Cytogenetic methods

František Šťáhlavský
Cytogenetic is a branch of genetics that is concerned with the study of the structure and function of the cell, especially the chromosomes.

1842 – first observations of chromosomes
1888 – used term chromosome
  \((chroma=colour, soma=body)\)
1902-04 – chromosomal inheritance theory
1950s – progress in methods (hypotonization, ...)
1956 – final determination of 2n in human
1968 – banding techniques
1990s – FISH techniques
EUCHROMATIN x HETEROCHROMATIN

- genetic activity
- no genetic activity

Functions of chromosomes
- spatial distribution of genes
- transport of genetic information during cell division
- crossing-over during meiosis, new genetic combinations

Drosophila melanogaster (2n = 8)
Melters et al. 2012

Holocentric chromosomes

Typical monocentric chromosomes

scorpion *Tityus bahiensis* (2n=5-19)

Pseudoscorpion: *Olpium turcicum*: 2n = 7, X0
Monocentric chromosomes

Holocentric (holokinetic) chromosomes

www.metasystems-international.com/ikaros

www.lucia.cz
https://karyotyper.com/

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www.lucia.cz
Karyogram of scorpion *Bothriurus rochensis*

Karyogram of *Astyanax fasciatus* deduced after conventional Giemsa staining and double FISH using 5S (green) and 18S rDNA (red) probes.

Idiogram (ideogram) of scorpion *Tityus trivittatus*
number of chromosomes

from $2n=2$ *Parascaris univalens*,
ants *Myrmecia pilosula, M. croslandi,*
(males $n=1$) to $2n = 446$ *Plebicula atlantica*

Márquez-Corro et al. 2018
number of chromosomes

Modal number of chromosomes
Odonata n=13
Lepidoptera n=28-32
Birds n=39-42
Diptera n=2-10

**Graphs and Diagrams**

- **Salticidae**
  - Number of cases
  - 2n values ranging from 14 to 30

- **Spiders**
  - Different families

- **Pseudoscorpions**
  - Number of species
  - 2n values ranging from 14 to 30

- **Iguanidae**
  - Different families
Zima 2000

<table>
<thead>
<tr>
<th></th>
<th>Palaeartic region</th>
<th>Europe</th>
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<tr>
<td></td>
<td>total species</td>
<td>studied species</td>
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<tr>
<td>Insectivora</td>
<td>81</td>
<td>72 (88.9%)</td>
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<tr>
<td>Chiroptera</td>
<td>73</td>
<td>57 (78.1%)</td>
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<tr>
<td>Rodentia</td>
<td>299</td>
<td>240 (80.3%)</td>
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</table>

Fish app. 30000 species  
- 1700 karyotyped

http://coleoguy.github.io/karyotypes/

Arachnids Karyotypes
http://www.arthropodacytogenetics.bio.br/index.html
Štundlová et al. 2019

Euscorpius (Alpiscorpius) germanus group

chromosome speciation?

“hybrid-sterility model” predicted that the recombination among rearranged chromosomes in heterokaryotypic hybrids generate unbalanced gametes and thus reduce fertility.

“suppressed-recombination model” suggest that the rearrangements reduce recombination between chromosomes and lead to the divergence and speciation.
types of chromosomal rearrangements

Single Chromosome Structural Changes
- Deletion
- Duplication
- Inversion

Two Chromosome Structural Changes
- Insertion
- Translocation

Loss
Gain
Copy Number Neutral Events

Pericentric Inversion
- Breaks in Chromosome
- Centromere
- Reinserted Piece of DNA with Centromere

Paracentric Inversion
- Breaks in Chromosome
- Centromere
- Reinserted Piece of DNA
types of chromosomal rearrangements

Scorpion *Gint gaitako*, 2n=30, Kovařík et al. 2019
Centric fusions - Robertsonian translocations or fissions

Mus musculus domesticus

Tandem fusion

Gonostome (E.) tetrachelatus
2n = 35

Chthonius (E.) sp. 1
2n = 29

Phillips & Ráb 2001

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<thead>
<tr>
<th>Species</th>
<th>Common name</th>
<th>2n</th>
<th>NF</th>
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<tr>
<td>Ball trout</td>
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<tr>
<td>Pink salmon</td>
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<td>Chthonius</td>
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<td>Dolly char</td>
<td>Chthonius</td>
<td>82</td>
<td>98</td>
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<td>White char</td>
<td>Chthonius</td>
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<td>Arctic char</td>
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<td>Smallmouthed char</td>
<td>Chthonius</td>
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<td>Boganid char</td>
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<td>Chthonius sp.</td>
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Mitosis

- S-phase
- DNA synthesis

G2

9 h
4 h
0.9 h

G1

Interphase
- centrioles
- Nucleus
- Nucleolus

Prophase
- Developing spindle
- centrioles
- Chromatids

Prometaphase
- Nuclear envelope
- Kinetochores
- Microtubules

Metaphase
- Equatorial plate
- Chromatids

Anaphase

Telophase
- and Cytokinesis
Meiosis

Meiosis is not a cycle

Gametes / plant and fungi meiospores

Zygote

Interphase $G^4$
$(2c, n)$

Interphase $S$
$(2c \times 2 = 4c, 2n)$

Prophase I
$(4c, 2n)$

Metaphase I
$(4c, 2n)$

Anaphase I
$(2c, n + 2c, n)$

Telophase I
$(2c, n + 2c, n)$

Zygote

Cytokinesis
$(c, n)$
$(c, n)$
$(c, n)$

Anaphase II
$(c, n + c, n)$
$(c, n + c, n)$

Metaphase II
$(2c, n)$

Telophase II
$(2c, n)$
$(2c, n)$

Sex chromosome

Mother cells of gametes or meiospores

Crossing-over
Cytogenetic techniques

The most important – good quality of chromosome preparation

- Dividing cells
  - bone marrow, blood, amniotic fluid, cord blood, tumor, and tissues (including skin, umbilical cord, chorionic villi, liver, and many other organs)
  - In invertebrates very often salivary gland, embryo, testis
  - A mitotic inhibitor (colchicine, colcemid) is added to the culture. This stops cell division at mitosis which allows an increased yield of mitotic cells for analysis.

- Hypotonic solution
  - Potassium chloride (KCl), Citric acid (Na$_3$C$_6$H$_5$O$_7$)

- Fixation
  - methanol (or ethanol) : glacial acetic acid (3:1)
  - Carnoy's fixative - ethanol : chloroform : glacial acetic acid (6:3:1)
  - always fresh !! -

- Spreading
  - good quality of microscope slides !!
  - "dropping"
  - "squashing"
  - "plate spreading"
Conventional staining – homogeneous staining

**Giemsia**
Haematoxylin
Acid-Schiff staining
Carbol fuchsin

Number, morphology and size of chromosomes

Scorpion: *Bothriurus rochensis*

Chthonius (*E.*) *fuscimanus*
2n = 35

Chthonius (*E.*) *tetrachelatus*
2n = 35

Chthonius (*E.*) sp. 1
2n = 29

Chthonius (*E.*) sp. 2
2n = 21

Štáhlavský & Král 2004
Conventional staining – homogeneous staining

Giemsa

sex chromosomes, rearrangements

Scorpion: *Hottentotta judaicus*

Spiders:
- *Spermophora senoculata*
- *Pholcus phalangioides*
- *Diguetia albolineata*
- *Holocnemus caudatus*

Harvestmen: *Gagrellopsis nodulifera* (Gorlov & Tsurusaki 2000)

- *Qumsiyeh et al. 2013*

no heteromorphic bivalent during pachytene

mistake

Kral et al. 2006
Conventional staining – homogeneous staining

Giemsa

B chromosomes

(a) 2n = 6 + XX; (b–e) 2n = 6 + XX + 2–5B (Špakulová et al. 2002)

Acanthocephalus lucii. Chromosome sets of 5 female individuals. (a) 2n = 6 + XX; (b–e) 2n = 6 + XX + 2–5B (Špakulová et al. 2002)
Interphase

Mitotic metaphase

Prophase I

Metaphase I

Metaphase II

Nemastoma bidentatum

A

B

C

D

E
Conventional staining – homogeneous staining

Ancestral state

Mesquite
http://www.mesquiteproject.org/

ChromEvol v. 2.0
http://www.tau.ac.il/~itaym/CP/chromEvol/

Conventional staining – homogeneous staining

R package chromePlus to estimate rates of chromosome number evolution
Selective staining – for spécifique regions, large blocks

**C- banding** - constitutive hetereochromatin

0.2 M HCl for 20-45 min (depurination)
- Rinse with DI water
4% Ba(OH)₂ (barium hydroxid) at 60 °C (denaturation)
- Rinse with DI water
2x SSC at 60 °C for 20-75min (renaturation)
- Rinse with DI water

*Podysma krylonensis*
Bugrov et al. 2004

*Ovis orientalis anatolica*  
Arslan & Zima 2004
Selective staining – for spécifique regions, large blocs

C- banding - constitutive heterochromatin

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2x SSC at 60 °C for 20-75min (renaturation)
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Ovis orientalis anatolica
Arslan & Zima 2004

Steropleurus martorelli (Orthoptera)
Fernandez-Piqueras et al. 1983, Genetica
Selective staining – for specific regions, large blocks

Ag-NOR staining - NOR = Nucleolar Organizing Region

The region contains several tandem copies of ribosomal DNA genes.

- 1 g of AgNO₃ in 1 mL of 0.02 g sodium citrate (C₆H₅Na₃O₇·2 H₂O) per 500 mL distilled water, adjusted to pH 3.0 with formic acid.
- Add 1-2 drops of the above solution onto the slides and place a cover slip over the preparation.
- Incubate slides in a moist chamber at 55–60°C for app. 30 min.

_Ovis orientalis anatolica_  Arslan & Zima 2004
Selective staining – for specific regions, large blocks

G- banding
- obtained by the action of trypsin (10-20s at room temperature in a fresh 0.25% trypsin and than washed in PBS to block the action of trypsin)
similar pattern also in Q-banding (stained by quinacrin)

*Sorex araneus*
2n=20-33
FN=40
Chrom. races=72
Selective staining – for specific regions, large blocks

G- banding
- obtained by the action of trypsin (10-20s at room temperature in a fresh 0.25% trypsin and then washed in PBS to block the action of trypsin)
similar pattern also in Q-banding (saturated by quinacrin)
Selective staining – for spécifique regions, large blocks

R- banding – bands reverse to G-banding
- the thermal denaturation of chromosomes (30-90 minutes at 87°C)

Fluorochrome staining
AT rich regions: DAPI (4',6-diamidin-2-fenylindol), chinakrin, Hoechst 33258
GC rich regions: chromomycin A₃, mithramycin, olivomycin
Transmission electron microscopy (TEM)

Ultrastructure of pairing of the X univalents with acrocentric chromosomes of the trivalent, *Malthonica ferruginea* male. (Král 2007)
FISH - Fluorescence In Situ Hybridization

- **Probe**

- **Target**

**a**

- **Indirect labeling**
- **Direct labeling**

**b**

- **Excitation**
- **Emission**
- **Non-radiative (quenching)**
- **Ground state**

**c**

- **Excited state**

**d**

- **Excited state**

**e**

- **Anti avidin**
- **FITC-avidin**
- **biotin**
- **ciévá DNA**

**Diagram:**

- **Detector**
- **Ocular lens**
- **Excitation filter**
- **Emission filter**
- **Dichroic mirror**
- **Sample**
(a) RNase pre-treatment and formaldehyde post-fixation

1. Dehydrate the slide in ethanol series and air dry
2. Incubate in RNase A solution
3. Wash 3 times in 2× SSC
4. Post-fixation in formaldehyde
5. Wash 3 times in 2× SSC
6. Dehydrate the slide in ethanol series and air dry

(b) Denaturation and hybridization

1. Denature the hybridization solution and place on ice
2. Drop the hybridization solution on the slide, cover and denature the sample
3. Incubate slides overnight

(c) Post-hybridization washes and blocking

1. Wash 2 times in 2× SSC
2. Wash 2 times in 0.1× SSC
3. Wash 1 time in 2× SSC
4. Wash 1 time in 2× SSC
5. Incubate in WBB for blocking

(d) Immunological probe detection and counterstaining

1. Mix the antibody in WNB
2. Place the solution on the slide, cover and incubate
3. Wash 2 times in WBB
4. Counterstain with DAPI and mount the slide
NICK translation
- DNA Polymerase I is used to replace some of the nucleotides of a DNA sequence with their labeled analogues

Primed *in situ* Labelling (PRINS)
Types of probes

- Satellite DNA
  - centromeric
  - telomeric

- Painting probes

- Locus specific

telomere
Insects (TTAGG)n
Vertebrates, Anellida, Mollusca (TTAGGG)n
Nematoda (TTAGGC)n

Ravatsos et al. 2015

Pokorná et al. 2011
Centromere-FISH (ACM-FISH)
armFISH
Catalyzed Reporter Deposition-FISH (CARD-FISH)
Cellular Compartment Analysis of Temporal (Cat) Activity by Fish (catFISH)
Cytochalasin B (CB-FISH)
Chromosome Orientation (CO)-FISH
Combined Binary Ratio (COBRA)-FISH
Chromosome Orientation and Direction (COD)-FISH
Combinatorial Oligonucleotide (COMBO)-FISH
Comet-FISH
Cryo-FISH
Double Fusion FISH (D-FISH)
DNA Breakage Detection FISH (DBD-FISH)
e-FISH
Fiber-FISH
Flow-FISH
Fusion-Signal FISH
Halo-FISH
Harlequin-FISH
Immuno-FISH
Locked Nucleic Acids (LNAs)-FISH
Multiplex (M)-FISH
Multilocus or ML-FISH
Premature Chromosome Condensation (PCC)-FISH
Peptide Nucleic Acid (PNA)-FISH
Quantitative-FISH (Q-FISH)
Quantum Dot (QD)-FISH
Rainbow-FISH
Raman-FISH
Replicative Detargeting FISH (ReD-FISH)
Reverse-FISH
Recognition of Individual Genes (RING)-FISH
RNA-FISH
Cross Species Color Banding (Rx)-FISH
Split-Signal FISH
Stellaris RNA FISH (Single-Molecule RNA FISH)
T-FISH
3-D FISH
Zoo-FISH
Ribosomal DNA (rDNA) - loci encoding 5S and 45S (18S-5.8S-28S) rRNAs

18S rDNA probe

Borba et al. 2014  Nguyen et al. 2010
locus specific

Excess rRNA genes organized in heterochromatin

Chromosome arm

NOR

Repeated rRNA genes

Intergenic spacer

Coding sequences

Ribosomal DNA (rDNA)
- loci encoding 5S and 45S (18S-5.8S-28S) rRNAs

Sember et al. 2015

Nemacheilidae

Ribosomal DNA (rDNA)
- loci encoding 5S and 45S (18S-5.8S-28S) rRNAs

Sember et al. 2015
Ribosomal DNA (rDNA)
- loci encoding 5S and 45S (18S-5.8S-28S) rRNAs

Sochorová et al. 2018

Number of 5S and 45S rDNA sites in different animal taxa

http://www.animalrdnadatabase.com

De Barros et al. 2017
Ribosomal DNA (rDNA)
- loci encoding 5S and 45S (18S-5.8S-28S) rRNAs

De Barros et al. 2017

Position of rDNA sites on chromosomes

45S
- Fish: 18%, 17% interstitial, 56% pericentromeric, 26% terminal
- Mammals: 16%, 24% interstitial, 52% pericentromeric, 34% terminal
- Arthropods: 42%, 24% interstitial, 34% pericentromeric, 24% terminal
- Mollusks: 16%, 17% interstitial, 67% pericentromeric, 16% terminal

5S
- Fish: 39%, 34% interstitial, 30% pericentromeric, 36% terminal
- Mammals: 27%, 27% interstitial, 49% pericentromeric, 14% terminal
- Arthropods: 23%, 28% interstitial, 49% pericentromeric, 18% terminal
- Mollusks: 36%, 36% interstitial, 36% pericentromeric, 28% terminal

Sochorová et al. 2018

http://www.animalrdnadatabase.com
M-FISH
multiplex FISH or multicolour FISH

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<th>chrom. #</th>
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<td>1</td>
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<td>2</td>
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<td>7</td>
<td>FITC, Cy3, Cy5</td>
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completed karyotype

metaphase chromosome spread
M-FISH

Multicolour banding (mBAND)
Chromosome sorting (flow cytometer)

- Sheath fluid
- Flow chamber
- Light source
- Deflection plates
- Collection tube
- Charged droplet
- Waste
- Chromosome suspension
- Fluorescence detector
- Forward scatter detector
- Drop charging signal
- Sorted chromosomes

Microdissection

- Taq DNA polymerase
- DOP primers
- dNTPs

DOP-PCR (degenerated-oligonucleotide-primed PCR)

FISH labeling
Pokorná et al. 2015, Chrom. Res.
ZOO-FISH - cross-species chromosome painting, which uses painting probes specific for whole chromosomes, enables detecting homologous synteny blocks, the occurrence of which is evidence that species share a common ancestry and are related.

Ancestral karyotype of the genus *Sorex*. Used painting probes of human.

Ferguson-Smith & Trifonov 2007

Biltueva et al. 2011
CGH – comparative genomic hybridization

Sex chromosomes
CGH in pachytene of Galleria mellonella female genomic probes were labelled with Alexa Fluor 488 (green) male-derived genomic probes with Cy3 (red)
Arrow indicates a region of the W chromosome exclusively stained by female genomic probe.
Vitková et al. 2007
**GISH – genomic *in situ* hybridization**

- a type of FISH, uses total genomic DNA from one species as the labeled probe and unlabeled genomic DNA from another species at a much higher concentration as blocking DNA, substantially increasing the hybridization specificity

GISH on chromosomes of the water frog *Pelophylax esculentus* obtained from bone marrow of a single female.

**B.** Metaphase chromosomes hybridized with the Alexa Fluor 488-labeled genomic probe from *P. lessonae* (*green* signals); chromosomes were counterstained with PI (red)

**D.** Metaphase chromosomes hybridized with the Cy3-labeled genomic probe from *P. ridibundus* (*red* signals); chromosomes were counterstained with DAPI (blue). (Zalesna et al. 2011)
Thank you for your attention