilute 4 g orcein in 100 mL propionic acid and 100 mL lactic acid. Filter twice.
13. Paraformaldehyde 4% solution: Add 4 g paraformaldehyde to 80 mL water and stir while heating at 60°C in a fume hood until translucent. Then add 0.5 mL 4M NaOH to make the solution transparent. Cool the solution and complete to 100 mL.
14. PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4.
15. PBT: Add 10 mL of 10× PBS and 200 μ L Tween 20 to 90 mL distilled water.
16. Pepsin 50 μ g/mL in HCl 0.01N: Dilute a 5 mg/mL pepsin stock solution in HCl 0.01 N.
17. Phosphate buffer: 34 mM KH₂PO₄, 36 mM Na₂PO₄, pH 6.8
18. RNase stock solution: 10 mg/mL RNase in 10 mM Tris-HCl (pH 7.5), 15 mM NaCl. Heat the solution at 100°C for 15 seconds and allow to cool. Make aliquot and store at -20°C.
19. Salmon sperm: Dilute in distilled water from a stock solution.
20. 10% SDS: Make the solution in distilled water and filter it to sterilize.
21. 3 M Sodium acetate: Adjust pH with acetic acid and filter to sterilize.
22. 2× SSC: Dilute from a 20× SSC stock solution.
23. 20× SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0.
24. 0.4× SSC/0.3% Tween 20: Dilute from 20× SSC and add 0.3% (v/v) of Tween 20.
25. 2× SSC/0.1% Tween 20: Dilute from 20× SSC and add 0.1% (v/v) of Tween 20

26. 4× SSC/0.2% Tween 20: Dilute from 20× SSC and add 0.2% (v/v) of Tween 20.
27. TE (Tris-EDTA): 10 mM Tris of the desired pH, 1 mM EDTA, pH 8.0.

TABLE 11.5
Technical Problems and Possible Solutions

Technique	Problem	Solution
Colchicine treatment	Scarce mitotic metaphase cells	Increase concentration or time of colchicine treatment.
Squash chromosome preparations	Scarce cells	Use a lower volume of acetic acid and increase time lapse till coverslip separation.
	Cells too aggregated and mounted on one another	Stronger squashing 5 minutes treatment in 50% acetic acid just before squashing will soften the material.
Spreading chromosome preparations	Opened chromatids	Shorter time of osmotic shock or colchicine treatment.
Chromosome microdissection	Difficulty to identify the chromosome to be microdissected	2% Giemsa staining for 1 minute
Chromosomes for Fiber-FISH	Aggregated cells	Improve cell homogenization
C-Banding	No bands and chromosomes with normal appearance	Add fresh fixative for 1 hour at RT.
	No bands and faint chromosomes	Increase time or temperature of barium hydroxide treatment Decrease time or temperature of barium hydroxide treatment
Silver impregnation	No visible nucleoli	Immerse the material in fresh fixative for 1 hour at RT. Check that oven temperature is at 60°C, and that solution pH is appropriate.
	Nucleoli are stained but chromosomes are scarcely visible	2% Giemsa for 1 minute after silver impregnation.
Triple fluorescent CMA ₃ -DA-DAPI Staining	No fluorescence	Be sure that preparations are not too old and were stored in the dark
		Increase DA counterstaining time
DNA amplification from microdissected chromosomes	Low amount of DNA obtained	Increase the number of chromosomes microdissected

TABLE 11.5 (Continued)
Technical Problems and Possible Solutions

Technique	Problem	Solution
FISH	No or scarce material in the slide after FISH	Increase the amplification time ($\Phi 29$)
		Take a look to the preparation under the microscope, before FISH, to be sure of the presence of enough cells
	No or faint fluorescence signal	Reduce denaturation temperature.
		Check probe fluorescence under the microscope before using it
Background		Check probe concentration and use a positive control slide
		Increase the time of pepsin treatment
		Reduce washing temperature
		Try a different antifading batch
		Increase FISH stringency
		Reduce probe concentration in the hybridization reaction
Feulgen reaction and image analysis	Scarcely stained cells	Improve slide preparation protocol and the post-pepsin washings
		Use new autoclaved solutions.
	Absence of repeatability in the measurements	Adjust time of hydrolysis
		Be sure that Schiff's reagent is freshly prepared and stored in the dark
	Be sure that fixation and image capture were done in the same conditions for both the sample and the standard	
	Check the linearity, uniformity, and stability of camera of microscope	

28. 1 M Tris-HCl: dissolve 121.1 g Tris-base in 800 mL distilled water. Adjust pH adding HCl until required. Complete to a final 1000 mL volume.
29. Unlabeled ACG nucleotide mixture: dATP, dCTP, and dGTP, 0.2 mM each in 100 mM Tris-HCl (pH 7.5) or ultrapure water.
30. Wash-blocking buffer: 0.4× SSC, 0.1% Triton X, 1% BSA or skimmed milk.

11.2.11 Troubleshooting

During the realization of the various techniques described earlier, some problems can occur impoverishing the final result and some solutions are provided in [Table 11.5](#).

11.3 DISCUSSION

11.3.1 Integration of Cytogenetic, Linkage, and Physical Maps and Genome Sequences

Currently, the available techniques have provided rather biased information on grasshopper genomes, with extensive data at the chromosome level, rather scarce information at molecular and genomic levels, and no information for genetic maps obtained from linkage analysis. Grasshopper genomes are the material of choice for cytogenetic studies because of their accessibility and low cost. But, the huge size of grasshopper genomes poses additional difficulty to the implementation of most molecular techniques. For instance, we tried to develop AFLP (amplified fragment length polymorphism) markers for population studies in *E. plorans*, but after 2 years, we had to abandon the project because we were unable to reproduce the observed AFLP patterns. The large genome of this species (10^{10} bp) (Ruiz-Ruano et al. 2011) and the presumed presence of many uncontrolled pseudogenes may have contributed to the lack of repeatability. However, we were more fortunate in developing inter-simple sequence repeat (ISSR) markers in this species, which showed much better reproducibility and allowed us to analyze population genetic structure and gene flow (Manrique-Poyato et al. 2013). Other authors have successfully used random amplified polymorphism DNAs (Sesarini and Remis 2008). This situation will change in the few next years, as NGS becomes increasingly cheap and accessible.

An appropriate combination of many of the techniques described here could advance some genome sequencing projects. The complete assembly of a giant genome, which is 2–5 times larger than the human genome, is a very difficult task because grasshopper genomes contain many copies of mobile elements (Montiel et al. 2012), satellite DNAs, and other repetitive elements (Cabrero et al. 2003; Cabrero and Camacho 2008; Cabrero et al. 2009; Cabral-de-Mello et al. 2011a,b). Many of these paralogous copies are scattered over the entire genome and especially abundant in euchromatin. Thus, the assembly of any given chromosome has many uncertainties. However, we can combine microdissection with PCR amplification of selected DNA sequences to map genes (or DNA sequences) into chromosomes. This type of approach is especially easy in grasshoppers because of their large meiotic chromosomes, which are excellent for microdissection. In this way, we inferred that the B chromosome in *L. migratoria* most likely arose 750,000 years ago (Teruel et al. 2010).

Physical maps of several repetitive DNAs, such as 45S rDNA (Cabrero and Camacho 2008), histone genes (Cabrero et al.

2009), 5S rDNA (Cabral-de-Mello et al. 2011a,b), or mobile elements such as *Gypsy*, *RTE*, and *Mariner* (Montiel et al. 2012), have been obtained in grasshoppers. Phylogenetic tracing of the physical maps should be used to compare species and to infer possible evolutionary patterns at the level of different subfamilies. This analysis could detect phylogenetic incongruences that suggest horizontal transfer, which is especially probable for mobile elements.

The physical maps will be helpful for completing genomic studies because they identify chromosomes. This task is difficult because some chromosomes are nearly the same size, but the mapping of all markers to chromosomes will solve the problem of distinguishing nonhomologous chromosomes of very similar size. Microdissection PCR mapping will help in chromosome identification, provided that one has sequence information.

11.3.2 Chromosome and Genome Organization

Chromosome organization in grasshoppers is rather uniform, at least in the Acrididae family, with two main patterns of karyotypes. The immense majority have 23 acro/telocentric chromosomes in males (24 in females); part of the Gomphocerinae subfamily has 17 (18) by the fixed occurrence of three centric fusions, yielding three long meta-submetacentric pairs (Hewitt 1979). Molecular phylogenetic studies in grasshoppers have shown that the ancestral condition in the Gomphocerinae is the 23 acro/telocentric pattern typically found in *Docicostaurus* and other genera (Bugrov et al. 2006; Contreras and Chapco 2006).

One interesting question is whether all 17 karyotypes in gomphocerine grasshoppers from several different genera (e.g., *Chorthippus*, *Omocestus*, *Stenobothrus*) are monophyletic. In this case, we expect similar gene content in each of the long metacentric pairs from different species; the three centric fusions may have occurred as separate events to yield, polyphyletically, long metacentric chromosome pairs that are not homologous between species. Many of these species carry 45S rDNA genes in the L₂ and L₃ chromosomes at similar interstitial locations, which support the monophyletic hypothesis. Similarly, the chromosome location of histone H3 and H4 genes supports this hypothesis. In species with 23 chromosomes, the location of these genes is highly conserved; they are interstitially located in the eighth autosome, in order of decreasing size. It is also highly conserved in species with 17 chromosomes, where it is located in the short arm of the smallest metacentric autosomes (L₃). This change is most parsimoniously explained by common ancestry, that is, the involvement of the H3–H4-carrying acrocentric (M₈) chromosome in the

centric fusion created the L₃ metacentric autosome (Cabrero et al. 2009). However, synteny for other markers should be analyzed in these species to test the monophyletic hypothesis. Today, we have appropriate tools for the tests: microdissection of separate chromosomes and molecular analysis of the gene content of each chromosome in different species. This approach can be extremely useful in groups where chromosome numbers have experienced dramatic changes. For instance, whereas most acridid grasshoppers show $2n\delta = 22 + X0$ chromosomes, the genus *Dichroplus* includes species with $2n\delta = 18 + X0$, such as *Dichroplus pratensis* (Bidau et al. 1991), and even with $2n\delta = 6 + XY$, such as *Dichroplus silveiraguidoi* (Cardoso et al. 1974). Unveiling how syntenic relationships among genes have changed in parallel with the complex chromosome rearrangements taking place in this and similar cases, will be an interesting topic to investigate in next years, by using the methodology described earlier.

11.3.3 Chromosome and Genome Evolution

The interplay between chromosome and genome evolution is, for the moment, a black box in grasshoppers. We know that acridid chromosomes are rather conserved because most species within a subfamily have very similar karyotypes. This picture changes a little when the location of heterochromatin (Cabrero and Camacho 1986a) or nucleolus organizer regions (Cabrero and Camacho 1986b) are considered, and it changes even more after physical mapping of a comprehensive number of species (Cabrero and Camacho 2008; Cabrero et al. 2009; Cabral-de-Mello et al. 2011a,b). These kinds of studies have revealed a remarkable evolutionary trend in grasshopper genomes for the two families of ribosomal RNA genes (i.e., 45S and 5S). In many species, both families have experienced an intragenomic spread to reach most chromosomes. This spread has been shown at both the intra- and interspecific levels. For instance, the eastern populations of *E. plorans* in Dagestan (Caucasus, Russia) carry 45S rDNA only in the S₉ and S₁₁ chromosomes, whereas Spanish and Moroccan populations carry it in almost all chromosomes (López-León et al. 2008). At the interspecific level, a similar pattern is observed with a broad range of species carrying this gene family, from a single chromosome pair to copies in all chromosomes (Cabrero and Camacho 2008). Similarly, the 5S rRNA gene family is located in a single chromosome pair in some species but in all chromosomes in others (Cabral-de-Mello et al. 2011b). This huge variation in chromosome location could be due to an inherent mobility of rDNA (Schubert 1984; Schubert and Wobus 1985). Remarkably, *Pezotettix giornae* and *Oedipoda caerulescens* carry a single cluster for 45S and 5S rDNA located at

two different chromosome pairs. In contrast, *Omocestus bolivari* carries both types of rDNA in all chromosomes, which suggests that they have common mechanisms for intragenomic mobility (e.g., association with the same type of mobile element). However, species where all chromosomes carry one of the rDNA types but only one carries the other type (e.g., *Stauroderus scalaris* carries 45S rDNA in all chromosome pairs but 5S rDNA in a single chromosome pair, whereas *Chorthippus nevadensis* shows the opposite pattern) (Cabral-de-Mello et al. 2011b) indicate that both rDNA types show independent mobility.

Even with conventional staining, numerous polymorphisms have been described for centric shifts (White 1973; Hewitt 1979), supernumerary segments (Camacho and Cabrero 1982), and B chromosomes (Hewitt 1979; Camacho 2005). We are only beginning to understand—with very scarce and partial details—the relationship between variation at chromosome level and genome evolution. For instance, B chromosomes in *E. plorans* are among the most polymorphic in any animal or plant (Camacho 2005). More than 50 variants had been described by López-León et al. (1993), but B chromosome classification in this species has not been continued. New types appear in nearly every new population that is exhaustively sampled. However, there are few widespread B variants; B₁ is the most common in the Iberian Peninsula, Balearic Islands, Morocco, Tunisia, and Sicily, suggesting that this was the ancestor variant for the entire western Mediterranean region (Cabrero et al. 2014). The majority of B chromosome variants in this species carry rDNA. In eastern populations (Dagestan, Armenia, Turkey, and Greece), B chromosome variants have the repetitive DNA and small amounts of 180-bp DNA tandem repeats (satDNA). Whereas rDNA is also the major component of the B₁ variant, all other variants in some Spanish populations (e.g., B₂, B₅, and B₂₄) carry more satDNA than rDNA, suggesting that B₁ was replaced by variants with higher relative amount of satDNA and lower amount of rDNA. The possibility that this difference could have influenced the replacement of B₁ for the other variants was noted by Cabrero et al. (1999).

A sequence-characterized amplified region (SCAR) marker found in *E. plorans* is specific to the B chromosomes because it is amplified only from B-carrying individuals (Muñoz-Pajares et al. 2011). The 1510 bp sequence of this marker is remarkably similar in B-carrying individuals from Spain, Morocco, Greece, Turkey, and Armenia. Because B chromosomes are dispensable (i.e., B-lacking individuals survive without them), it is unlikely that sequence conservation is due to selective constraints. The high similarity of the SCAR sequence between so distant regions thus suggests that B chromosomes are very young in this species. This example shows how the joint analysis of chromosome and genome evolution can

allow hypothesis testing about the origin of certain genomic compartments, such as a B chromosome. A similar approach, based on the comparison of the internal transcribed spacers 45S rDNA sequences, suggests that the B chromosome arose from the smallest autosome (S_{11}) (Teruel et al. 2014). Similarly, we inferred that the B chromosome in *L. migratoria* could have been derived from the M_8 autosome because the H3 and H4 histone genes mapped to only these two chromosomes (Teruel et al. 2010). As in *L. migratoria*, the mapping of a repetitive DNA (U2 snDNA) indicates the possible origin of the B chromosome from the longest autosome pair in *Abracris flavolineata*. This hypothesis is based on isochromosome formation because of the distribution of the U2 snDNA clusters in both arms of the B chromosome (Bueno et al. 2013). Sex chromosomes have also been recently analyzed by FISH mapping. These data suggested an independent origin of sex-derived systems in related Melanoplinae genera, that is, *Eurotettix* and *Dichromatos*, and a common origin and subsequent differential accumulation of multigene families in neo- X_1X_2Y *Dichromatos* sex chromosomes (Palacios-Gimenez et al. 2013).

These examples show the information that can be obtained by combining cytogenetics with molecular tools. Undoubtedly, there will be new developments that allow further testing of interesting hypotheses on the evolution of different genomic compartments—chromosomes, chromosome segments, mobile genomic elements, repetitive DNAs, and so forth—that lead to the first complete grasshopper genome sequence.

11.4 CONCLUSIONS

The recent publication of the first draft genome for a grasshopper species (*L. migratoria*) by Wang et al. (2014) will represent a qualitative change in the kind of molecular approaches that can be applied at all levels. The availability of a reference genome will undoubtedly allow designing primers for PCR amplification of many genomic regions in other species, with higher easiness for conserved regions, and higher availability for close relative species such as those belonging to the Oedipodinae subfamily. The locust genome is not yet complete because, as in other genomes, the regions being rich in repetitive DNA need additional work, a subject where chromosome studies as those reported here will be of great aid.

With this reference genome, a variety of comparative studies to infer evolutionary changes of many genomic regions can now be performed, thus opening new avenues for cytogenetical work determining the correspondence between physical maps and genome location. In addition, multitude of phylogenetic studies can now be carried out to unveil the evolutionary history of grasshoppers

by using many nuclear markers. So far, phylogenomic studies in grasshoppers have been performed only with mitochondrial genes, but next years will surely witness the inclusion of multiple nuclear genes, as soon as other genomes are available.

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