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**
   \[(chroma\text{-}colour, \ soma\text{-}body)\]
1902-04 – chromosomal inheritance theory

1950s – progress in methods (hypotonization,...
1956 – final determination of 2n in human
1968 – banding techniques
1990s – FISH techniques
Functions of chromosomes
- spatial distribution of genes
- equal transport of genetic information during cell division
- crossing-over during meiosis, new genetic combinations

EUCHROMATIN x HETEROCHROMATIN

Genetic activity | No genetic activity

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

Holocentric chromosomes

- microtubules
- kinetochore
- Irradiation
- Single-stranded break
- Next cell division - Replication
- Mitotic anaphase
- 'Stable'
- Kinetic
- Inherited

Typical monocentric chromosomes

- microtubules
- centromere
- Irradiation
- Single-stranded break
- Next cell division - Replication
- Mitotic anaphase
- 'Unstable'
- Kinetic
- Acentric
- Lost
- Dicentric
- Anaphase bridge

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

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

- holocentrics
- 6-eyed spiders
- mites / ticks
- centipedes
- insects
- nematodes
Holocentric chromosomes

Microtubules

Kinetochore

Irradiation

Single-stranded break

Next cell division - Replication

Mitotic anaphase

'Stable'

Colonization of the land 500 Mya

Monocentric chromosomes

Dicentric

Acentric

Lost

Anaphase bridge

Melters et al. 2012

Pseudoscorpion: *Olpium turcicum*: $2n = 7$, $X_0$

Zedek & Bures 2018, *Annals of Botany*
Monocentric chromosomes

Holocentric (holokinetic) chromosomes

<table>
<thead>
<tr>
<th>Arm Ratio</th>
<th>Levan et al. (1964)</th>
<th>Green &amp; Sessions (1991)</th>
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<tr>
<td>1.0</td>
<td>M (Metacentric)</td>
<td>m (Metacentric)</td>
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<td>1.0 ≤ x &lt; 1.7</td>
<td>m (Metacentric)</td>
<td>m (Metacentric)</td>
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<td>1.7 ≤ x &lt; 3.0</td>
<td>sm (Submetacentric)</td>
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<td>t (Telocentric)</td>
</tr>
<tr>
<td>∞</td>
<td></td>
<td></td>
</tr>
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</table>

www.metasystems-international.com/ikaros

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

www.metasystems-international.com/ikaros

www.lucia.cz
http://imagej.nih.gov/ij/

http://www.drawid.xyz
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 $n=2-10$

![Graph showing the modal number of chromosomes for different groups, including Odonata, Lepidoptera, birds, and Diptera, with specific chromosome numbers mentioned for each category.](image)
Zima 2000

<table>
<thead>
<tr>
<th></th>
<th>Palaeartic region</th>
<th>Europe</th>
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</thead>
<tbody>
<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>
</tr>
<tr>
<td>Chiroptera</td>
<td>73</td>
<td>57 (78.1%)</td>
</tr>
<tr>
<td>Rodentia</td>
<td>299</td>
<td>240 (80.3%)</td>
</tr>
</tbody>
</table>

Fish app. 30000 species
- 1700 karyotyped

http://coleoguy.github.io/karyotypes/
### Zima 2000

<table>
<thead>
<tr>
<th>Class</th>
<th>Palaeartic region</th>
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</tr>
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</table>

### Arachnids Karyotypes

https://arthropodacytogenetics.bio.br/
Scorpiones

Štundlová et al. 2019

Euscorpius (Alpiscorpius) germanus group

Cryptocercus

Che et al. 2006
“hybrid-sterility model” predicted that the karyotypic hybrids generate unbalanced gametes and thus reduce fertility.

“suppressed-recombination model” suggests that the rearrangements reduce recombination between chromosomes and lead to the divergence and speciation.

Štundlová et al. 2019
types of chromosomal rearrangements

Single Chromosome Structural Changes:
- Deletion
- Duplication
- Inversion

Two Chromosome Structural Changes:
- Insertion
- Translocation

Copy Number Neutral Events:
- Loss
- Gain

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

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

- Single Chromosome Structural Changes
  - Deletion
  - Duplication
  - Inversion

- Two Chromosome Structural Changes
  - Insertion
  - Translocation

Mitotic metaphase

Meiotic pachytene

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

Mus musculus domesticus

Tandem fusion

Phillips & Ráb 2001

<table>
<thead>
<tr>
<th>Species</th>
<th>Common name</th>
<th>2n</th>
<th>NF</th>
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<tr>
<td>Salmoninae (cont.)</td>
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<tr>
<td>Salvelinus confluentes</td>
<td>Ball trout</td>
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<td>fontinalis</td>
<td>Brook trout</td>
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<td>100</td>
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<td>nanouxi</td>
<td>Lake trout</td>
<td>84</td>
<td>100</td>
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<tr>
<td>rutilus</td>
<td>Potted char</td>
<td>84</td>
<td>100</td>
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<tr>
<td>planius</td>
<td>Japanese char</td>
<td>84-86</td>
<td>100</td>
</tr>
<tr>
<td>alpinus/fulmis complex</td>
<td>Dolly char</td>
<td>82</td>
<td>98</td>
</tr>
<tr>
<td>albus</td>
<td>Varilen char</td>
<td>78</td>
<td>98</td>
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<td>alpinus</td>
<td>Arctic char</td>
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<td>elgicus</td>
<td>Small mouthed char</td>
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<td>aganidae</td>
<td>Bogani char</td>
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<td>98</td>
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<tr>
<td>arcticus</td>
<td>Stone char</td>
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<td>100</td>
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<td>Eastern Arctic char</td>
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<td>98</td>
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<tr>
<td>m. krasenzi</td>
<td>Varilen char</td>
<td>82</td>
<td>98</td>
</tr>
<tr>
<td>Salvelinus scheetzatovi</td>
<td>Longfinned char</td>
<td>56</td>
<td>98</td>
</tr>
</tbody>
</table>

Chthonius (E.) tetrachelatus
2n = 35

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

Number fundamental
Mitosis

S-phase
DNA synthesis

G1

Interphase
Prophase
Prometaphase

Metaphase
Anaphase
Telophase

and Cytokinesis
Meiosis

- Interphase G¹ (2c, 2n)
- Interphase S (2c × 2 = 4c, 2n)
- Prophase I (4c, 2n)
- Metaphase I (4c, 2n)
- Anaphase I (2c, n + 2c, n)
- Telophase I (2c, n + 2c, n)

- Interphase G² (4c, 2n)
- Crossing-over

- Cytokinesis
  - (c, n)
  - (c, n)
  - (c, n)
  - (c, n)

- Meiosis is not a cycle

- Zygote
  - (c + c = 2c, n + n = 2n)

- Gametes / plant and fungi meiospores

- Mother cells of gametes or meiospores

- Sex chromosome
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$_2$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)

  allways fresh !! -

- Spreading
  - (good quality of microscope slides !!)
    - „dropping“
    - „squashing“
    - „plate spreading“

The different steps of the karyotyping procedure

- Amniocentesis
- or
- Venipuncture (peripheral blood)
- PhA
  (= mitogen) phytohaemagglutinin
  Embryonic cells or Lymphocytes

- KCl
  (= hypo-osmotic)

- Colchicine
  (= Mitosis blocker)

- Hypotonic solution
  - Potassium chloride (KCl), Citric acid (Na$_2$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)

  allways fresh !! -

- Spreading
  - (good quality of microscope slides !!)
    - „dropping“
    - „squashing“
    - „plate spreading“

Fixation

- Pipette transfer
- Microscope glass slide
- Methylalcohol/ Acetic Acid Mix (3:1)

Heating & Drying

- Chromosome staining

- Microscopy (250x)

Graphiced E. Schmid 2002
Conventional staining – homogeneous staining

**Giemsa**

Haematoxylin

Acid-Schiff staining

Carbol fuchsin

Number, morphology and size of chromosomes

Scorpion: *Bothriurus rochensis*

*Chthonius (E.) fuscimanus*

2n = 35

*Chthonius (E.) tetracherche*

2n = 35

*Chthonius (E.) sp. 1*

2n = 29

*Chthonius (E.) sp. 2*

2n = 21

Šťáhlavský & Král 2004
Conventional staining – homogeneous staining

Giemsa

sex chromosomes, rearrangements

Scorpion: *Hottentotta judaicus*

no heteromorphic bivalent during pachytene

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

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

Qumsiyeh et al. 2013

Kral et al. 2006
Conventional staining – homogeneous staining

Giemsa

B chromosomes

\[ \begin{array}{c}
\text{a} \\
\text{b} \\
\text{c} \\
\text{d} \\
\text{e}
\end{array} \]

\[ \begin{array}{c}
\text{1} \\
\text{2} \\
\text{3} \\
XX \\
B
\end{array} \]

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

Hervestmen Metagagrella tenuipes Tsurusaki 1993
Conventional staining – homogeneous staining

Ancestral state

Mesquite
http://www.mesquiteproject.org/

ChromEvol v. 2.0
http://www.tau.ac.il/~itym ay/cp/chrom Evol/

Conventional staining – homogeneous staining

R package chromePlus to estimate rates of chromosome number evolution

Sylvester et al. 2020, Proc. R. Soc. B
Selective staining – for spécifique regions, large blocks

C-banding - constitutive heterochromatin

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
Selective staining – for specifique regions, large blocs

C- banding - constitutive heterochromatin

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

*Ovis orientalis anatolica* Arslan & Zima 2004

*Steropleurus martorelli* (Orthoptera) Fernandez-Piqueras et al. 1983, Genetica

*X* *Y*
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 AgNO$_3$ in 1 mL of 0.02 g sodium citrate $(C_6H_5Na_3O_7\cdot2H_2O)$ 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.
Selective staining – for spécifique 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 (saturated 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 than washed in PBS to block the action of trypsin)
similar pattern also in Q-banding (saturated by quinacrin)

Comparison of G-banded chromosomes of *Sorex minutus* and *S. granarius*

Biltueva et al. 2011
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

**a**
- Probe
- Target

**b**
- indirect labeling
- direct labeling

**c**

**d**

**e**
- target DNA
  - biotin
  - FITC - avidin
  - anti avidin
  - biotin
  - FITC - avidin

**Diagram details**
- Detector
- Ocular lens
- Emission filter
- Dichroic mirror
- U.V. lamp
- Excitation filter
- Sample

**Excitation**
- excited states
- non-radiative (quenching)
- ground state
(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 WWB
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)
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
Types of probes

- Satellite DNA
  - centromeric
  - telomeric

- Painting probes

- Locus specific

Insects
Vertebrates, Anellida, Mollusca
Nematoda

(TTAGG)n
(TTAGGG)n
(TTAGGC)n

Eremias velox Pokorná et al. 2011
**Types of probes**

- Satellite DNA
  - centromeric
  - telomeric

- Painting probes

- Locus specific

**Telomeres**

- Insects
  - Vertebrates, Anellida, Mollusca
  - Nematoda

- (TTAGG)n
- (TTAGGG)n
- (TTAGGC)n

*Anolis distichus* Ravatsos et al. 2015

Augstenová et al. 2018, *Genes*
18S rDNA probe

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

Nemacheilidae

Sember et al. 2015

<table>
<thead>
<tr>
<th>Zn</th>
<th>Karyotype description</th>
<th>NF</th>
<th>Partial karyotypes with rDNA-bearing sites</th>
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<tr>
<td>60</td>
<td>8m - 16m - 21st-a</td>
<td>74</td>
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<tr>
<td>60</td>
<td>10m - 12m - 28st-a</td>
<td>72</td>
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<tr>
<td>60</td>
<td>8m - 16m - 24st-b</td>
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<td>60</td>
<td>8m - 12m - 30st-a</td>
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<tr>
<td>60</td>
<td>21m - 18m - 20st-a</td>
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<td>50</td>
<td>16m - 24m - 10st-a</td>
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<table>
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<tr>
<th>44-46</th>
<th>21m - 18m - 6st-a (NF=859)</th>
<th>19m - 14m - 16st-a (NF=859)</th>
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<tr>
<td>50</td>
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<tr>
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<td>6m - 18m - 26st-a</td>
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<td>14m - 16m - 14st-a</td>
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<td>5m - 24m - 19st-a</td>
<td>62</td>
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<tr>
<td>60</td>
<td>10m - 20m - 14st-a</td>
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<td>10m - 16m - 16st-a</td>
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<td>50</td>
<td>6m - 14m - 30st-a</td>
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<td>50-51</td>
<td>8m - 22m - 20st-a</td>
<td>60-62</td>
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</table>

Red bars indicate 45S rDNA sites; Green bars indicate 18S rDNA sites; Black squares indicate variable polymorphic rDNA loci
Ribosomal DNA (rDNA)
- loci encoding 5S and 45S (18S-5.8S-28S) rRNAs

http://www.animalrDNAdatabase.com

2500 species
1068 Arthropods
653 fish

Sochorová et al. 2018
Sochorová et al. 2021

Sochorová et al. 2018
- Number of publications
- 1st release vs 2nd release
- No. publications

Schizodon fasciatus
54 loci 45S/2C

De Barros et al. 2017

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

Sochorová et al. 2021
Ribosomal DNA (rDNA)
- loci encoding 5S and 45S (18S-5.8S-28S) rRNAs

Sochorová et al. 2018
- 2500 species
- 1068 Arthropods
- 653 fish

Position of rDNA sites on chromosomes

http://www.animalrDNAdatabase.com

De Barros et al. 2017
M-FISH
multiplex FISH or multicolour FISH

<table>
<thead>
<tr>
<th>chrom. #</th>
<th>probe fluor composition</th>
<th>chromosome paint</th>
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<tbody>
<tr>
<td>1</td>
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<tr>
<td>2</td>
<td>Cy3</td>
<td><img src="image" alt="Red Paint" /></td>
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<td>3</td>
<td>Cy5, FITC</td>
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<tr>
<td>4</td>
<td>Cy5</td>
<td><img src="image" alt="Blue Paint" /></td>
</tr>
<tr>
<td>5</td>
<td>Cy3, Cy5</td>
<td><img src="image" alt="Pink Paint" /></td>
</tr>
<tr>
<td>6</td>
<td>FITC, Cy3</td>
<td><img src="image" alt="Yellow Paint" /></td>
</tr>
<tr>
<td>7</td>
<td>FITC, Cy3, Cy5</td>
<td><img src="image" alt="White Paint" /></td>
</tr>
</tbody>
</table>

completed karyotype

![Karyotype Image](image)
M-FISH

Multicolour banding (mBAND)
Chromosome sorting
(flow cytometer)

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

Microdissection

1. Initial microdissection
2. Microdissection after sorting
3. Final microdissection

- Taq DNA polymerase
- DOP primers
- dNTPs

FISH labeling

DOP-PCR
(degenerated-oligonucleotide-primed PCR)
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

Chelonida expansa

**CGH** – comparative genomic hybridization

**Sex chromosomes**

CGH in pachytene of *Galleria mellonella*  
female genomic probes were labelled with Alexa Fluor 488 (green)  
and male-derived genomic probes with Cy3 (red)  
Arrow indicates a region of the W chromosome exclusively stained by female genomic probe.  
Vítková 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