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Molecular cytogenetics of selected actinopterygian fishes: insight from repetitive sequences to whole genome analyses

Molekulární cytogenetika paprskoploutvých ryb: od repetitivních sekvencí po analýzu celého genomu

Ph.D. thesis

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I hereby declare that this Ph.D. Thesis is exclusively my own work, and that it has not been submitted (or any of its part) in order to obtain any academic degree earlier or at another institution. My contribution to the research presented in the Thesis is reflected by the order of authors of the included publications. All publications and other sources used in the thesis have been properly cited.

Prague, 1. 11. 2016

Zuzana Majtánová

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CONTENTS

PODĚKOVÁNÍ **4**
ABSTRAKT **6**
ABSTRACT **7**
LIST OF PUBLICATIONS INCLUDED INTO THE THESIS **8**
1. INTRODUCTION **10**
2. AIMS OF THE THESIS **13**
3. LITERATURE REVIEW **14**
3.1. Characterization of fish genomes 14
3.2. Major events shaping fish genome evolution 17
3.3. Fish cytogenetics – application and future prospects 19
4. MATERIAL AND METHODS **23**
4.1. Material 23
4.2. Methods 23
5. RESULTS AND DISCUSSION **24**
5.1. Lepisosteidae 24
5.2. Amiidae 26
5.3. Pantodontidae 27
5.4. Cobitidae 29
5.5. Coregonidae 30
6. CONCLUSION **32**
7. PUBLICATIONS **34**
7.1. Publication I 34
7.2. Publication II 53
7.3. Publication III 81
7.4. Publication IV 119
7.5. Publication V 131
7.6. Publication VI 141
8. REFERENCES **157**

ABSTRAKT

Paprskoploutvé ryby, Actinopterygii, skupina s nejvyšší taxonomickou diverzitou mezi všemi obratlovci, představují v posledním období atraktivní objekt evolučních studií. S pokrokem v oblasti molekulární cytogenetiky a cytogenomiky roste i význam těchto metod ve studiu uspořádání a evoluce rybích genomů jako základu této diverzity. Má dizertační práce se zabývá studiem evoluce genomu vybraných skupin ryb, a to jak bazálních linií parskoploutvých (Lepisosteidae a Amiidae) a kostnatých (Pantodontidae), tak i "vyšších" kostnatých ryb z čeledí Cobitidae a Coregonidae. Využila jsem metody konvenční a molekulární cytogenetiky společně s fylogenetickými a statistickými přístupy. V rámci své dizertační práce popisuji cytogenetickou variabilitu blízce příbuzných druhů síhů (*Coregonus*), a roli repetitivních úseků genomu v ekologické speciaci. V kontrastu s recentní cytotaxonomickou diverzitou síhů, jsme odhalili relativně dlouhodobou karyotypovou stabilitu ve spojení s asexuálním rozmnožováním u sekavců rodu *Cobitis*. V další publikaci detailně popisujeme překvapivé uspořádání AT/GC bazí na chromozomech a v genomu kostlínů (*Atractosteus* a *Lepisosteus*) a bioinformaticky analyzujeme jejich uspořádání v genomu těchto bazálních paprskoploutvých ryb v kontextu hlavních linií obratlovců. V návaznosti na práci o kostlínech, přinášíme detailní cytogenetická data u kaprouna obecného (*Amia calva*), jediného recentního představitele řádu Amiiformes. Ačkoliv patří kaproun mezi nejbližší žijící příbuzné kostlínů, ukazujeme jeho karyologickou a cytogenetickou podobnost kostnatým rybám. Dále jsem se podílela na cytogenetické analýze motýlkovce afrického (*Pantodon buchholzi*), jediného představitele čeledi Pantodontidae, řádu Osteoglossiformes. Výsledky této dizertační práce ukazují, jak dynamický a komplexní systém představují genomy ryb a že i v době masivně využívaných sekvenací nové generace, mohou cytogenetické přístupy poskytnout data nezískatelná jinými metodami.

ABSTRACT

Actinopterygian fishes exhibit the greatest taxonomical diversity of all vertebrates, making this group attractive to address numerous evolutionary questions. The role of molecular cytogenetics and cytogenomics further increase because recent advances in these fields provide more comprehensive view of fish genome organization and evolutionary dynamics, responsible for this amazing diversity. My Thesis investigates the genome organization of selected fish lineages, namely basal lineages of Actinopterygians (Lepisosteidae and Amiidae) and Teleosts (Pantodontidae), together with "modern" fishes Cobitidae and Coregonidae. I have integrated conventional and molecular cytogenetic techniques together with phylogenetic and statistical approaches. Publications included into the Thesis describe e.g. the cytogenetic variability and dynamics in closely related fish species of the genus *Coregonus* and the impact of repetitive sequences on the ecological speciation. In contrast to the recent cytotaxonomical diversity of Coregonids, we have detected a karyotype stability associated with asexual reproduction in spined loaches of the genus *Cobitis.* In the subsequent publication, we describe a surprising AT/GC genome organization in gars (*Atractosteus* and *Lepisosteus*) and summarize the knowledge of genome organization and karyotype differentiation of these basal actinopterygian lineages in the context of other major vertebrate lineages. Also we provide first reliable chromosomal data for the bowfin *Amia calva*, the only representative of fishes from order Amiiformes. Although bowfin represents the closest extant lineage of gars, we show its karyological and cytogenetic similarities with Teleosts. I have participated on cytogenetic analyses of the African butterfly fish *Pantodon buchholzi*, the only representative of the family Pantodontidae (Osteoglossiformes). The outcomes of this Thesis reveal the dynamic nature and the complexity of the fish genome, as well as the importance of cytogenetic and cytogenomic methodologies in the age of the next generation sequencing.

LIST OF PUBLICATIONS INCLUDED INTO THE THESIS

I. **Majtánová Z.**, Choleva L., Symonová R., Ráb P., Kotusz L., Pekárik & Janko P. 2016. Asexual reproduction does not apparently increase the rate of chromosomal evolution: karyotype stability in diploid and triploid clonal hybrid fish (*Cobitis*, Cypriniformes, Teleostei). PLoS ONE. 11:e0146872. IF_{2015}: 3.057

II. **Majtánová Z.**, Symonová R., Arias-Rodriguez L., Sallan L. & Ráb P. 2016. "Holostei versus Halecostomi" problem: insight from cytogenetics of ancient non-teleost actinopterygian fish, bowfin *Amia calva*. Journal of Experimental Zoology Part B: Molecular and Develomental Evolution. Invited manuscript to the special issue „Genome of the spotted gar". IF_{2015}: 2.083

III. Symonová R., **Majtánová Z.**, Arias-Rodriguez L., Mořkovský L., Kořínková T., Cavin L., Johnson Pokorná M., Doležálková M., Flajšhans M., Normandeau E., Ráb P., Meyer A. & Bernatchez L. 2016. Unique genome organisation in gars (Lepisosteidae: Actinopterygii) shows more similarities to mammals than to any other ray-finned fishes. Journal of Experimental Zoology Part B: Molecular and Develomental Evolution. Invited manuscript to the special issue „Genome of the spotted gar". IF_{2015}: 2.083

IV. Symonová R., **Majtánová Z.**, Sember A., Staaks GBO., Bohlen J., Freyhof J., Rábová M. & Ráb, P. 2013. Genome differentiation in a species pair of coregonine fishes: an extremely rapid speciation driven by stress-activated retrotransposons mediating extensive ribosomal DNA multiplications. BMC Evolutionary Biology. 13:42. IF_{2013}: 3.407

V. Ráb P., Yano C., Lavoué S., Jegede O., Bertollo L., Ezaz T., **Majtánová Z.**, Oliveira E. & Cioffi M. 2016. Karyotype and repetitive DNAs mapping in the African butterfly fish *Pantodon buchholzi*, the sole species of the family Pantodontidae. Cytogenetic and Genome Research. DOI: 10.1159/000450534. IF_{2015}: 1.638

VI. Symonová, R., Sember, A., **Majtánová Z.** & Ráb P. 2015. Characterization of fish genomes by GISH and CGH. Pp. 118-131. In: Fish Cytogenetic Techniques (Chondrichthyans and Teleosts). Eds: Catherine Ozouf-Costaz, Eva Pisano, Fausto Foresti, Lurdes Foresti de Almeida Toledo CRC Press, Inc., Enfield, NH 03748, USA.

1. INTRODUCTION

Actinopterygians (ray-finned fishes) is the largest and most diverse vertebrate group, dominating modern aquatic ecosystems. The number of taxonomically recognized species is approaching 29 600 (67 orders, 474 families, 4621 genera) (Bemis et al. 1997; Suzuki et al. 2010; Nelson et al. 2016) but the eventual number of extant fish species is estimated to be close to 32 000 (Faircloth et al. 2013; Nelson et al. 2016). Ray-finned fishes have undergone vast radiation events which are unparalleled to other vertebrate taxa (Venkatesh 2003; Alfaro et al. 2009) and roughly represent the half of the extant vertebrate species (Clark 2003; Near et al. 2012; Nelson et al. 2016). Actinopterygians show vast differences in their life strategies and adaptations for life; they occupy essentially all aquatic and semi aquatic habitats that have liquid water at least part of the year, including thermal and alkaline springs, hypersaline lakes, sunless caves, anoxic swamps, temporary ponds, torrential rivers, wave-swept coasts, and high-altitude and high-latitude environments (Helfman et al. 2009). Fishes consume a variety of resources from microplankton to mammals and display an enormous diversity in morphology, ecology and behaviour. Since Actinopterygians are essential components of most ecosystems in which they occur, investigation of their evolutionary history is vital for interpreting not only their living biodiversity but also that of vertebrates in general (Hurley et al. 2007; Near et al. 2012; Sallan 2014).

The vast majority of living Actinopterygians belong to the group Teleostei derived from the actinopterygian stem about 450-350 MYA (Venkatesh 2003). The success of Teleosts is often attributed to an additional genome duplication event (teleost specific genome duplication, TSGD) next to the two basal ones and to TSGD-related morphological novelties (Sallan 2014). Fossil record of diverse Teleosts has been recorded from Jurassic and Cretaceous periods (Arratia 2000; Venkatesh 2003). The most ancient teleost fossils dated

approximately 235 MYA and are poorly classified into Pholidophorids and Ichthyokentemids lineages (Maisey et al. 1996).

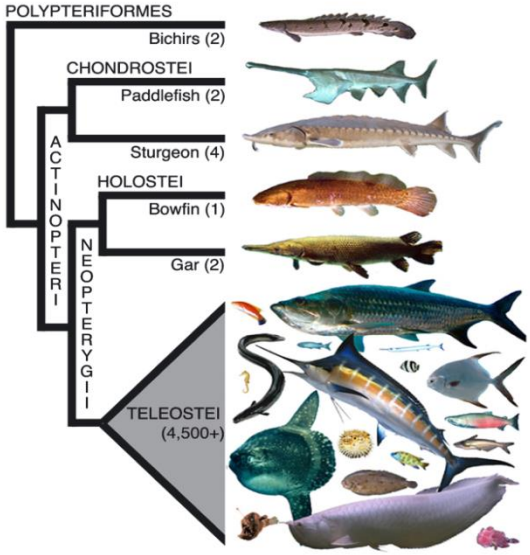


Figure 1. Consensual phylogeny of living actinopterygian clades after Sallan (2014). Numbers indicate living genera. Drawings represent the major lineages of Actinopterygians. Basic topology after Grande (2010) and Faircloth et al. (2013).

The remainders of Actinopterygians belong to five lineages with long-retained ecomorphologies: Polypteriformes (bichirs), Lepisosteiformes (gars), Amiiformes (bowfin), Polyodontiformes (paddlefish) and Acipenseriformes (sturgeons and shovelnoses) (Figure 1.). Despite over a century of systematic research and the rapid progress toward reconstructing the Vertebrate Tree of Life, the phylogenetic relationships among the major actinopterygian clades still remain unresolved (Thomson and Shaffer 2010). In fact, the inconsistence in species richness of extant lineages prohibits a reliable reconstruction of the ancestral state. Therefore, although 29,585 living species of Teleosts have been described (Nelson et al. 2016), the species richness of extant basal Actinopterygians is limited to 12 living species of bichirs (with genera *Polypterus* and *Erpetoichthys*) (Suzuki et al. 2010), 25 species of sturgeons (*Acipenser*, *'Huso'*, *Scaphirhynchus, Pseudoscaphirhynchus)* (Bemis et al. 1997), two species of paddlefishes (*Polyodon, Psephurus*) (Bemis et al. 1997), 7 species of gars

(with genera *Lepisosteus*, *Atractosteus*), and a single species of bowfin (*Amia calva*). Consequently, with the exception of Teleosts and Tetrapods, the pattern of vertebrate phylogeny close to the base of the tree shows very long phyletic branches that lack intervening divergences leading to extant descendants (Figure 2) (Broughton et al. 2013).

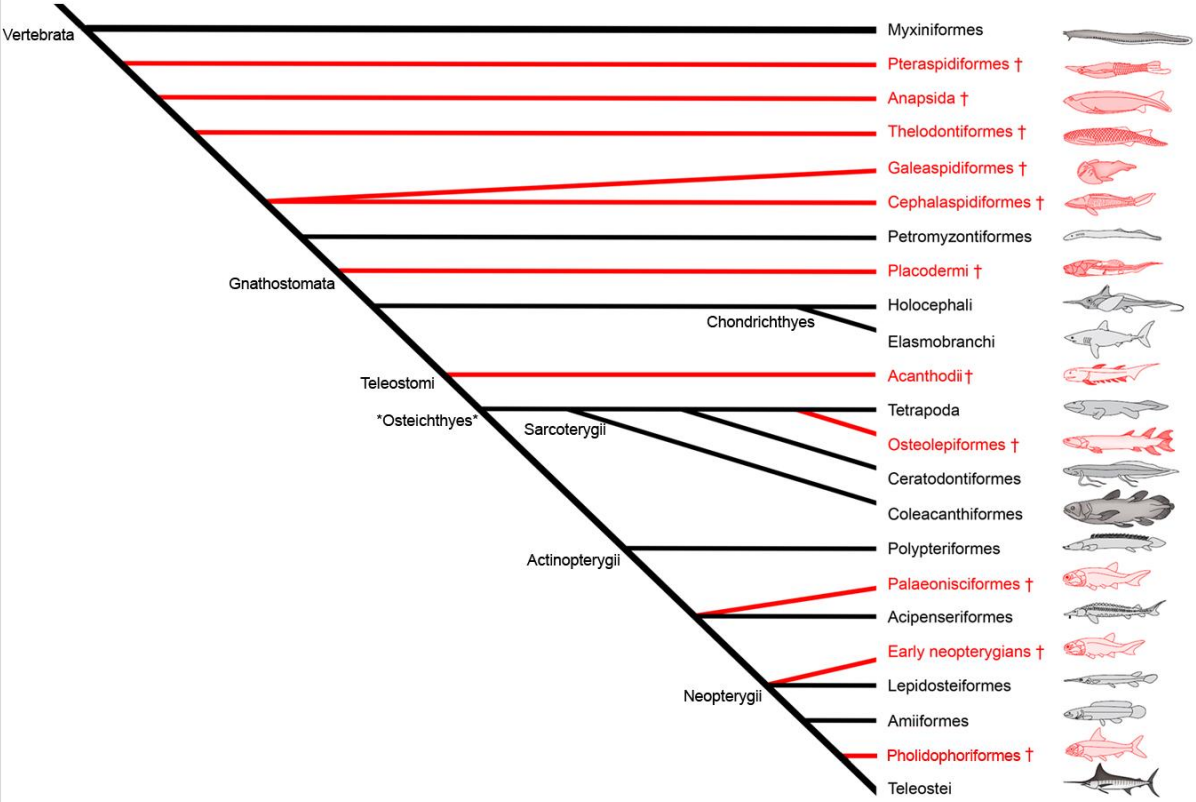


Figure 2. Hypothesized phylogenetic relationships among living (black colour) and extinct (†, red colour) vertebrate groups. Picture is adjusted after Helfman et al.(2009)

An extensive review of literature has been recently made by Sallan (2014) who stated that the molecular consensus is now coalescing around the nested Polypteriformes + Actinopteri, Chondrostei + Neopterygii, and Holostei + Teleostei topology (Figure 1). However a large amount of conflicts between molecular and morphological hypotheses, driven by choice of characters, taxa and genes still exists. Most working concepts of actinopterygian relationships are based on morphological data, and unlike other clades of vertebrates, there has been no comprehensive effort to resolve the phylogeny of

Actinopterygians and Teleosts using molecular data that sample multiple nuclear genes and include taxa that span the major lineages (Near et al. 2012). In this conflicting area, cytogenetics is becoming an important biodiversity-detection tool for estimating biodiversity-related evolutionary aspects. The synergy between chromosomal and molecular biology analysis promotes cytogenetics to a powerful tool in the integration of knowledge in genetics, genomics, taxonomy and evolution.

2. AIMS OF THE THESIS

Presented Thesis is composed of five scientific articles and one methodical book chapter published in Fish Cytogenetic Techniques. The Thesis is concerning cytogenetics and genome evolution of fishes from different actinopterygian groups: Cobitidae, Salmonidae, Pantodontidae, Lepisosteidae and Amiidae. Main aims of these studies could be summarized in following points:

- Characterize whether and how karyotype evolution in asexual fish complex of hybrid spined loaches occurs. Test the integrity of parental chromosomes in 2n and 3n hybrid genomes. Provide new molecular dating including both sexual and asexual lineages (**publication I**).
- Examine chromosomes of archaic fish *Amia calva* by using conventional and fluorescence cytogenetic methods. Compare these results with others basal lineages of actinopterygian fishes and expanding our knowledge on the phylogenetic relationships of these lineages (**publication II**).
- Use molecular cytogenetic methods to describe the genome organization of gars, followed by combining cytogenetic with bioinformatic analyses (**publication III**).

- Examine chromosome structure and genome differentiation in sympatric species of ciscoes (genus *Coregonus*) from the Lake Stechlin (Germany). Describe the impact of repetitive DNA sequences on the process of adaptive speciation (**publication IV**).
- Provide the first detailed cytogenetic analysis on chromosomes of African butterfly fish *Pantodon buchholzi* and thus to gain insight into its evolution within Osteoglossiformes, one of the most basal teleost lineages (**publication V**).
- Optimize the protocol that allows successful application of the genomic *in situ* hybridization (GISH) and comparative genomic hybridization (CGH) methods in cytogenetic analyses of actinopterygian fishes. Describe the principle and limitations of these methods (**publication VI**).

3. LITERATURE REVIEW

3.1. Characterization of fish genomes

Fish genomes could be characterised by several features, e.g. genome size, chromosomal characteristics or genome organization. In general, the available information about structure and organization of fish genome mainly relates to the structure and evolution of chromosomes (Oliveira et al. 2002, Martins and Wasko 2004). The diploid chromosome number (2n) and fundamental number (NF = nombre fondametal, number of major chromosome arms originally designed to quantify the centric translocations or fissions of the Robertsonian type in domestic mouse; Matthey 1945) is remarkably stable in Actinopterygians, and this evolutionary conservatism seems to contradict with the striking diversity of actinopterygian lineages in terms of genome size, composition, and synteny (Mank and Avise 2006). The

hypothesized ancestral teleost protokaryotype (2n = 48 - 50) (Jaillon et al. 2004; Kohn et al. 2006; Kasahara et al. 2007; but see also Nakatani et al. 2007) is conserved in approximately 50% of examined taxa (Mank and Avise 2006; Arai 2011). However, polymorphisms at the chromosomal level are frequently recorded (Mank and Avise 2006; Arai 2011), involving the presence supernumerary chromosomes, polyploidy and structural rearrangements. Chromosome number thus can vary from very low counts, e.g. 2n = 12 reported in Spark anglemouth *Sigmops bathyphilus* (Stomiformes) (Post 1974) or 2n = 16 in Chocolate gourami *Sphaerichthys osphromenoides* (Perciformes) (Calton and Denton 1974) to extremely high numbers, such as e.g. 2n = 446 in the cyprinid snowtrout *Ptychobarbus dipogon* (Cypriniformes) (Yu and Yu 1990) or 2n = 437 in Siberian sturgeon *Acipenser baeri* (Acipenseriformes) (Havelka et al. 2016).

Table 1. Summary of traits and factors involved in genome and chromosomes organisation of Actinopterygii. Data are collected from the manuscript II (Majtánová et al. *in press*); * lineages with undergone WGD: Salmoniformes, Cypriniformes, Synbranchiformes, Siluriformes (Soltis and Soltis 2012), ** Pessia et al. (2012)

Group	2n chromosome counts (basic features)	Micro-chromosomes	CG heterogeneity	Whole genome duplication (WGD) after R1 and R2	C-value/haploid DNA content (pg) www.genomesize.com	Specific features in the genome history and chromosomal evolution
Acipenseriformes (sturgeons, paddlefish)	~ 120 – 240 – 360	YES ~ 50%	only NOR	multiple in sturgeons, one in paddlefish	*Acipenser* 1.8 – 9.3 *Polyodon* 1.6 – 2.4	multiple WGD, ploidy diversity
Lepisosteiformes (gars)	56 – 58	small sized chromosomes	in both extant genera	not observed	*Atractosteus* 1.2 *Lepisosteus* 1.4	regionally high recombination rate **
Amiiformes (bowfin)	46	NO	only NOR	not observed	*Amia calva* 1.2	convergent genome evolution with teleosts?
Polypteriformes (bichirs)	36 – 38 biarmed, extremelly large	NO	only NOR	not observed	*Erpetoichthis* 4.5 *Polypterus* 3.6 – 7.2	not investigated
Teleostei	~ 50 (exceptionally up to 100 – 150)	Micro B-chromosomes	only NOR	TGD and lineage specific WGDs	0.4 – ~ 1.0 for exceptions see *	from genome compaction to lineage specific WGD

The number of chromosomes nevertheless is not related with the size of the actinopterygian genome. According to Gregory (2015; the database Genomesize), the genome size (DNA content; C-value) of haploid fish cells varies from 0.39 pg in *Tetraodon fluviatilis* to 7.25 pg reported in *Polypterus bichir*. C-values of major actinopterygian clades are given in Table 1. However, only a limited fraction (roughly 1050) of actinopterygian genomes has been examined and therefore, the genome size variation is probably underestimated (Oliveira

et al. 2002; Gregory 2005) This huge span of genomes sizes in actinopterygian fishes is several-fold greater than those in most other major vertebrate groups (Venkatesh 2003; Gregory 2005; Mank and Avise 2006) and possibly indicates the plasticity of fish genomes.

Differences of nucleotide composition within genomic sequences from a variety of lineages revealed several decades ago by thermal melting and gradient centrifugation experiments (Inman 1966; Filipski et al. 1973). Since the early studies, a generally accepted concept has arisen about the genome compositional heterogeneity in homeotherms (birds, mammals) and a substantially decreased heterogeneity to homogeneity in poikilotherms (fishes, amphibians) with some transitional states in several reptile lineages (Bernardi 2005). Fish genomes, in general, show a lower AT/GC heterogeneity, while the GC-rich regions are less abundant than in warm-blooded vertebrates (Bucciarelli et al. 2002). This phenomenon was explained by the "thermodynamic stability hypothesis" stating that the compositional heterogeneity was an adaptation to homeothermy (reviewed by Costantini et al. 2009). One of the proposed mechanisms probably responsible for AT/GC heterogeneity is GC-bias gene conversion (gBGC). The gBGC is recombination-associated segregation distortion, favoring G and C over A and T bases (Marais 2003). Different banding techniques enable cytogenetic visualization of AT/CG heterogenity on chromosomes (e.g. CMA_3 staining, G-banding). Based on these banding techniques and analyses of GC-profiles of the linkage groups originating from the publicly available *Lepisosteus oculatus* genome, Symonová et al. (*in press*) (**publication III**) have demonstrated the AT/GC compartmentalization in gar genomes. So far, gars represented the only ray-finned lineage with the genome compositional heterogeneity supported by cytogenetic as well as bioinformatic data.

Access to entire genome sequences revolutionize our understanding of how genetic information is stored and organized in DNA molecules, how it has evolved over time and provides important details of the gene catalogue within a species. The first full genome

sequence information of a teleost fish - pufferfish *Takifugu rubripes* was published in 2002 (Aparicio et al. 2002). Since then the complete genome of roughly 60 species of Actinopterygians has been published (Sayers et al. 2011; Flicek et al. 2014). Sequence information from many other teleost species will be available in the near future as a result of modern sequencing technologies (Bernardi et al. 2012). Besides the sequencing of entire genomes, scanning approaches that give a comprehensive picture of the main characteristics of a genome, such as restriction site-associated DNA (RAD) sequencing and transcriptome sequencing have been applied to fishes (Sun et al. 2016).

It is important to note that even high quality whole genome sequencing do not offer a complete picture of the genome, due to technical restrictions on accurately assembling large genomic scaffolds, especially from genomic regions rich on repetitive elements. However, combining DNA sequence data with chromosomal mapping and visualization of constitutive heterochromatin by means of cytogenetic techniques can still provide a more comprehensive picture of the genome, which is not yet clearly defined, even in completely sequenced genomes (Cioffi and Bertollo 2012; Dion-Côté et al. 2016).

3.2. Major events shaping fish genome evolution

Fish genomes have undergone numerous changes throughout their evolutionary history. More than 45 years ago, Susumo Ohno (1970) proposed that duplications of genes and even entire genomes could be one of the major mechanisms responsible for increasing complexity during evolution (Meyer and Van de Peer 2005). Localized (or tandem) duplications resulting from unequal crossing over and whole genome duplications are believed to be the two dominant mechanisms contributing to vertebrate genome evolution (Lu et al. 2012). Several rounds of whole-genome duplication (WGD) have occurred during the fish evolution, including the two

most basal genome duplications that preceded the origin of vertebrates (referred to as 1R) and jawed vertebrates (2R) (Ohno 1970; Putnam et al. 2008; for review see Kasahara 2007). These ancient duplication events have provided an evolutionary substrate for diversification of gene functions, origin of evolutionary novelties, reduced probability of extinction and for speciation in Actinopterygians. A third round of whole genome duplication, termed ′teleost-specific genome duplication′ (TSGD or 3R), occurred shortly before the origin of Teleosts (Amores et al. 1998; Taylor et al. 2003; Vandepoele et al. 2004; Inoue et al. 2005). Subsequently, some fish lineages have undergone independently additional rounds of WGD later in the evolution of the Teleosts that might have led to their further speciation. Additional WGD occurred e.g. in Salmonidae (Phillips and Ráb 2001; Crête-Lafrenière et al. 2012; Macqueen and Johnston 2014), Cyprinidae (Xu et al. 2014; Yang et al. 2015), Botiidae (Šlechtová et al. 2007), Catostomidae (Uyeno and Smith 1972), Cobitidae (Saitoh et al. 2010), and Callichthyidae (Taylor and Alexandrou 2012). Moreover, evidence of multiple genome duplications and subsequent reduction in functional ploidy has been reported also in a non-teleost actinopterygian lineage: independent WGD events in sturgeons and paddlefishes (Ludwig et al. 2001; Crow et al. 2012). Empirical support for these WGDs came from studies of the evolution of Hox gene clusters in deuterostomes, where a general pattern from a single protostome or early deuterostome Hox cluster to four Hox clusters in Tetrapods was observed (Hoegg and Meyer 2005; Kuraku and Meyer 2009). In Teleosts, seven to eight Hox clusters have been observed (Amores et al. 1998; Meyer and Van de Peer 2005). Evidence that duplication events have contributed significantly to gene families expansion and to genome evolution was later confirmed by analyses of conserved gene order and/or synteny within complete vertebrate genome sequences (e.g. Amores et al. 1998; Aparicio et al. 2002; Vandepoele et al. 2004; Braasch et al. 2016).

3.3. Fish cytogenetics – application and future prospects

Fish cytogenetics is a very specific field of study. Since chromosomes of the highest quality are a prerequisite for the success of further cytogenetic techniques, fish chromosomes and karyotypes are often difficult to obtain in comparison to other vertebrates. The study of each fish group requires the optimization and development of specific protocols adjusted to field and/or laboratory conditions. Moreover, actinopterygian species often have karyotypes consisting of numerous small-sized chromosomes, harden their examination and interpretation. Although several cytogenetic methods have been developed and commonly used to explore the fish karyotypes, due to limitations of space, the methods applied in the publications associated to this Thesis will be further presented.

Traditionally, cytogenetic studies often examine the topology of repetitive sequences on fish karyotypes, including multigene families, such as ribosomal DNAs (rRNA genes) and histone genes, satellites, micro- and minisatellites, and transposable elements (TEs), in order to expand our knowledge on karyotype differentiation and evolution (Charlesworth et al. 1994). C-banding, a technique specific for detecting the constitutive heterochromatin, was the first method which was used to explore the karyotype differentiation in fishes. C-positive regions are regarded as sites of highly and moderately repetitive DNA (Sumner 2003). The size and position of the C-bands are very valuable chromosome markers in karyological studies and are particularly useful in karyotyping. C-bands can be highly variable and significant polymorphism can be detected among individuals and/or populations of the same species (Salvadori et al. 2015).

Another conventional cytogenetic technique widely used on fish cytogenetics is the detection of nucleolar organizing regions (NORs) with Ag-NOR staining, as described by Howell and Black (1980; for review see Pisano et al. 2007). This staining is based on the silver impregnation of the remnants of the rRNA-protein complex synthesized by active major

ribosomal genes during interphase or prior to mitosis (Howell 1982). In vertebrates, ribosomal genes (rDNA) are organized as two distinct multigene families (major and minor ribosomal genes) comprising a large number of copies of a single transcription unit, clustered in long direct tandem arrays. The major ribosomal genes (45S rDNA), coding for the 18S, 5.8S and 28S rRNAs are localized in NORs. The other family (5S rDNA) codes for the 5S rRNA and is usually localized on different chromosome pairs from those bearing major rRNA genes. The rRNA genes can be found clustered in one or several regions of the genome (Martins and Wasko 2004). In the past decades distribution and number of rDNA clusters have been described in hundreds of fish species not only by Ag-NOR staining but mainly by fluorescence *in situ* hybridization (FISH) with rDNA probes (reviewed in Martins & Wasko 2004, Cabral-de-Mello & Martins 2010, Gornung 2013). Despite that sequence of rDNA loci is evolutionarily conservative across vertebrates; their topology on chromosomes can be variable and highly informative for tracking the history of the karyotype evolution across related taxa (Hillis & Dixon 1991). Therefore, rDNA loci are often used as molecular marker for comparative cytogenetic studies in fishes (e.g. Martins et al. 2000; Fontana et al. 2003; Ocalewicz et al. 2008; Pereira et al. 2013). Furthermore, the variability in the rDNA sequences and its chromosomal sites provides evidence about genome evolution at chromosomal and molecular levels (Fontana et al. 2003). Evolutionarily conserved GC-rich DNA blocks, characteristic of rDNA sites of Teleosts (Mayr et al. 1985; Amemiya and Gold 1986; Ráb et al. 1996) and reference therein), can be cytologicaly identified by fluorescent staining with fluorochromes such as Chromomycin (CMA_3), mythramycin and olivomycin (Schmid and Guttenbach 1988) that binds preferentially to GC-rich chromatin segments and counterstained with AT-specific DAPI fluorescence (Rábová et al. 2015). Correlations between Ag-NOR, CMA_3 positive sites and FISH with rDNA probes have been reported for a

number fish species (Ráb et al. 1999; Deiana et al. 2000; Galetti et al. 2005; Morescalchi et al. 2007).

In contrast to conserved sequences of rDNA genes, TEs are considered to be drivers of genome evolution and they have been involved in chromosome rearrangements of a wide variety of organisms. The TEs interact with other genomic locus, due to their ability to move and replicate, thereby generating plasticity (Wicker et al. 2007). They fall into two main classes according to their structural organization and transposition mechanism within the genome – DNA transposons and retrotransposons (for unified classification system of TEs see Wicker et al. 2007). All types of eukaryotic TEs have been described in teleost genomes (Aparicio et al. 2002) but a variability in TE content is observed among diverse lineages: the genome coverage of TEs is 10x higher in zebrafish (55% of the genome) than in the pufferfish *Tetraodon* (6%) (Chalopin et al. 2015). Regarding chromosome organization, TEs can be organized in clusters or dispersed throughout the genome. Almost all categories of TEs generally co-localize with other types of repeats (duplicated pseudogenes, minisatellites) in specific heterochromatic regions of the genome (Ferreira et al. 2011). So far, the genomic distribution of fish TEs has been extensively analysed, particularly by sequence analysis and FISH experiments. De Boer et al. (2007) have studied transposon activity during genome reorganization process in Atlantic salmon *Salmo salar*. Analysis of sequence similarities between copies within the families of these bursts exhibits several waves of transposition activities, correlated to salmonid species divergence. Thus, a large scale transposon activity and translocation has been identified as a key factor in the genome reorganization process of salmonids. Dion-Côté et al. (2014) have suggested reactivation of TEs as one of factors that caused genomic instability through aneuploidy in dwarf and normal lake whitefish leading to reproductive isolation between these forms. Symonová et al. (2013b) (**publication IV**) have detected extensive genome re-arrangements in 45S rDNA associated with TEs (re)activation

in sympatric European cisco *Coregonus albula* and Stechlin cisco *C. fontanae* leading to a rapid genome divergence and possible intralacustrine speciation. Recent studies thus suggests that TEs have a significant influence on genome architecture as part of chromosome rearrangements, contribute to pre- and post-mating reproductive isolation, and therefore might be involved in the formation of new species (Werren 2011; Arkhipova et al. 2012; Belyayev 2014; Chalopin et al. 2015).

Using whole genomes as hybridization probes enables the genomic *in situ* hybridization (GISH) and comparative genomic hybridization (CGH). Both GISH and CGH are derived FISH methodologies. Although initially, CGH had been developed as a diagnostic tool for cancer cytogenetics and GISH had been mostly used in plant cytogenetics, both methods are nowadays regarded as valuable tools in fish cytogenetics. The efficiency of both methods is largely affected by the presence of genome-specific repetitive sequences (Kato et al. 2005). GISH uses total genomic DNA from one species as the labelled probe and unlabelled genomic DNA from another species at much higher concentrations as blocking DNA, substantially increasing the hybridization specificity. This technique can distinguish chromosomes from different genomes and is very useful for studying the genome affinity between polyploid species and their progenitors (Zhu and Gui 2007; Valente et al. 2009; Rampin et al. 2012; Knytl et al. 2013; Symonová et al. 2013a). In CGH, test and reference genomic DNAs are independently labelled with distinct fluorochromes and then co-hybridized onto metaphase chromosomes (Symonová et al. 2015). Following hybridization, the chromosomes are scanned to compare the fluorescence intensities along the lengths of the chromosomes in order to detect intensity ratio differences which subsequently pinpoint genomic imbalances. In fish cytogenetics, CGH was applied mainly to localize sex-specific chromosome regions and track early stages of sex chromosome differentiation (Traut and

Winking 2001), genomic comparison within sympatric species pairs of *Coregonus* (Symonová et al. 2013b) and chromosome elimination in hybrids (Fujiwara et al. 1998; Sakai et al. 2007).

4. MATERIAL AND METHODS

4.1. Material

Fishes from different localities and countries (Bulgaria, Czech Republic, Germany, Hungary, Mexico, Nigeria, Poland, Romania and Slovakia), have been used for the purpose of this Thesis. Chromosomal and tissue material was collected over the course of several years as part of a collaborative effort between several laboratories (Charles University in Prague, Slovak Academy of Sciences, University of Wrocław, Polsko; Leibniz-institut of freshwater biology and inland fisheries, Germany; Universidad Juárez Autónoma de Tabasco, Mexico; Adamawa State University, Nigeria). All experimental procedures were conducted with relevant approvals and national guidelines. Certification numbers for permissions and approvals are included in relevant publications. Tissue, and chromosome preparations and DNA samples are stored in collection of Laboratory of Fish Genetics, IAPG CAS, v.v.i.

4.2. Methods

Molecular techniques: Extraction of genomic DNA (gDNA) from fin, muscle or blood tissue either by Phaenol-chlorophorm method or by the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), gel electrophoresis, concentration measurement, PCR amplification, DNA purification, DNA probe labelling via labelling PCR or Nick Translation (using a Nick

Translation Mix; Roche), whole genome amplification using (GenomiPhi kit, GE Healthcare Life Sciences), Sanger sequencing in our facilities (ABI 3700 sequencer) or external service by Macrogen, DNA cloning.

Cytogenetic techniques: Preparation of chromosomal suspension from kidneys, blood and fin regenerates, Giemsa staining, C-banding, G-banding, CMA_3 staining, Ag-NOR staining, FISH, GISH, CGH.

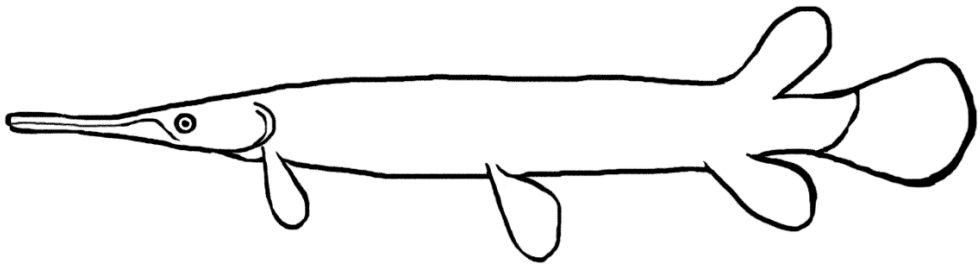
Microscopy and image analyses: Examination of chromosomal preparations by light and fluorescence microscopy, analyses of chromosomes in the IKAROS and ISIS imaging programs (Metasystems, Altlussheim, Germany), graphic adjustment of the captured digital images in Microimage and Adobe Photoshop software (version CS5).

5. RESULTS AND DISCUSSION

5.1. Lepisosteidae

Gars are a unique and archaic group of ray-finned fishes. Extant gars (Actinopterygii, Holostei, and Lepisosteidae), represented by only seven species, classified in the genera *Lepisosteus* and *Atractosteus*, are the only living representatives of one of the earliest radiations of Neopterygii. The first investigation on gar cytogenetics has performed Ojima and Yanamo (1980) who described the chromosome number and karyotype of longnose gar *Lepisosteus osseus.* Almost nothing has changed in the level of knowledge on gar cytogenetic characteristics since the study of Ráb et al. (1999) who have found a cytologically detectable association of major ribosomal DNA (28S rDNA) sites with GC-rich blocks of DNA in *L. osseus*. They concluded that such an association is likely to be evolutionary conserved

because this feature is common in all studied Neopterygii and sturgeons. In 2009, the karyotype description of the tropical gar, *A. tropicus* was published, based on conventional Giemsa staining (Arias-Rodríguez et al. 2009). Recently in 2014, the genome of *L. occulatus* has been sequenced (Flicek et al. 2014), and therefore, cytogenomic studies are expected to flourish in gars in the near future.



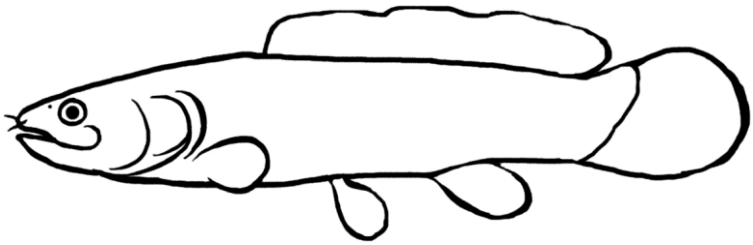
In the **publication III**, we followed up the work of Ráb et al. (1999) by analysing *L. occulatus* and another, unstudied species *Atractosteus tropisus* using molecular cytogenetic methods. We applied molecular cytogenetic methods which revealed the extraordinary similarity of gar AT/GC genome organization with the mammalian one. We further supported our findings with bioinformatic analyses which unequivocally confirmed the cytogenetic findings and quantified the purely qualitative results of molecular cytogenetics. Since our findings deny the so far generally accepted onset of the AT/GC compartmentalization only in lineages leading to birds and mammals, we discuss the importance of these findings for vertebrate genome evolution in a wider phylogenetic spectrum.

Recently published work of Braasch et al. (2016) surprisingly documented a synteny between spotted gar and chicken linkage groups with only 17 large fissions, fusions or translocations major chromosomal re-arrangements. Their work also demonstrated that the spotted gar gene expression pattern exhibits many similarities with those of tetrapod genomes. Moreover, based on the fossil evidences, gars and early tetrapods probably had faced similar selective pressures which resulted to comparable genome organisation (Sallan, *pers.com.*). An alternative view may be the similarities on genome organisation of gars and birds are not the

result of convergence evolution, but rather the inheritance of the genome structure from the common ancestor of Gnathostomata (Meyer, *pers. com.*).

5.2. Amiidae

Bowfin *Amia calva* (Actinopterygii, Holostei, Amiidae) is the only one extant survivor of once widely diversified halecomorph fishes (Grande and Bemis 1998). Despite its uniqueness, the karyotype of the bowfin (2n = 46) was reported in only in two studies based on conventionally Giemsa-stained chromosomes (Ohno et al. 1969; Suzuki and Hirata 1991). Genome organization was never studied by molecular methods. After the surprising findings in gars, I tried to explore the cytogenomic features of the bowfin, the putative the most closely related extant lineage of gars.



In the **publication II**, we explored the chromosomal characteristics of an ancient non-teleost ray-finned fish, bowfin *Amia calv*a, as revealed by means of conventional (Giemsa staining, C-banding, CMA_3 staining, Ag-NOR staining) and molecular (FISH with rDNA probes) cytogenetic methods. Our results demonstrated that bowfin possesses the typical teleostean genome organization and karyotype features, e.g. teleost-like chromosomal number and AT/GC homogenously stained chromosomes. Furthermore, the NOR regions are localized on a single pair of chromosomes and are extremely GC-rich. In addition, we also reviewed the "Holostei versus Halecostomi" problem from the cytogenetic point view based on available

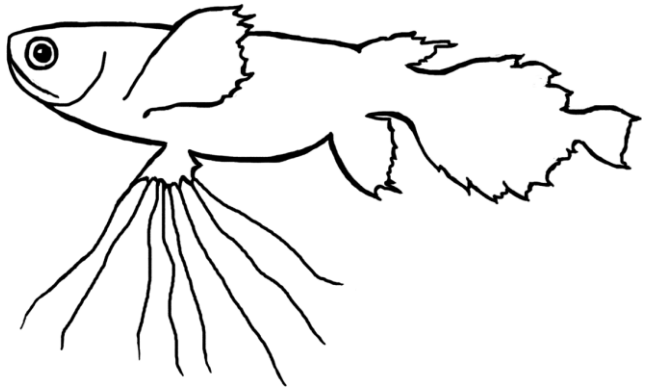
data of non-teleost Actinopterygians namely Polypteridae, Acipenseridae, Polyodontidae, Lepisosteidae and representatives of Teleosts.

According to our results, the bowfin strongly resembles the teleost pattern of genome organization, suggesting the Halecostomi topology, a phenomenon also observed for several morphological characteristics. However, recent phylogenomic analyses convincingly support the monophyletic relationship of the spotted gar and bowfin (Braasch et al. 2016). This controversy should be evaluated in a broader sense. The fossil record supporting the Holostei topology remains unsettled because the reliable placement of fossils near the base of the lineage stems is questionable (discussed in Sallan 2014). Gars, bowfins, and teleost lineages either diverged very rapidly after the end-Triassic extinction, or were indistinguishable prior to that, leaving little evidence of their transformation series. On top of that, gars were highly diversified and species-rich lineage at early Mesozoic, but that diversity has gradually lost during evolutionary time (Grande 2010). On the contrary, crown Teleosts underwent whole genome duplication during the first 100 million years of the Mesozoic, and have subsequently demonstrated an extreme speciation and diversification. Therefore, gars and bowfin might appear more closely related than Teleosts in molecular studies, which can only sample the living subset of those lineages (Sallan, *pers. com*). Thus, it is really hard to reconstruct the evolutionary events surrounding the origins of these groups, despite the apparent universal support for Holostean monophyly in phylogenies based on nuclear genes.

5.3. Pantodontidae

The African freshwater butterfly fish *Pantodon buchholzi* is the only species of the family Pantodontidae, order Osteoglossiformes. The phylogeny of the extant osteoglossiforms is relatively well-known, with morphological and molecular characters supporting the same

topology (Lavoué et al. 2010). However, the phylogenetic position of *Pantodon* is poorly supported, since molecular and morphological characters lead to contradictory topologies. Molecular characters support the hypothesis in which *Pantodon* is the sister group of the rest of Osteoglossiformes (Lavoué and Sullivan 2004; Inoue et al. 2009; Mu et al. 2012; Lavoué 2016), but morphological characters support an alternative hypothesis that *Pantodon* is closely related to Osteoglossidae and Arapaimidae (e.g. Li and Wilson 1996; Hilton 2003; Wilson and Murray 2008). Surprisingly, the karyotype of *P. buchholzi* was studied only by conventional stain (Uyeno 1973), and molecular cytogeentic approaches, which could expand our understanding on the evolution of Osteoglossiformes, are greatly lacking.



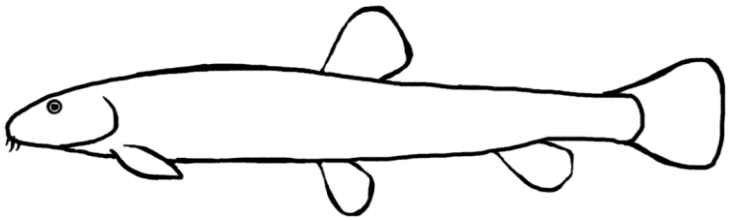
In the **publication V** we present a detailed cytogenetic analysis of *P. buchholzi* from the lower Niger population (Lavoué et al. 2010). In addition to conventional chromosomal markers, we examined the topology on karyotype of nine classes of repetitive DNA sequences by FISH, including the multigene families 5S and 18S rDNAs and U2 snRNA, di- and tri-nucleotide microsatellites and the retrotransposable element *Rex6*.

In 2010, Lavoué et al. (2010) determined two genetically well differentiated allopatric populations of *P. buchholzi* from Niger and Congo River basins respectively. They estimated their divergence time to be at least 57 MYA. It is surprising that with such intraspecific genetic differentiation between these two *Pantodon* lineages, no significant morphological differentiation has been established. For comparison, two additional species from the family

Osteoglossiformes, the South American arowana *Osteoglossum bicirrhosum* and the Asian arowana *Scleropages formosus*, demonstrate comparable divergence times to the lineages of *P. buchholzi*. However, there is apparent morphological differentiation between these two arowana species. Given the large genetic differentiation between the African populations of *Pantodon*, it is expected that the two populations should demonstrate distinct karyotypes.

5.4. Cobitidae

European spined loaches of the *Cobitis* hybrid complex represent a favourite evolutionary model to understand dynamics of animal hybridization, asexuality and polyploidy (e.g. Janko et al. 2007a; Choleva et al. 2008; Janko et al. 2012; Choleva et al. 2014). Sexual parental species have wide distribution over Europe where *C. elongatoides* (2n=50) was documented to hybridize with closely related species of *C. taenia* (2n=48), *C. tanaitica* (2n=50) and/or *C. pontica* (2n=50), giving rise to virtually all-female di-, tri- and tetraploid hybrids. Hybrid females are assumed to have strictly clonal gynogenetic reproduction, whereas males seem to be sterile (Janko et al. 2007a; Choleva et al. 2012). The karyotypes traits of the spined loaches of the genus *Cobitis* are commonly explored by cytogenetics, providing valuable information to morphological descriptions and phylogenetic reconstructions (Boroń 1992; Rábová et al. 2001; Janko et al. 2005, 2007b, Vasil'ev and Vasil'eva 2008, 2011). Advanced cytogenetic studies analysed karyotypes by banding patterns or FISH (Boroń 1999, 2003a,b; Boroń et al. 2003; Rábová et al. 2007). Based on my experience obtained during the preparation of **publication I** the GISH methodology described in **publication VI** have been further optimized.



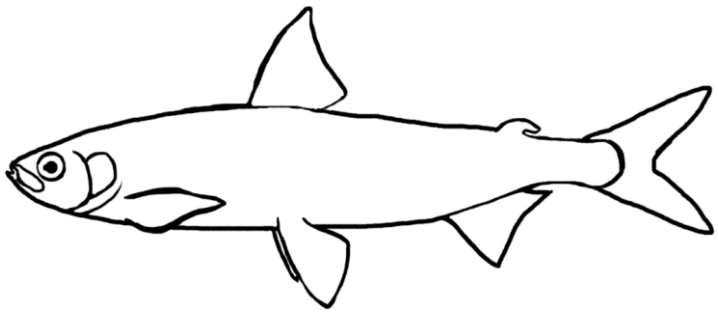
In the **publication I** we tested whether asexual reproduction and/or hybridization have notably increased the rate of chromosomal changes in comparison with sexual lineages. We compared chromosomal sets of diploid or polyploid clonal hybrids to those of their direct sexual progenitors using GISH. We tested whether the karyotypes of sexual species and hybrids notably diverged since the primary hybrid formation, whether the parental chromosome sets maintain their integrity in the hybrid clonal lineage or whether recombination between chromosomal sets occurs. Our data do not indicate that clonally transmitted karyotypes accumulate morphological structural changes at any notably higher rate in comparison with sexual species. It appears therefore, that the karyotypes of parental species remain rather static in clonal lineages for long periods, spanning over hundred thousands of generations.

Our results are contradictory to previously published studies on the rate of karyotype evolution in asexual invertebrates. In these studies, authors revealed accelerated accumulation of morphological structural changes in clonal genomes of apomictic organisms (Sunnucks et al. 1996; Normark 1999; Welch et al. 2004; Schneider and Cella 2010). Since hybrid *Cobitis* reproduce via automictic parthenogenesis, we conclude that the effect of asexuality on the rate of chromosomal evolution may depend on particular type of asexual reproduction.

5.5. Coregonidae

Coregonids represent a paleoautopolyploid group (Allendorf and Thorgaard 1984; Timusk et al. 2011; Crête-Lafrenière et al. 2012; Macqueen and Johnston 2014) with advanced but not

fully completed re-diploidization (Wolfe 2001), enabling sexual reproduction with occasional quadrivalent formations during meiosis (Timusk et al. 2011). Generally, a robust amount of descriptive cytogenetic data is available for this lineage (e.g. Phillips and Ráb 2001; Rossi and Gornung 2005; Kirtiklis and Jankun 2006; Dion-Côté et al. 2015). European Coregonid species in temperate lakes that have been established since the last glaciation (12 000 – 15 000 YA), providing suitable model systems for studying postglacial evolution, adaptive radiation and ecological speciation (Schluter and Rambaut 1996; Taylor 1999). One such model system occur in the Lake Stechlin (Germany), where the otherwise broadly distributed, autumn-spawning European cisco *C. albula* coexists with the endemic, smaller, spring-spawning Stechlin cisco *C. fontanae* (Schulz and Freyhof 2003). The ecological and evolutionary aspects of these sympatric species have been extensively studied, including morphology (Helland et al. 2009), temperature-dependent metabolic adaptations (Ohlberger et al. 2008a,b) or genetic structure (Mehner et al. 2010).



In the **publication IV** (Symonová et al. 2013b) we have described an extensive variation in the number of 45S rDNA sites between *C. albula* and *C. fontanae* genomes and a co-localization of the 45S rDNA and the transposable element *Rex1* in both species. We demonstrated cytogenomic background of a rapid ecological speciation, which remained undetectable by both sequence and karyotype analysis. We provided indirect evidence that rDNA probably utilized the spreading mechanism of TEs and subsequently affected

recombination rates in both genomes, thus, contributed to reproductive isolation and lead to a rapid genome divergence. We attribute these extensive genome re-arrangements associated with speciation events to stress-induced TEs (re)activation.

It is speculated that transposable elements can specifically target rDNA loci (Jakubczak et al. 1991; Eagle and Crease 2012). rDNA units with incorporated TEs are nonfunctional, suggesting selective pressure against these insertions. However, the rDNA-specific TEs are highly successful (Zhang et al. 2008). The total number of functional rDNA units on genome usually exceeds the minimum required for host viability, and thus insertion of TEs into rDNA units may have little impact on host fitness (Eickbush 2002; Zhang et al. 2008). The publication IV has been cited in the context of other studies in diverse fish lineages, e.g. Characiformes, Siluriformes, Perciformes, Salmoniformes (Pucci et al. 2014; Cioffi et al. 2015; de Oliveira et al. 2016; Maneechot et al. 2016).

6. CONCLUSION

Ray-finned fishes exhibit the most diversified vertebrate group, promoting them to the biggest and most exciting challenge in cytogenetic studies. Over the past decades, fish cytogenetics has matured into a multidisciplinary science that draws heavily on theoretical and technical advances associated with developments in molecular biology, flow-cytometery, bioinformatics and phylogenetics (Robinson and Yang 2012). Cytogenomics (or modern cytogenetics) is combining set of methodologies, including conventional (banding), molecular (chromosome painting, gene mapping and sequencing) and genomic tools broadening the perspectives in the study of karyotypes and chromosomes (Pereira 2013). As a result, it now provides crucial analytical approaches for countless questions in evolutionary biology

(Robinson and Yang 2012). Furthermore, its increasing reliance on sequence data to confirm and refine syntenies, identify breakpoints, neocentromeres and other chromosomal features has irrevocably cemented the association between molecular cytogenetics, karyotypic change and genomics (Robinson and Yang 2012). In summary, the development and improvement of molecular cytogenetic methods have substantially expanded the spectrum of chromosome studies and have played an important role in the precise characterization of the genomic features related to chromosome biology. The current availability of a still increasing number of completely sequenced eukaryotic genomes has opened new "avenues" for advancing cytogenetics (Cioffi et al. 2012).

7. PUBLICATIONS

7.1. Publication I

Majtánová Z., Choleva L., Symonová R., Ráb P., Kotusz L., Pekárik & Janko P. 2016. Asexual reproduction does not apparently increase the rate of chromosomal evolution: karyotype stability in diploid and triploid clonal hybrid fish (*Cobitis*, Cypriniformes, Teleostei). PLoS ONE. 11:e0146872. IF_{2015}: 3.057

Author contribution: collecting and raising the material, direct chromosomal preparation, chromosomal preparation from regenerated caudal fins, karyotype analyses, probe preparation, GISH experiments, image processing, analysing of the data, writing the paper

I declare that the contribution of Zuzana Majtánová to the publication Majtánová et al. (2016), as stated here, is true.

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RESEARCH ARTICLE

Asexual Reproduction Does Not Apparently Increase the Rate of Chromosomal Evolution: Karyotype Stability in Diploid and Triploid Clonal Hybrid Fish (Cobitis, Cypriniformes, Teleostei)

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Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

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Abstract

Interspecific hybridization, polyploidization and transitions from sexuality to asexuality considerably affect organismal genomes. Especially the last mentioned process has been assumed to play a significant role in the initiation of chromosomal rearrangements, causing increased rates of karyotype evolution. We used cytogenetic analysis and molecular dating of cladogenetic events to compare the rate of changes of chromosome morphology and karyotype in asexually and sexually reproducing counterparts in European spined loach fish (*Cobitis*). We studied metaphases of three sexually reproducing species and their diploid and polyploid hybrid clones of different age of origin. The material includes artificial F1 hybrid strains, representatives of lineage originated in Holocene epoch, and also individuals of an oldest known age to date (roughly 0.37 MYA). Thereafter we applied GISH technique as a marker to differentiate parental chromosomal sets in hybrids. Although the sexual species accumulated remarkable chromosomal rearrangements after their speciation, we observed no differences in chromosome numbers and/or morphology among karyotypes of asexual hybrids. These hybrids possess chromosome sets originating from respective parental species with no cytogenetically detectable recombinations, suggesting their integrity even in a long term. The switch to asexual reproduction thus did not provoke any significant acceleration of the rate of chromosomal evolution in *Cobitis*. Asexual animals described in other case studies reproduce ameiotically, while *Cobitis* hybrids described here produce eggs likely through modified meiosis. Therefore, our findings indicate that the effect of asexuality on the rate of chromosomal change may be context-dependent rather than universal and related to particular type of asexual reproduction.

ncn.gov.pl; project number DEC-2011/03/B/NZ8/02095) to JK. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Introduction

Most species differ by their karyotype, which are routinely defined by the variation in number, size and morphology of chromosomes. Differences originating during karyotype evolution play an important role in speciation [1]. Simultaneously, the rate at which chromosome rearrangements accumulate varies greatly from one species to another. For example, changes in chromosome number have occurred roughly 20 times faster in mammals than in frogs [2] (although even there one can find lineages with exceptionally fast rate of chromosomal evolution, e.g. [3]). With more than 27.000 taxonomically known species [4], teleost fishes show rather conserved karyotypes with a uniform haploid chromosome number n = 24–25 or close to it [5,6], suggesting a slow rate of karyotype change, mostly by intrachromosomal rearrangements [7]. However, a number of exceptions exist, especially in lineages of polyploid origin (see [8]).

The reasons why karyotypes remain conservative in some groups while they readily mutate and rearrange in others continue to be the focus of ongoing and lively debate (e.g. [9]). At least some factors have been proposed to modify rates of karyotype evolution. One of them, polyploidization, has been shown to instantaneously increase the chromosome numbers [10] but also to directly increase rates of chromosome breakages and aneuploidy incidence [11]. Asexuality (defined in a broader sense by the lack of efficient recombination and segregation; e.g. [12]) is another factor that is supposed to significantly influence the rate of chromosomal changes [13]. Relaxed constraints on the pairing of homologous chromosomes during gamete formation in clones may allow faster accumulation of chromosomal mutations than in sexual species (e.g. [14]). An absence of effective recombination ultimately leads to an accumulation of independent mutations on any locus including whole chromosomes (Meselson effect; [15]). Chromosomal complements of asexual organisms, where most of them are also polyploids, may therefore acquire strongly aneuploid or structurally changed characteristics relatively easily. The hypothesis of asexuality-linked increase in the rate of karyotype evolution is supported by studies of apomictic arthropods [13,14,16–18] and root knot nematodes [19] which suggest that once sex is lost, chromosomal structure can become irregular extremely quickly; e.g. chromosomal fissions in *Sitobion miscanthi* aphids occurred over several dozens of generations during seven years in laboratory culture [13].

However, number of uncertainties remains. Especially, it is unclear whether the aforementioned indications of increased rates of karyotype changes could be generally attributed to asexual reproduction *per se* because other features of those organisms, especially the holokinetic nature of their chromosomes, may also influence resulting patterns [20]. Moreover, since abovementioned studies were focused on apomictic parthenogens (i.e. with eggs formed by mitosis) it is unknown whether other types of asexual reproduction have the same effect on the rate of morphological change of karyotype. Actually, asexuality can be achieved by many cytological mechanisms, which have profoundly different predictions about the heterozygosity of resulting individuals [21,22]. This may affect the rate of chromosomal evolution differently [14]. Certain types of automixis may be exactly equivalent to apomixis [23], e.g. premeiotic endoduplication. In that case, the genetic material is duplicated before entering meiosis while segregation and recombination take place on bivalents that form between sister chromatids, the genetically identical copies derived from endoduplication event. The progeny is therefore genetically identical to its mother [24]. Other types of automixis may lead to intragenomic recombination or exclusions of large genomic parts (e.g. [25]). Therefore, it is important to test the general applicability of the hypothesis of asexuality-driven increase of karyotype mutation rate.

Spined loaches from the *Cobitis taenia* hybrid complex (*sensu* Janko *et al.* [26]) are fish model with included automictic reproduction, allowing us to study karyotype changes in

vertebrate automicts as an alternative to published cases from invertebrate apomicts. Primary hybridization between *C. elongatoides* and either *C. taenia* or *C. tanaitica* (two sister species distantly related to *C. elongatoides*; [27]) leads to diploid hybrid clonal lineages reproducing via gynogenesis. In this reproductive mode, the sperm-dependent parthenogenetic females produce unreduced eggs that are only activated but not truly fertilized by sperm from males of one of the parental species. Therefore, males serve as hosts for these so called clonal sexual parasites [28–30]. Clonal spined loaches have retained meiosis [31] but unreduced egg production is ensured by premeiotic genome doubling [32], a similar mechanism to the clonal pond loach *Misgurnus anguillicaudatus* [33]. Interestingly, having originated as diploid hybrids, these lineages expanded over large parts of the European continent (for distribution of particular *Cobitis* hybrid population types in Europe see [34,35]). They also largely tend to increase the ploidy level by accidental incorporation of sperm nucleus into unreduced clonal eggs at fertilization via genome addition, resulting in polyploid (mostly 3n) clonal lineages [26,28,29].

Considering that interspecific hybridization with subsequent clonal formation has been proceeding continuously at least since the last interglacial [26], contemporary clonal assemblages represent a mixture of very recent as well as relatively old clonal hybrid lineages. The oldest asexual lineage (referred to as the 'Hybrid clade I') apparently predates the last glacial maximum and its age was estimated to be roughly 0.3 MYA [26].

In the present study we compare rates of morphological evolution of karyotypes of three sexual species (*C. elongatoides*, *C. taenia* and *C. tanaitica*) and their wild-caught diploid and triploid hybrid biotypes with asexual reproduction. Hybrid biotypes differ by age represented by three classes: F1 laboratory-produced clonal hybrids; individuals derived from recent (Holocene) hybridizations; individuals derived from ancient (interglacial) hybridizations. Using conventional (Giemsa staining) and molecular (genomic *in situ* hybridization; GISH) cytogenetic methods we test following tasks: i) whether visible changes in chromosome number and/or morphology occurs in karyotypes of hybrid biotypes; ii) whether the original parental chromosomal sets can be unambiguously identified in hybrid karyotypes of clones of different age and iii) whether large-scale recombinations and/or rearrangements can be detected. Karyological observations were put into phylogenetic and time-based context with a newly dated phylogeny of parental species as well as hybrids by using available calibration point. This point was studied both on a relative and absolute time scales by visualizing the changes in karyotypes of parental species onto dated phylogeny. The dating allowed testing of the hypothesis whether asexual reproduction and/or hybridity have notably increased the rate of chromosomal changes in comparison with karyologically diversified sexual lineages. We particularly focused on whether karyotypes of older clonal lineages differ from contemporary parental species more than those recently derived.

Materials and Methods

Background knowledge about karyotypes of parental species

Sexual parental species included in this study are *C. elongatoides*, *C. taenia* and *C. tanaitica* and hereafter we use the acronyms E, T and N for their haploid genomes, respectively. These are used to designate species and also hybrids with various genomic combinations (e.g. triploid hybrid biotype containing two haploid genomes of *C. elongatoides* and one haploid genome of *C. tanaitica* is encoded EEN). To evaluate rates of karyotype evolution in sexual species and hybrid clones, we take an advantage of previous karyological studies of European spined loaches. While no intraspecific karyotype differences has been discovered so far, it has been shown that karyotypes of parental species have diverged from each other by various chromosomal rearrangements involving one probable fusion and numerous pericentric inversions

[34,36]. In contrast with the substantial karyotype differentiation among parental species, several studies demonstrated consistency of karyotypes within species across their distribution ranges [34,36–38]. Multilocus phylogenetic study [27] suggested the sister relationship of sexual *C. taenia* and *C. tanaitica* species with *C. elongatoides* being their distant relative. In concordance with this hypothesis, *C. elongatoides* (2n = 50) possesses the most differentiated karyotype dominated by meta- and submetacentric chromosomes, while karyotypes of *C. tanaitica* and *C. taenia* are more similar to each other and dominated by subtelo- and acrocentric chromosomes. They differ, however, in number of chromosomes and their centromere positions with *C. taenia* (2n = 48) having more acrocentrics than *C. tanaitica* (2n = 50). A detailed karyotype characteristic of sexual species is given in Table 1.

Animals used in this study

Altogether 57 *Cobitis* individuals of nine biotypes were used in the study (Table 2). The examined animals included di- and triploid clonal forms of various genomic constitutions, i.e. EN, ET, EEN, ENN, EET and ETT. Since the genomic composition of species and hybrid forms may not be identified by external morphology because of their cryptic nature, the genome of each individual was determined by species-specific allozyme markers (*Aat*, *Mdh*, *Pgm*, *Gpi-A* and *Ldh*) according to Janko *et al.* [28]. Interspecific hybrids mostly came from native populations in Europe from Bulgaria (2 localities, 4 individuals), Germany (3 localities, 4 individuals), Poland (2 localities, 10 individuals), Romania (4 localities, 18 individuals), Slovakia (1 locality, 8 individuals) and Czech Republic (1 locality, 1 individual). In addition, one laboratory-born first-generation (F1) ET and three backcrossed (B1) ETT hybrids were analysed as control specimens to test the karyotype stability and to standardize the GISH approach for correct classification of chromosomes between hybrid and parental complements. The maternal ancestry of a subset of studied hybrid individuals and their divergence from parental species were determined using mitochondrial cytochrome *b* gene (cyt *b*) according to Janko *et al.* [30].

Ethics statement

All specimens were collected in accordance with the national legislation of the countries concerned. The experimental procedures involving fish were approved by the Institutional Animal Care and Use Committee of the Institute of Animal Physiology and Genetics, CAS, v.v.i, according with directives from State Veterinary Administration of the Czech Republic, permit number 217/2010, and by the permit number CZ 00221 issued by Ministry of Agriculture of the Czech Republic. LC has Certificate of competency according to §17 of the Czech Republic Act No. 246/1992 coll. on the Protection of Animals against Cruelty (Registration number: CZU 955/06), provided by Central Commission for Animal Welfare, which authorizes animal experiments in the Czech Republic.

Chromosome preparation

Metaphase chromosomes were prepared according to Ráb and Roth [39] with slight modifications. Briefly, fish were injected with 0.1% colchicine solution (1 ml/100 g of body weight) 45 min before being sacrificed using an overdose of 2-phenoxyethanol anaesthetics agent (Sigma). The kidneys were removed, dissected in 0.075 M KCl at room temperature and the cell suspension free of tissue fragments was hypotonized for 8 min in 0.075 M KCl, fixed in fresh prepared fixative (methanol: acetic acid 3:1, v/v), washed twice in fixative, and finally spread onto slides. We also used a preparation of chromosomes from regenerated caudal fins according to Völker and Ráb [40].

Table 1. Karyotype characteristics of *Cobitis* genomes with various ploidy levels and genomic compositions of biotypes involved in this study.

ploidy level	(n)			(2n)					(3n)			
genome composition	E	T	N	EE	TT	NN	ET	EN	EET	ETT	EEN	ENN
metacentric chromosomes	11	5	5	22	10	10	16	16	27	21	27	21
submetacentric chromosomes	13	9	13	26	18	26	22	26	35	31	39	39
subtelocentric chromosomes	1	1	3	2	2	6	2	4	3	3	5	7
acrocentric chromosomes	-	9	4	-	18	8	9	4	9	18	4	8
total number of chromosomes	25	24	25	50	48	50	49	50	74	73	75	75

Abbreviations: Capital letters represent sets of haploid genomes: E, *Cobitis elongatoides*; T, *C. taenia*; N, *C. tanaitica*.

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Table 2. Individuals used in this study.

Biotype	Country	Locality	Lat	Long	AOC	NOI	NOMKA	NOGE	NOAGM
EE	Hungary	Szodrakosz Cr.	47°43'58.8"N	19°07'58.8"E	-	1	5	-	-
	Poland	Budkowiczanka R.	50°50'50.1"N	18°11'07.1"E	-	1*	-	-	-
TT	Germany	Haaren Cr.	53°04'58.8"N	7°49'58.8"E	-	2* + 1	4	-	-
NN	Romania	Danube R.	44°04'47.9"N	26°43'51.2"E	-	2*	-	-	-
	Romania	Sinoe at Histria	44°37'58.8"N	28°52'58.8"E	-	1	4	-	-
ET	Czech Rep.	Laboratory F1	50°24'37.6"N	14°27'16.9"E	F1 generation	1	9	1	4
	Poland	Dolna Barycz R.	51°36'59.1"N	16°30'49.1"E	Holocene	4	27	2	4
total						5	36	3	8
EN	Bulgaria	Jantra R.	43°09'58.0"N	25°55'53.8"E	hybrid clade I	2	22	1	1
	Romania	Danube R.	44°04'47.9"N	26°43'51.2"E	hybrid clade I	3	15	2	9
total						5	37	3	10
EET	Germany	Neisse R.	51°51'28.8"N	14°36'34.9"E	Holocene	1	12	1	8
	Poland	Polska Woda R.	51°31'17.0"N	17°30'07.0"E	Holocene	3	16	-	-
	Poland	Dolna Barycz R.	51°36'59.1"N	16°30'49.1"E	Holocene	2	5	1	2
	Czech Rep.	Pšovka Cr.	50°22'11.8"N	14°33'06.8"E	Holocene	1	2	-	-
total						7	35	2	10
ETT	Germany	Issel R.	51°51'00.0"N	06°15'00.0"E	Holocene	2	13	2	3
	Germany	Ilmenau R.	53°22'34.0"N	10°14'36.5"E	Holocene	1	2	-	-
	Czech Rep.	Laboratory B1	50°24'37.6"N	14°27'16.9"E	B1 generation	3	17	2	10
total						6	32	4	13
EEN	Bulgaria	Vit R.	43°15'47.0"N	24°19'30.1"E	hybrid clade I	2	56	2	6
	Poland	Polska Woda R.	51°31'17.0"N	17°30'07.0"E	hybrid clade I	1	7	1	1
	Romania	Barlat R.	46°40'23.8"N	27°40'07.5"E	Holocene	3	6	-	-
	Romania	Comana R.	44°10'07.1"N	26°08'54.9"E	hybrid clade I	1	2	-	-
	Romania	Adjud R.	46°04'21.0"N	27°12'32.2"E	Holocene	1	2	-	-
	Slovakia	Cierna voda R.	48°36'27.0"N	21°59'34.1"E	hybrid clade I	8	51	5	17
total						16	124	8	24
ENN	Romania	Danube R.	44°04'47.9"N	26°43'51.2"E	Holocene	10	35	3	12
total						10	35	3	12

Abbreviations: Capital letters represent sets of haploid genomes: E, *Cobitis elongatoides*; T, *C. taenia*; N, *C. tanaitica*. Shortcuts in the column captions: AOC, age of clones; NOI, number of individuals; NOMKA, number of metaphases karyologically analysed; NOGE, number of GISH experiments; NOAGM, number of analysed GISH metaphases.

In order to prepare probes for GISH experiments only gDNA was used from individuals marked with *.

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Karyotype analysis

Metaphases of each individual were inspected by Giemsa or DAPI staining in order to confirm number and morphology of their chromosomes. In total 299 metaphases of 49 hybrids were inspected (Table 2). Metaphases were classified using the nomenclature proposed by Levan *et al.* [41] and classified into four categories: metacentrics, submetacentrics, subtelocentrics and acrocentrics. Subset of metaphases from 23 hybrid individuals was arranged in a decreasing size order to demonstrate consistency of karyotypes (Fig 1A–1F and S1A–S1R Fig, S1 and S2 Tables).

Probe preparation, GISH

A subset of 23 hybrid individuals was used for further GISH analyses to determine whether the original parental chromosomal sets are distinguishable in hybrid karyotypes. At least two GISH experiments with different individuals were successfully performed *per* biotype (S1 and S3 Tables). Probes used in GISH experiments were prepared from whole genomic DNA (gDNA) of pure parental species *C. elongatoides*, *C. taenia* and *C. tanaitica*. gDNA was extracted from muscles or fins using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. gDNA samples were labelled with biotin-16-dUTP (Roche, Mannheim, Germany) and digoxigenin-11-dUTP (Roche) using a Nick Translation Mix (Roche) following the protocol supplied by the manufacturer. The best results were obtained after 1.5–5 h (time strongly depends on the starting gDNA quality and fragment length) of nick translation until labelled DNA fragments were approximately 200–500 bp long. Species-specific hybridization probes combined gDNA of sexual species (*C. elongatoides* and *C. taenia*, or *C. elongatoides* and *C. tanaitica*) to perform GISH experiments on chromosomes of hybrids (Table 2). Salmon sperm was used as blocking reagent for repetitive DNA. The hybridization and detection procedure were carried out under conditions described by Symonová *et al.* [42]. The biotin-dUTP labelled probes were detected using streptavidin-FITC (Invitrogen, San Diego, Calif., USA). The digoxigenin-dUTP labelled probes were detected using anti-digoxigenin-rhodamin (Roche). The chromosomes were counterstained with Vectashield/DAPI (1.5 mg/ml) (Vector, Burlingame, Calif., USA).

Image processing

Chromosomal preparations were examined by an Olympus Provis AX 70 epifluorescence microscope. Images of metaphase chromosomes were recorded with a cooled Olympus DP30BW CCD camera. The IKAROS and ISIS imaging programs (Metasystems, Altlussheim, Germany) were used to analyse grey-scale images. The captured digital images from GISH experiments were pseudocoloured (blue for DAPI, red for anti-digoxigenin-rhodamin, green for streptavidin-FITC) and superimposed using Microimage and Adobe Photoshop software, version CS5, respectively.

Estimates of clonal ages and speciation times in mutational-time units using the coalescent methods

We have applied molecular clocks in order to evaluate the absolute time scale over which observed karyotype changes have accumulated among parental sexual species and their hybrids. For estimates of ages of clonal lineages we used cytoplasmic markers (mtDNA) because it is haploid and traces the clonal evolution to its original formation event. We note that estimation clonal ages from nuclear markers is much more complicated since nucleus of clones is of hybrid nature and subject of polyploidization through subsequent genome

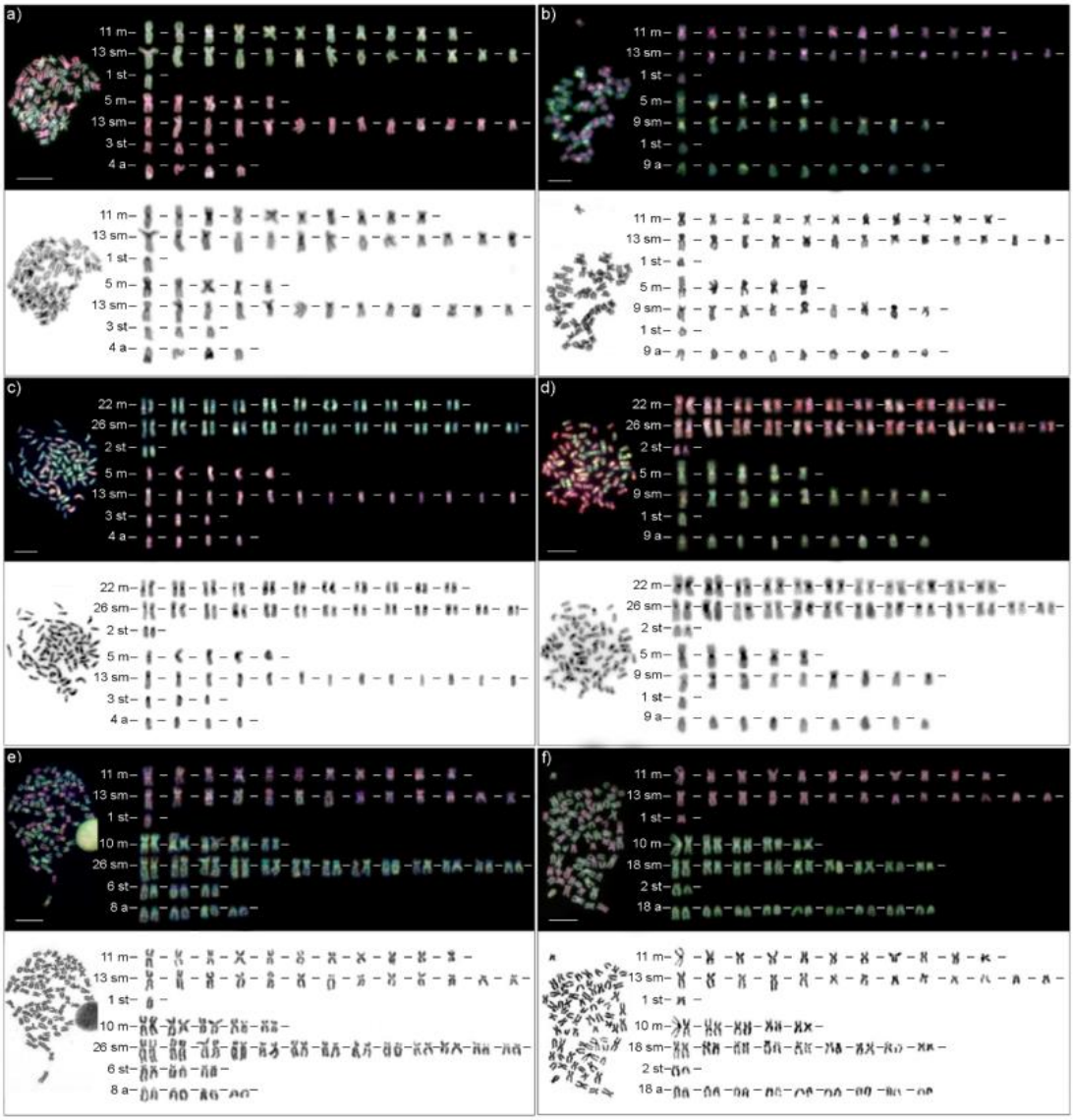


Fig 1. Representative karyotypes of hybrid biotypes after GISH and/or DAPI/Giemsa staining. (A) EN hybrid metaphase with hybridization pattern of *Cobitis elongatoides* gDNA in green, *C. tanaitica* gDNA in red. (B) ET hybrid with *C. elongatoides* in red, *C. taenia* in green. (C) EEN hybrid with *C. elongatoides* in green, *C. tanaitica* in red. (D) EET hybrid with *C. elongatoides* in red, *C. taenia* in green. (E) ENN hybrid with *C. elongatoides* in red, *C. tanaitica* in green. (F) ETT hybrid with *C. elongatoides* in red, *C. taenia* in green. Capital letters represent haploid genome sets: E, *C. elongatoides*; N, *C. tanaitica*; T, *C. taenia*. Chromosomes were arranged in a decreasing size order and classified in four morphological groups: metacentric (m), submetacentric (sm), subtelocentric (st) and acrocentric (a). Probes labelled with biotin-16-dUTP were detected with FITC-streptavidin (green signals on chromosomes); probes labelled with digoxigenin-11-dUTP were detected with anti-digoxigenin-rhodamin (red signals on chromosomes). To visualize the morphology of chromosomes DAPI (A, B, C, D) or Giemsa (E, F) stained karyotype was used. Captured DAPI stained karyotypes were inverted. Bars equal 5 μm. Detail information about individuals used is provided in S1 Table.

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incorporations ('leaky gynogenesis' *sensu* Janko *et al.* [28]). On the other hand, for the estimation of parental species speciation time, nuclear markers (nDNA) had to be used since Choleva *et al.* [27] demonstrated massive mtDNA introgression from *C. elongatoides* to *C. tanaitica* suggesting its mtDNA diversity reflects secondary hybrid introgression rather than its speciation from sister species *C. taenia*.

Given the aforementioned necessity to use distinct sets of markers for divergence time estimates of clones and sexual species, respectively we performed two-step analysis to obtain, comparable time estimates from both types of markers. First (1), we applied coalescent method to independently estimate the clonal ages and speciation times on the relative time scale (expressed in mutational-time units that are scaled by locus-specific mutation rates). In a second step (2), we translated such estimates into absolute-time units by consistently calibrating both markers (mtDNA and nDNA) with the same outgroup species.

1) Coalescence analysis: The age of the oldest known *Cobitis* clonal lineage was estimated as the time to the most recent common ancestor (TMRCA) for all cyt *b* sequences of asexual individuals sampled by Janko *et al.* [26] that cluster in the so called 'Hybrid clade I'. TMRCA was estimated by coalescent method of Griffiths and Tavare [43] and it was implemented in the Genetree software by R. C. Griffiths, which is particularly suitable because the observed pedigree of the Hybrid clade I conformed to the infinite-allele model (we observed no evidence for multiple mutational hits on sequence positions within this clade). We first determined the Maximum Likelihood (ML) value of θ ($\theta = 2N_f\mu$ for haploid maternally inherited mitochondrial locus; N_f represents the effective population size of females, and μ represents the mutation rate per locus per generation). Using the ML estimate of θ (1.156) we performed one million simulations of the coalescent process providing us with an estimate of TMRCA and 95% confidence intervals in coalescent units.

Speciation times in mutation units of *C. elongatoides*, *C. taenia* and *C. tanaitica* as well as their contemporary and ancestral population sizes, θ_{TN}, θ_N, θ_E, θ_{TNE} and θ_{TN} ($\theta = 4N_e\mu$) were estimated from sequences of nine nuclear loci from Choleva *et al.* [27]. The calculation was performed with the Bayesian Markov chain Monte Carlo (MCMC) algorithms implemented in BPP Version 2.0b [44], which allow for the incorporation of relative mutation rates among loci and multiple haplotypes per species. The analysis was performed under the assumption of (TT, NN) monophyly in the true species tree (see Choleva *et al.* [27]). As recommended in Yang [45], the mutation rate scalars (i.e. the information about the relative substitution rates of individual loci) were obtained by among-locus comparison of average distances between ingroup and outgroup species. As an outgroup we used the sexual species *C. paludica*, which is a phylogenetically distant taxon suggesting that the coalescent error (i.e. the deviations among loci caused by polymorphism within the common ancestor of in-group and outgroup taxa) would not be of a large magnitude [45]. With this outgroup the MCMC was run under conditions described in Choleva *et al.* [27].

2) In the second step, we converted abovementioned estimates into absolute-time units, which require a calibration point on *Cobitis* phylogeny that is suitable for employed nuclear markers. Since *Cobitis* phylogeny has been mostly addressed by mtDNA loci we first constructed an ultrametric tree of published cyt *b* sequences covering all major *Cobitis* lineages (dataset from Tang *et al.* [46]) using previously published calibration point, i.e. the opening of the Gibraltar strait (~5.33 MYA), which separated two sister species, i.e. *C. maroccana* and *C. paludica* [47]. Although the usage of this calibration point may have limitations due to its recency, obtained ultrametric tree provided two relevant facts: i) it allowed estimating the branch specific mutation rate of mitochondrial cyt *b*, which was used to convert relative estimates of clonal age into absolute time units, and ii) it provided an estimate of absolute divergence time between ingroup species and *C. paludica* outgroup. This time estimate was

subsequently used to convert relative estimates of speciation times from nuclear markers into absolute time units and hence, both mtDNA and nDNA markers were calibrated consistently.

The data of Tang *et al.* [46] were used for ultrametric tree construction with two modifications. Firstly, we excluded sequences of taxonomically problematic species belonging to *Misgurnus* and *Paramisgurnus* genera (see Šlechtová *et al.* [48] discussion of this topic). Secondly, we replaced the *C. paludica* sequence from Tang *et al.* [46] with the *C. paludica* specimen from the Valle River, Spain (GenBank accession No: AY860180.1), as recommended by Doadrio and Perdices [47] because this population represents a phylogenetically closer lineage to the African *C. maroccana* and thus minimizes the coalescent error. Using such a modified dataset, we reconstructed the ML tree of cyt *b* sequences with RAxML [49] and ultrameterised it with the penalized likelihood method (PL) [50] implemented in R package Ape ([51]; S2 Fig). PL is a computationally inexpensive method that is nonetheless more robust against violations of model assumptions than strict-clock and relaxed-clock models [52]. ML estimates of branching times and branch-specific substitution rates depend on the lambda parameter which controls the variation of substitution rate change among branches (lambda may oscillate between zero -saturated model with one distinct rate for each branch—and infinity—model converging to a strict clock). Cross validation criterion removing one tip each time was used to select the optimal lambda value to construct the final ultrametric tree and obtain the ML estimates of branching times and substitution rates for each branch.

Having obtained the lineage-specific mutation rates, the TMRCA of the Hybrid clade I was translated into the time in years (t) as $t = \mathrm{TMRCA}^{*} N_f^{*} g$ using the generation time (g) of two years (N_f was estimated from θ using the ML estimate of substitution rates provided by PL). In order to accommodate the uncertainty around mutation rate of clonal hybrids, which combine genetic material from two or more species, clonal age was estimated using an average rate over the branches leading to the species that participate in the origin of asexual *Cobitis* hybrids. Speciation times were translated into absolute time units by taking the ML estimate of the divergence time of *C. paludica* and ingroup species from the PL analysis.

Results

Karyotype analysis of hybrid metaphases

Giemsa stained metaphases of two diploid (EN, ET) and four triploid (EEN, ENN, EET and ETT) hybrid biotypes were analysed in order to evaluate the morphological stability of their karyotypes. Chromosomes were sorted out into one of four categories (meta-, submeta-, subtelo- and acrocentric chromosomes) based on their morphology. Without exception, all complete metaphases of all hybrid biotypes contained chromosomal numbers corresponding to appropriate combinations of haploid parental genomes involved, namely EN hybrid had 2n = 50, ET had 2n = 49, ENN and EEN had 3n = 75, ETT had 3n = 73, and EET had 3n = 74 (Fig 1, S1 Fig, Table 1). Also, numbers of chromosomes in particular morphological categories of all hybrids matched those expected by combining the karyotypes of parental species (e.g. in diploid hybrid of EN genomic constitution we observed 16 metacentrics– 11 metacentrics derived from E genome and 5 from the N genome; other categories could be derived similarly). These data are summarized in Table 1 and we further provide Giemsa or DAPI stained karyotypes of 23 individuals from all biotypes in Fig 1 and S1 Fig to document the consistency of such observations. Information about individuals used for karyotyping is given in S1 and S2 Tables.

We further focused on the differences among hybrids along the time gradient of their historical origin. We observed no detectable changes in karyotypes of hybrid clones irrespective of their age. In particular, Fig 1A and 1C, S1A–S1E Fig display representatives of ancient Hybrid

clade I, Fig 1B and 1E, and S1F–S1O, S1Q and S1R Figs show representatives of lineage originated in the Holocene epoch; Fig 1D and 1F and S1P Fig shows the first generation hybrid obtained from recent laboratory crosses of parental species (S1 and S2 Tables).

Identification of parental chromosomes in karyotypes of hybrid individuals based on GISH

Cytogenetic analyses were performed on metaphases of all 2n and 3n hybrid biotypes in order to identify parental chromosomal sets, and to detect potential large-scale recombinations and/or rearrangements. Reliability of conventional approach was validated by GISH analysis of first generation hybrids in which we clearly sorted karyotypes into haploid sets categorized after parental species (Fig 1D and 1F, S3H and S3L Fig). After having applied GISH to wild caught hybrids, we got the same result and clearly assigned chromosomes to haploid chromosomal sets of sexual species involved in the primary hybridizations (i.e., to *C. elongatoides*, *C. taenia*, or *C. tanaitica*; Fig 1 and S3 Fig). For example, in GISH experiment to EN hybrid metaphases, the *C. elongatoides* signals were green and *C. tanaitica* signals were red (Fig 1A). According to GISH signal ratio we observed 11 meta-, 13 submeta- and 1 subtelocentric chromosome in green colour. Number and morphology of these chromosomes correspond to haploid set of chromosomes of pure *C. elongatoides* (Table 1). The rest of 50 chromosomes from EN hybrid metaphase were coloured in red and contained 5 meta-, 13 submeta-, 3 subtelo- and 4 acrocentric chromosomes which exactly matched to the haploid chromosome number of pure *C. tanaitica* (Fig 1A, Table 1). Analogously, after GISH experiments with all other hybrid biotypes (Fig 1B–1F and S3 Fig), the chromosomes matched by numbers and by morphology to combination of haploid and diploid chromosome set of respective sexual species. The summary of these observations is provided in Table 1.

In concordance with observation from Giemsa stained metaphases no detectable differences in karyotypes were observed among hybrids of different age of origin (Fig 1, S3 Fig, S1 and S3 Tables). No visible evidence for large-scale intergenomic exchanges was observed in the diploid and triploid hybrid metaphases of hybrids under this study. In some metaphases we found parts of individual chromosomes with balanced signals from both parental species but such observation resulted from non-specific hybridization rather than from recombination events and was never confirmed in other metaphases from the same individual including the same slide. GISH hybridization pattern was not uniform over the whole chromosomal length. The hybridization signals were stronger in the pericentromeric regions, particularly in metacentric chromosomes and in the telomeric regions of many other chromosomes (Fig 1 and S3 Fig).

Age estimates of studied clones and speciation times of parental species

The cyt *b* tree topology reconstructed with RAxML matched Tang *et al.* [46]. The cross-validation selected a relatively high lambda value (10^2), which is consistent with Bohlen's *et al.* [53] finding of no significant deviation from strict molecular clock in *Cobitis* cyt *b*. Using such a lambda value for PL method, we found relatively older cladogenetic events compared to Tang *et al.* [46] since these authors used a more distant *C. paludica* population as a putative sister lineage to *C. maroccana* for calibration, while we used a more closely related sequence from the Valle River, Spain (see [47]). The average cyt *b* substitution rate found by the PL method in the clade of hybridizing *Cobitis* species equalled 0.0065 per site per MYA. Having applied this rate to the ML estimate of the TMRCA of the Hybrid clade I provided a clonal age equal to 0.371 MYA (95% C I, 0.172–0.670 MYA) (Fig 2).

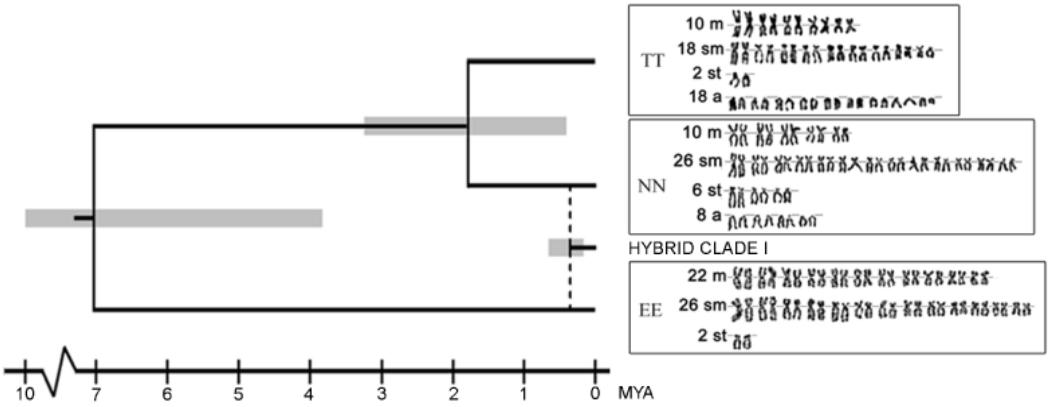


Fig 2. Ultrametric phylogenetic tree demonstrating estimated speciation times of parental species and the Hybrid clade I. Species-specific karyotypes arranged from Giemsa stained chromosomes are shown along the right side of cladogram. Confidence intervals of nodes of interest are in grey colour. TT, *C. taenia*; NN, *C. tanaitica*; EE, *Cobitis elongatoides*. Chromosomes were arranged in a decreasing size order and classified in four morphological groups: metacentric (m), submetacentric (sm), subtelocentric (st) and acrocentric (a).

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The ML estimate of a divergence time between *C. paludica* and the studied species within the *C. taenia* hybrid complex equalled 16.17 MYA. Using this divergence time as a calibration point for each nuclear locus from [27], the BPP software suggested that *C. elongatoides* diverged from the *C. taenia*–*C. tanaitica* ancestor during Miocene about 7 MYA (3.83–10.28 MYA), while both latter species diverged from each other for the period of Pleistocene-Pliocene about 1.8 MYA (0.42–3.25 MYA) (Fig 2).

Altogether, the results represent the hybrid biotypes under this study as representatives of a wide gradient of evolutionary ages (Table 2). The youngest hybrids are clearly represented by the first-generation individuals arisen by laboratory crosses. mtDNA analysis further revealed that natural hybrids formed two types with respect to their age. First type of hybrids shared haplotypes with the maternal species, demonstrating their recent origin during the Holocene epoch (see [26,30] for details). Second type of natural hybrids possessed haplotype E29 or E30 (*sensu* Janko *et al.* [26]) representing the oldest known *Cobitis* clonal lineage, the Hybrid clade I (Table 2).

Discussion

Stability of parental chromosomes in hybrid metaphases and their identification based on GISH

Analyses of Giemsa stained metaphases of hybrid clones enabled sorting of all chromosomes into morphological categories whose counts corresponded to expected numbers upon combining haploid complements of parental species. These observations were confirmed by GISH because chromosomal staining by species specific probes allowed unambiguous distinguishing of parental components in genomes of all studied hybrids. The observed pattern shows that chromosomes of hybrids did not undergo large-scale restructuring otherwise one would expect a failure to assign them to the morphological categories corresponding to karyotypes of respective parental species. Karyotypes of particular biotypes were uniform, regardless of whether these were of recent or ancient origin (including the oldest known *Cobitis* clonal lineage—the Hybrid clade I). This indicates the long-term stability of karyotypes of these asexuals. Our

findings are not trivial from several reasons. Application of GISH to identify parental components in hybrids of cold-blooded vertebrates often faced technical hurdles stemmed from their small sized chromosomes. Hence, only a few previous studies reported successful results allowing further interpretations ([25] and subsequent references on *Ambystoma* salamanders, [54–62]). Moreover, our findings contrast other asexual organisms, e.g. *Sitobion miscanthi* where extra chromosomal element (originated by fission) was observed after seven years or less in laboratory culture [13].

Although various cytological mechanisms are supposed to maintain the long-term integrity of clonally inherited chromosomes, asexual organisms may have so called 'minimal sex' (i.e. occasional incorporation of low amount of alien genetic material into ova [63]) or sometimes switch between phases of sexual and asexual reproduction [64]. Such 'sexual periods' are expected to produce recombinant patterns of hybrid asexuals lineages, which were not observed in *Cobitis* hybrids studied here. Moreover, asexual transmission of genomes is seldom perfect, especially in automictic asexual lineages where limited recombinations between co-inherited genomes have been found (e.g. [25,63]). This may lead to a notable introgression of one parental genome onto the genomic background of the other one. Such intragenomic recombinations have the consequence in less heterozygous progeny. Arai and Mukaino [65] found that genetically variable progeny may also be formed by automictic organisms with premeiotic endoduplication and attributed such an observation to occasional formation of bivalents between the chromosomes from different parental taxa. Mitotic intraclonal gene conversion or recombination was also considered as one of possible explanations for exceptionally high rate of homozygosity in species-diagnostic SNP markers of hybrid clonal fish *Poecilia formosa* [66].

In contrast to aforementioned studies, this study showed that asexually transmitted *Cobitis* genomes maintain their integrity even in relatively old clonal lineages. We found no evidence of recombination between homeologous chromosomes from different parental species despite the fact that gametogenesis in hybrid *Cobitis* involves quasi normal meiosis [31,32]. Data from this and previous studies of *Cobitis* (see above) suggest that intraclonal recombinations among chromosomal complements originating from hybridizing parental species seem to be either absent or rare as they have not accumulated to a notable extent in hybrid clonal lineages whose ages span over hundreds of thousands of generations (Hybrid clade I). Although cytogenetic approach has a limitation in resolution power, there is possibility of detection of large-scale recombination as observed in unisexual salamanders [25]. On the other hand there is a case of *Pelophylax* hybrid complex where ongoing and site-specific interspecific genetic transfer mediated by hybridogenetic hybrids has been evidenced [63,67]. GISH experiments on metaphases of diploid hybrid *P. esculentus* which originate through hybridization between *P. ridibundus* and *P. lessonae* have detected two clear groups of chromosomal constituents, each originating from one of the sexual parental species but no intergenomic exchanges in metaphase chromosomes [54]. Similarly in *Cobitis* hybrid complex, if potential recombination occurred, these are small-scale changes and undetectable by conventional karyotype analyses or by GISH method. Our finding of integrity of karyotypes in *Cobitis* hybrids is well in line with previous cytological studies [31,32], experimental crossing experiments (e.g. [29]), and multilocus genotyping [28,30]. The result congruently indicates that *Cobitis* clones maintain a fixed heterozygosity in essayed species-specific loci. The only source of intra-lineage variation known so far stems from post formational mutations and incorporations of entire paternal genomes leading to increased ploidy of the clonally reproducing progeny. The integrity of clonal *Cobitis* genomes may theoretically be explained by high sequence divergence between parental species which prevents large-scale chromosomal recombinations. However, Bi and Bogart [25] described intergenomic recombination blocks on chromosomes of *Ambystoma* hybrids composed of *A.*

laterale and *A. jeffersonianum* parental species. Despite authors did not provide divergence time of these species, Robertson *et al.* [68] showed that their divergence in cyt *b* (~ 10%) is more than twice as large than between *A. laterale* and *A. texanum* (~ 4%), which diversified about 10 MYA [69]. Recombination can therefore occur between genomes of species that are even more diverged than *Cobitis* under study (~ 7 MYA). Stability of Cobitid clonal genomes may, therefore, have another explanation.

Is there an evidence for increased rates of chromosomal evolution?

The genetic variability of extant asexuals is derived from the variability possessed by their sexual progenitors at the time of primary hybridization. It means the variability of sexual genomes became frozen in forming clonal lineages. The clonal progeny observed nowadays thus bears the combination of genomes derived from the first generation of an interspecific hybrid. Therefore, our finding that parental chromosomal complements in clones exactly match morphological categories of karyotypes in contemporary sexual relatives suggests that neither the karyotypes of clones, nor the karyotypes of sexual species have accumulated morphological changes since the origin of hybrid lineages sampled in natural populations.

According to the molecular dating, parental species diverged from each other during Miocene and Pleistocene, respectively. Since then, their karyotypes differentiated in both number and categories of chromosomes [34,36] (Fig 2, Table 1) including the large changes in the positions of centromeres (particularly dynamic regions of chromosomal evolution in most eukaryotes [70]), as well as centric fusion. We cannot distinguish whether these changes have accumulated gradually or have been acquired during or right after speciation events. However, taking into account the level of karyotype differentiation among sexual species and the fast mutation rates documented in some clonal organisms [13,14,16–19], one would expect to observe at least some morphologically visible mutations accumulated in karyotypes of the much older *Cobitis* Hybrid clade I (ca 0.37 MYA which represents over one fifth of the time since the separation of *C. taenia* and *C. tanaitica*). Instead of increased rates of karyotype evolution, our current data suggest that asexual karyotypes lacked any notable reorganization despite they have been clonally transmitted more than several hundreds of thousands of generations since the initial clonal formation.

Abovementioned molecular dating should be considered with a large grain of salt due to large confidence intervals around the age estimates and other well-known problems inherent in molecular clock dating. However, another point that strongly supports the conclusion that clonally transmitted karyotypes evolve conservatively over long periods comes from biogeographic data. These imply that the clonal Hybrid clade I has survived at least one and probably more interglacials in multiple separated refugee [26]. We compared Hybrid clade I individuals that were sampled from areas that belong to different Danubian refugial areas as identified by Janko *et al.* [26]. Here, samples from Bulgaria and Romania represented the Lower Danubian refugium, samples from Eastern Slovakia represented the Pannonian refugium, and samples from Poland aimed to cover Central European/upper Danubian refugium (Table 2). Yet, we did not find karyotype differentiation among Hybrid clade I individuals originating from those distinct populations, which most likely evolved in isolation [26] suggesting that their identical karyotypes cannot be explained by homogenizing gene flow. Rather, we prefer the explanation that karyotypes of different refugial populations did not accumulate any morphological changes at least since the last interglacial.

We are aware that our conclusions are valid only for the resolution power of applied methods and sample number. Also, we may not exclude the accumulation of small-scale chromosomal mutational changes. For example, we observed indications of the accumulation of

heterochromatin in the pericentromeric region in hybrid karyotypes. The phenomenon of the stronger signals in the centromeric regions is similar to the one described in *Ambystoma* salamanders [25], *Pelophylax* water frogs [54], and *Brassica* plants [71]. This may be explained by a higher degree of homeology of repetitive sequences on chromosome arms than at the centromeres with consequent weak blocking by the competitor DNA and a higher concentration of species specific repetitive sequences at centromeres [71]. Further investigation of the nature of karyotypic variation and genome evolution in *Cobitis* fishes will require the isolation and characterization of the major repetitive DNA sequences in the heterochromatin of each species to determine how homogeneous they are, and to map the sites of particular sequences on chromosomes by *in situ* hybridization. The same applies for mobile genetic elements such as retrotransposons, which are expected to degenerate in asexuals with the absence of meiosis [72,73], but there is nothing known about asexuals with modified meiosis, as for example in *Cobitis* hybrids.

Conclusion

Conventional karyological studies of apomictic parthenogens show that asexuality increases the rate of chromosomal evolution [13,14,16,18] and could lead to accumulation of visible chromosome changes on short evolutionary time scale [13]. We applied conventional as well as molecular methods on karyotypes of automictic *Cobitis* hybrids and observed no morphological structural changes even in longer time scales. This indicates an interesting possibility that the effect of asexuality on the rate of chromosomal evolution is rather context-dependent than universal, i.e. it may depend e.g. on particular type of asexual reproduction. Future studies should take this possibility explicitly into account.

Supporting Information

S1 Fig. Representative karyotypes of hybrid biotypes after DAPI/Giemsa staining. (A-C) EN hybrids. (D-F) EEN hybrids. (G-I) ENN hybrids. (J-L) ET hybrids. (M-O) EET hybrids. (P-R) ETT hybrids. Chromosomes were arranged in a decreasing size order and classified in four morphological groups: metacentric (m), submetacentric (sm), subtelocentric (st) and acrocentric (a). To visualize the morphology of chromosomes DAPI (K and Q) or Giemsa (A-J, L-P and R) stained karyotype was used. Captured DAPI stained karyotypes were inverted. Bars equal 5 μm. Detail information about individuals used is provided in S2 Table.
(TIF)

S2 Fig. Maximum likelihood phylogenetic tree of *Cobitis* and *Sabanejewia* species. ML phylogenetic tree constructed from cytochrome *b* gene sequences from Tang *et al.* [46] and from Doadrio and Perdices [47]. Calibration point follows Doadrio and Perdices [47]. Widths of branches are proportional to ML estimate of substitution rate. *Cobitis* species are highlighted (grey colour), species mentioned in this study are highlighted.
(TIF)

S3 Fig. Representative metaphases of hybrid biotypes after GISH experiments. (A, B) EN hybrids. (C, D) EEN hybrids. (E, F) ENN hybrids. (G, H) ET hybrids. (I, J) EET hybrids. (K, L) ETT hybrids. Probes labelled with biotin-16-dUTP were detected with streptavidin-FITC (green signals on chromosomes); probes labelled with digoxigenin-11-dUTP were detected with anti-digoxigenin-rhodamin (red signals on chromosomes). Bars equal 5 μm. Detail information about individuals and hybridization patterns is provided in S3 Table.
(TIF)

S1 Table. Hybrid individuals used for GISH experiments presented in Fig 1.
(DOCX)

S2 Table. Hybrid individuals used for karyotyping presented in S1 Fig.
(DOCX)

S3 Table. List of hybrid individuals used for GISH experiments presented in S3 Fig.
(DOCX)

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Author Contributions

Conceived and designed the experiments: RS. Performed the experiments: ZM. Analyzed the data: LC KJ ZM. Contributed reagents/materials/analysis tools: LC KJ PR ZM. Wrote the paper: ZM LC RS PR JK LP KJ. Designed the whole study: PR KJ LC. Collected and raised material: LC KJ ZM JK LP.

References

1. Rieseberg LH. Chromosomal rearrangements and speciation. Trends Ecol Evol. 2001; 16: 351–358. doi: 10.1016/S0169-5347(01)02187-5 PMID: 11403867
2. Wilson AC, Maxson LR, Sarich VM. Two types of molecular evolution. Evidence from studies of interspecific hybridization. Proc Natl Acad Sci U S A. 1974; 71: 2843–2847. PMID: 4212492
3. Schmid M, Steinlein C, Bogart JP, Feichtinger W, León P, La Marca E, et al. The chromosomes of terraranan frogs. Insights into vertebrate cytogenetics. Cytogenet Genome Res. 2010; 130–131: 1–568. doi: 10.1159/000301339 PMID: 21063086
4. Nelson JS. Fishes of the world. Fourth edition. Nelson, Joseph S.; Department of Biological Sciences, University of Alberta, Edmonton, Alberta T6G 2E9, Canada, Canada.: John Wiley & Sons, Inc.; 2006.
5. Kohn M, Högel J, Vogel W, Minich P, Kehrer-Sawatzki H, Graves JAM, et al. Reconstruction of a 450-My-old ancestral vertebrate protokaryotype. Trends Genet TIG. 2006; 22: 203–210. doi: 10.1016/j.tig.2006.02.008 PMID: 16517001
6. Mank JE, Avise JC. Phylogenetic conservation of chromosome numbers in Actinopterygiian fishes. Genetica. 2006; 127: 321–327. doi: 10.1007/s10709-005-5248-0 PMID: 16850236
7. Arai R. Fish karyotypes: a check list. 2011 edition. Tokyo ; New York: Springer; 2011.
8. Ravi V, Venkatesh B. Rapidly evolving fish genomes and teleost diversity. Curr Opin Genet Dev. 2008; 18: 544–550. doi: 10.1016/j.gde.2008.11.001 PMID: 19095434
9. Phillips R, Ráb P. Chromosome evolution in the Salmonidae (Pisces): an update. Biol Rev Camb Philos Soc. 2001; 76: 1–25. PMID: 11325050
10. Otto SP, Whitton J. Polyploid incidence and evolution. Annu Rev Genet. 2000; 34: 401–437. doi: 10.1146/annurev.genet.34.1.401 PMID: 11092833
11. Mayer VW, Aguilera A. High levels of chromosome instability in polyploids of *Saccharomyces cerevisiae*. Mutat Res Mol Mech Mutagen. 1990; 231: 177–186. doi: 10.1016/0027-5107(90)90024-X
12. Martens K, Loxdale HD, Schön I. The elusive clone—in search of its true nature and identity. In: Schön I, Martens K, Dijk P, editors. Lost Sex. Springer Netherlands; 2009. pp. 187–200. Available: http://link.springer.com/chapter/10.1007/978-90-481-2770-2_9
13. Sunnucks P, England PR, Taylor AC, Hales DF. Microsatellite and chromosome evolution of parthenogenetic *Sitobion aphids* in Australia. Genetics. 1996; 144: 747–756. PMID: 8889535

14. Schneider MC, Cella DM. Karyotype conservation in 2 populations of the parthenogenetic scorpion *Tityus serrulatus* (Buthidae): rDNA and its associated heterochromatin are concentrated on only one chromosome. J Hered. 2010; 101: 491–496. doi: 10.1093/jhered/esq004 PMID: 20231264

15. Welch MD, Meselson M. Evidence for the evolution of bdelloid rotifers without sexual reproduction or genetic exchange. Science. 2000; 288: 1211–1215. PMID: 10817991

16. Normark BB. Evolution in a putatively ancient asexual aphid lineage: recombination and rapid karyotype change. Evolution. 1999; 53: 1458. doi: 10.2307/2640892

17. Spence JM, Blackman RL. Inheritance and meiotic behaviour of a de novo chromosome fusion in the aphid *Myzus persicae* (Sulzer). Chromosoma. 2000; 109: 490–497. PMID: 11151679

18. Welch JLM, Welch DBM, Meselson M. Cytogenetic evidence for asexual evolution of bdelloid rotifers. Proc Natl Acad Sci U S A. 2004; 101: 1618–1621. doi: 10.1073/pnas.0307677100 PMID: 14747655

19. Triantaphyllou AC. Oogenesis and the chromosomes of the parthenogenic root-knot nematode *Meloidogyne incognita*. J Nematol. 1981; 13: 95–104. PMID: 19300730

20. Blackman RL, Spence JM, Normark BB. High diversity of structurally heterozygous karyotypes and rDNA arrays in parthenogenetic aphids of the genus *Trama* (Aphididae: Lachninae). Heredity. 2000; 84 (Pt 2): 254–260. PMID: 10762396

21. Suomalainen E, Saura A, Lokki J. Cytology in evolution of parthenogenesis. 1 edition. Boca Raton, Fla: CRC Press; 1987.

22. Stenberg P, Saura A. Cytology of asexual animals. In: Schön I, Martens K, Dijk P, editors. Lost Sex. Springer Netherlands; 2009. pp. 63–74. Available: http://link.springer.com/chapter/10.1007/978-90-481-2770-2_4

23. Normark BB, Judson OP, Moran NA. Genomic signatures of ancient asexual lineages. Biol J Linn Soc. 2003; 79: 69–84. doi: 10.1046/j.1095-8312.2003.00182.x

24. Lutes AA, Neaves WB, Baumann DP, Wiegraebe W, Baumann P. Sister chromosome pairing maintains heterozygosity in parthenogenetic lizards. Nature. 2010; 464: 283–286. doi: 10.1038/nature08818 PMID: 20173738

25. Bi K, Bogart JP. Identification of intergenomic recombinations in unisexual salamanders of the genus *Ambystoma* by genomic *in situ* hybridization (GISH). Cytogenet Genome Res. 2006; 112: 307–312. doi: 10.1159/000089885 PMID: 16484787

26. Janko K, Culling MA, Ráb P, Kotlík P. Ice age cloning—comparison of the Quaternary evolutionary histories of sexual and clonal forms of spiny loaches (*Cobitis*; Teleostei) using the analysis of mitochondrial DNA variation. Mol Ecol. 2005; 14: 2991–3004. doi: 10.1111/j.1365-294X.2005.02583.x PMID: 16101769

27. Choleva L, Musilova Z, Kohoutova-Sediva A, Paces J, Rab P, Janko K. Distinguishing between incomplete lineage sorting and genomic introgressions: complete fixation of allospecific mitochondrial DNA in a sexually reproducing fish (*Cobitis*; Teleostei), despite clonal reproduction of hybrids. PLoS ONE. 2014; 9: e80641. doi: 10.1371/journal.pone.0080641 PMID: 24971792

28. Janko K, Bohlen J, Lamatsch D, Flajshans M, Epplen JT, Ráb P, et al. The gynogenetic reproduction of diploid and triploid hybrid spined loaches (*Cobitis*: Teleostei), and their ability to establish successful clonal lineages on the evolution of polyploidy in asexual vertebrates. Genetica. 2007; 131: 185–194. doi: 10.1007/s10709-006-9130-5 PMID: 17216551

29. Choleva L, Janko K, De Gelas K, Bohlen J, Šlechtová V, Rábová M, et al. Synthesis of clonality and polyploidy in vertebrate animals by hybridization between two sexual species. Evol Int J Org Evol. 2012; 66: 2191–2203. doi: 10.1111/j.1558-5646.2012.01589.x

30. Janko K, Kotusz J, De Gelas K, Šlechtová V, Opoldusová Z, Drozd P, et al. Dynamic formation of asexual diploid and polyploid lineages: multilocus analysis of *Cobitis* reveals the mechanisms maintaining the diversity of clones. PLoS ONE. 2012; 7: e45384. doi: 10.1371/journal.pone.0045384 PMID: 23028977

31. Saat TV. Reproduction of the diploid and polyploid spinous loach (*Cobitis*, Teleostei), oocyte maturation and fertilization in the triploid form. Russ J Dev Biol. 1991; 22: 332–338.

32. Juchno D, Boroń A, Spóz A, Kujawa R, Kolczyńska J. Diplotene chromosomes of oocytes of polyploid hybrid *Cobitis* (Pisces, Cobitidae). Konferencja Embriologiczna, Acta Biologica Cracoviensia,series Botanica. Poznań; 2014. p. 64.

33. Itono M, Morishima K, Fujimoto T, Bando E, Yamaha E, Arai K. Premeiotic endomitosis produces diploid eggs in the natural clone loach, *Misgurnus anguillicaudatus* (Teleostei: Cobitidae). J Exp Zoolog A Comp Exp Biol. 2006; 305: 513–523. doi: 10.1002/jez.a.283

34. Janko K, Flajšhans M, Choleva L, Bohlen J, Šlechtová V, Rábová M, et al. Diversity of European spined loaches (genus *Cobitis* L.): an update of the geographic distribution of the *Cobitis taenia* hybrid complex

with a description of new molecular tools for species and hybrid determination. J Fish Biol. 2007; 71: 387–408. doi: 10.1111/j.1095-8649.2007.01663.x

35. Choleva L, Apostolou A, Ráb P, Janko K. Making it on their own: sperm-dependent hybrid fishes (*Cobitis*) switch the sexual hosts and expand beyond the ranges of their original sperm donors. Philos Trans R Soc B Biol Sci. 2008; 363: 2911–2919. doi: 10.1098/rstb.2008.0059

36. Rábová M, Pelikánová Š, Choleva L, Ráb P. Cytogenetics of bisexual species and their asexual hybrid clones in European spined loaches, genus *Cobitis*. II. Mapping of telomeric (TTAGGG)n sequences and DAPI-positive heterochromatins in four parental species. ECI XII European Congress of Ichthyology, Book of Abstracts. Cavtat, Croatia; 2007. p. 59.

37. Boroń A. Replication banding patterns in the spined loach, *Cobitis taenia* L. (Pisces, Cobitidae). Genetica. 2003; 119: 51–55. PMID: 12903746

38. Ráb P, Bohlen J, Rábová M, Flajšhans M, Kalous L. Cytogenetics as a tool in fish conservation: the present situation in Europe. In: Pisano E, Ozouf-Costaz C, Foresti F, Kapoor BG, editors. Fish Cytogenetics. Enfield, NH 03748, USA: Science Publishers; 2007. pp. 215–241.

39. Ráb P, Roth P. Cold-blooded vertebrates. In: Balíček P, Forejt J, Rubeš J, editors. Methods of chromosome analysis. Brno: Cytogenet Sect Cs Biol Soc Publishers; 1988. pp. 115–124.

40. Völker M, Ráb P. Direct chromosome preparation from regenerating fin tissue. In: Ozouf-Costaz C, Pisano E, Foresti F, Foresti de Almeida-Toledo L, editors. Fish Cytogenetic Techniques. Enfield, NH 03748, USA: CRC Press, Inc.; 2015. pp. 37–41.

41. Levan A, Fredga K, Sandberg AA. Nomenclature for centromeric position on chromosomes. Hereditas. 1964; 52: 201–220. doi: 10.1111/j.1601-5223.1964.tb01953.x

42. Symonová R, Sember A, Majtánová Z, Ráb P. Characterization of fish genomes by GISH and CGH. In: Ozouf-Costaz C, Pisano E, Foresti F, de Almeida L, editors. Fish Cytogenetic Techniques. CRC Press; 2015. pp. 118–131. Available: http://www.crcnetbase.com/doi/10.1201/b18534-17

43. Griffiths RC, Tavare S. Simulating probability distributions in the coalescent. Theor Popul Biol. 1994; 46: 131–159. doi: 10.1006/tpbi.1994.1023

44. Yang Z, Rannala B. Bayesian species delimitation using multilocus sequence data. Proc Natl Acad Sci. 2010; 200913022. doi: 10.1073/pnas.0913022107

45. Yang Z. Likelihood and Bayes estimation of ancestral population sizes in hominoids using data from multiple loci. Genetics. 2002; 162: 1811–1823. PMID: 12524351

46. Tang Q, Freyhof J, Xiong B, Liu H. Multiple invasions of Europe by East Asian cobitid loaches (Teleostei: Cobitidae). Hydrobiologia. 2008; 605: 17–28. doi: 10.1007/s10750-008-9296-1

47. Doadrio I, Perdices A. Phylogenetic relationships among the Ibero-African cobitids (*Cobitis*, cobitidae) based on cytochrome b sequence data. Mol Phylogenet Evol. 2005; 37: 484–493. PMID: 16150615

48. Šlechtová V, Bohlen J, Perdices A. Molecular phylogeny of the freshwater fish family Cobitidae (Cypriniformes: Teleostei): delimitation of genera, mitochondrial introgression and evolution of sexual dimorphism. Mol Phylogenet Evol. 2008; 47: 812–831. doi: 10.1016/j.ympev.2007.12.018 PMID: 18255319

49. Stamatakis A. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics. 2006; 22: 2688–2690. doi: 10.1093/bioinformatics/btl446 PMID: 16928733

50. Sanderson MJ. Estimating absolute rates of molecular evolution and divergence times: a penalized likelihood approach. Mol Biol Evol. 2002; 19: 101–109. PMID: 11752195

51. Paradis E. Analysis of phylogenetics and evolution with R. 1 edition. New York: Springer; 2006.

52. Paradis E. Molecular dating of phylogenies by likelihood methods: a comparison of models and a new information criterion. Mol Phylogenet Evol. 2013; 67: 436–444. doi: 10.1016/j.ympev.2013.02.008 PMID: 23454091

53. Bohlen J, Perdices A, Doadrio I, Economidis PS. Vicariance, colonisation, and fast local speciation in Asia Minor and the Balkans as revealed from the phylogeny of spined loaches (Osteichthyes; Cobitidae). Mol Phylogenet Evol. 2006; 39: 552–561. PMID: 16439160

54. Zaleśna A, Choleva L, Ogielska M, Rábová M, Marec F, Ráb P. Evidence for integrity of parental genomes in the diploid hybridogenetic water frog *Pelophylax esculentus* by genomic *in situ* hybridization. Cytogenet Genome Res. 2011; 134: 206–212. doi: 10.1159/000327716 PMID: 21555873

55. Rampin M, Bi K, Bogart JP, Collares-Pereira MJ. Identifying parental chromosomes and genomic rearrangements in animal hybrid complexes of species with small genome size using Genomic *In Situ* Hybridization (GISH). Comp Cytogenet. 2012; 6: 287–300. doi: 10.3897/CompCytogen.v6i3.3543 PMID: 24260669

56. Pereira CSA, Rab P, Collares-Pereira MJ. Chromosomes of Iberian Leuciscinae (Cyprinidae) revisited: evidence of genome restructuring in homoploid hybrids using dual-color FISH and CGH. Cytogenet Genome Res. 2013; 141: 143–152. doi: 10.1159/000354582 PMID: 24107574

57. Pereira CSA, Aboim MA, Ráb P, Collares-Pereira MJ. Introgressive hybridization as a promoter of genome reshuffling in natural homoploid fish hybrids (Cyprinidae, Leuciscinae). Heredity. 2014; 112: 343–350. doi: 10.1038/hdy.2013.110 PMID: 24220087

58. Knytl M, Kalous L, Symonová R, Rylková K, Ráb P. Chromosome studies of European cyprinid fishes: cross-species painting reveals natural allotetraploid origin of a *Carassius* female with 206 chromosomes. Cytogenet Genome Res. 2013; 139: 276–283. doi: 10.1159/000350689 PMID: 23652770

59. Symonová R, Flajšhans M, Sember A, Havelka M, Gela D, Kořínková T, et al. Molecular cytogenetics in artificial hybrid and highly polyploid sturgeons: An evolutionary story narrated by repetitive sequences. Cytogenet Genome Res. 2013; 141: 153–162. doi: 10.1159/000354882 PMID: 24051427

60. Pokorná M, Rens W, Rovatsos M, Kratochvíl L. A ZZ/ZW sex chromosome system in the thick-tailed Gecko (*Underwoodisaurus milii*; Squamata: Gekkota: Carphodactylidae), a member of the ancient gecko lineage. Cytogenet Genome Res. 2014; 142: 190–196. doi: 10.1159/000358847 PMID: 24603160

61. Zhu H-P, Gui J-F. Identification of genome organization in the unusual allotetraploid form of *Carassius auratus gibelio*. Aquaculture. 2007; 265: 109–117. doi: 10.1016/j.aquaculture.2006.10.026

62. Valente TG, Schneider HC, Gross CM, Feldberg E, Martins C. Comparative cytogenetics of cichlid fishes through genomic *in-situ* hybridization (GISH) with emphasis on *Oreochromis niloticus*. Chromosome Res Int J Mol Supramol Evol Asp Chromosome Biol. 2009; 17: 791–799. doi: 10.1007/s10577-009-9067-5

63. Uzzell T, Gűnther R, Berger L. *Rana ridibunda* and *Rana esculenta*: a leaky hybridogenetic system (Amphibia, Salientia). Proc Acad Nat Sci Phila. 1977; 128: 147–171.

64. Alves MJ, Coelho MM, Collares-Pereira MJ. Evolution in action through hybridisation and polyploidy in an Iberian freshwater fish: a genetic review. Genetica. 2001; 111: 375–385. PMID: 11841181

65. Arai K, Mukaino M. Clonal nature of gynogenetically induced progeny of triploid (diploid × tetraploid) loach, *Misgurnus anguillicaudatus* (Pisces: Cobitididae). J Exp Zool—J EXP ZOOL. 1997; 278: 412–421.

66. da Barbiano LA, Gompert Z, Aspbury AS, Gabor CR, Nice CC. Population genomics reveals a possible history of backcrossing and recombination in the gynogenetic fish *Poecilia formosa*. Proc Natl Acad Sci. 2013; 201303730. doi: 10.1073/pnas.1303730110

67. Mikulíček P, Kautman M, Demovic B, Janko K. When a clonal genome finds its way back to a sexual species: evidence from ongoing but rare introgression in the hybridogenetic water frog complex. J Evol Biol. 2014; 27: 628–642. doi: 10.1111/jeb.12332 PMID: 26227900

68. Robertson AV, Ramsden C, Niedzwiecki J, Fu J, Bogart JP. An unexpected recent ancestor of unisexual *Ambystoma*. Mol Ecol. 2006; 15: 3339–3351. doi: 10.1111/j.1365-294X.2006.03005.x PMID: 16968274

69. Bi K, Bogart JP. Time and time again: unisexual salamanders (genus *Ambystoma*) are the oldest unisexual vertebrates. BMC Evol Biol. 2010; 10: 238. doi: 10.1186/1471-2148-10-238 PMID: 20682056

70. Eichler EE, Sankoff D. Structural dynamics of eukaryotic chromosome evolution. Science. 2003; 301: 793–797. doi: 10.1126/science.1086132 PMID: 12907789

71. Snowdon RJ, Köhler W, Friedt W, Köhler A. Genomic *in situ* hybridization in *Brassica* amphidiploids and interspecific hybrids. Theor Appl Genet. 1997; 95: 1320–1324. doi: 10.1007/s001220050699

72. Hickey DA. Selfish DNA: a sexually-transmitted nuclear parasite. Genetics. 1982; 101: 519–531. PMID: 6293914

73. Arkhipova I, Meselson M. Transposable elements in sexual and ancient asexual taxa. Proc Natl Acad Sci. 2000; 97: 14473–14477. doi: 10.1073/pnas.97.26.14473 PMID: 11121049

7.2. Publication II

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I declare that the contribution of Zuzana Majtánová to the publication Majtánová et al. (2016), as stated here, is true.

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"Holostei versus Halecostomi" problem: insight from cytogenetics of ancient non-teleost actinopterygian fish, bowfin *Amia calva*

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Abbreviated title: **Molecular cytogenetics of *Amia calva***

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Abstract

Bowfin belongs to an ancient lineage of non-teleost ray-finned fishes (actinopterygians), and is the only extant survivor of a once diverged group, the Halecomorphi or Amiiformes. Due to the scarcity of extant non-teleost ray-finned lineages, also referred as "living fossils", their phylogenetic interrelationships have been the target of multiple hypotheses concerning their sister group relationships. Molecular and morphological datasets have produced controversial results; bowfin is considered as either the sister group to genome-duplicated teleosts (together forming the group of Halecostomi) or to gars (Lepisosteiformes; together forming the group of Holostei). However, any detailed cytogenetic analysis of bowfin chromosomes has never been performed to address this issue. Here we examined bowfin chromosomes by conventional (Giemsa-staining, C-banding, base specific fluorescence and silver-staining) and molecular (FISH with rDNA probes) cytogenetic protocols. We identified diploid chromosome number 2n = 46 with a middle-sized submetacentric chromosome pair as the major ribosomal DNA-bearing (45S rDNA), GC-positive and silver-positive element. The minor rDNA (5S rDNA) sites were localized in the pericentromeric region of one middle-sized acrocentric chromosome pair. Comparison with available cytogenetic data of other non-teleost actinopterygians (bichirs, sturgeons, gars) and teleost species including representative of basally branching lineages showed bowfin chromosomal characteristics more similar to the teleost type than to any other non-teleosts. Particularly striking differences were identified between bowfin and gars, the latter of which were found to mimic mammalian AT/GC genomic organisation. Such conclusion however contradicts the most recent phylogenomic results and raises the question what states are ancestral and what are derived.

Keywords: comparative and molecular cytogenetics, basal ray-finned fishes, gar-*Amia*-teleost problem, genome organization and divergence, teleost ancestral karyotype

55 **Introduction**

56 The bowfin *Amia calva*, a voracious freshwater predator occurring in eastern North America,
57 is the only extant survivor of a once taxonomically diverse and widespread halecomorph (or
58 amiiform) fish fauna from the Mesozoic and Paleogene periods (Grande and Bemis, '98;
59 Nelson, 2006). As a single extant halecomorph species belonging to a genus, which first
60 appeared over 100 million years ago (Grande and Bemis '98) and representing one of only
61 five living non-teleost actinopterygian lineages, bowfin represent exceptional example of
62 species with extremely long-retained ecomorphologies. The bowfin has also been of interest
63 to biologists due to its longevity, aestivation and air breathing (see Grande and Bemis, '98 and
64 reference therein). More recently, bowfin became an indispensable component of molecular
65 and morphological phylogenetic analyses of actinopterygians (in-depth review in Sallan,
66 2014). Multiple studies have sought to determine the interrelationships of non-teleost and
67 teleost ray-finned fishes in order to reconstruct deep branching events within the clade. These
68 have produced two contradictory hypotheses: i) molecular phylogenies overwhelmingly place
69 bowfin as the sister group to gars, Lepisosteiformes or Ginglymodi within the monophyletic
70 Holostei lineage (Inoue et al., 2003; Near et al., 2012; Faircloth et al., 2013; Betancur-R et al.,
71 2013; Broughton et al., 2013; Braasch et al., 2016; Pasquier et al., 2016); ii) the majority of
72 morphological studies favour a sister group relationship of bowfin with teleosts within the
73 monophyletic Halecostomi (in details reviewed by Sallan, 2014; see Grande, 2010 for another
74 view). This has produced the famous "gar – bowfin – teleost" or "Holostei versus

75 Halecostomi" problem (e.g. Grande, 2010; López-Arbarello, 2012; Sallan, 2014) and
76 obscured the circumstances of origins of teleosts and their innovations, such as genome
77 duplication and morphological novelties. To address this phylogenetic debate, numerous
78 osteological, paleontological as well as molecular characteristics and combinations thereof
79 have been investigated (Sallan, 2014), including, most recently, ultraconserved elements of
80 the genome (Faircloth et al., 2013) or analyses of the spotted gar genome and bowfin
81 transcriptome (Braasch et al., 2016). Available cytogenetic data, i.e. chromosome number,
82 karyotype and other (sub)chromosomal characteristics, have not been well investigated so far
83 though they might have shed light to the problem from another point of view. Previously, the
84 bowfin chromosome number and karyotype were reported merely in two studies, by Ohno et
85 al. ('69) and Suzuki and Hirata ('91), both based on conventionally Giemsa-stained
86 chromosomes.

87 In this study, we report on bowfin chromosomal characteristics, as investigated by
88 means of conventional (Giemsa staining, C-banding, CMA_3/DAPI staining, Ag-staining) and
89 molecular (FISH with rDNA probes) cytogenetic protocols. We address the "Holostei versus
90 Halecostomi" problem from the cytogenetic point of view based on available data of non-
91 teleost actinopterygians, namely Polypteriformes, Acipenseridae, Polyodontidae,
92 Lepisosteiformes and teleost representatives. We show that bowfin share more karyological
93 and cytogenetic similarities with teleosts than with gars or other non-teleost actinopterygians.
94

95 **Materials and Methods**

96 *Material/Specimen acquisition*

97 Blood samples of 2 unsexed juvenile individuals of the bowfin *Amia calva* were collected
98 from the broodstock belonging to the DACBiol, UJAT, Villahermosa, Tabasco, Mexico. All
99 institutional and national guidelines was covered by the "Valid Animal Use Protocols" Nr. CZ
100 00386 of the Laboratory of Fish genetics, the Institute of Animal Physiology and Genetics
101 issued by the Czech Ministry of Agriculture on 25 September 2014 and by the ethical and
102 research regulations at 2016 from the Universidad Juárez Autónoma de Tabasco, Mexico.

103

104 *Blood culturing and chromosome preparation*

105 Leucocytes from blood samples of *A. calva* were cultivated and chromosome spreads were
106 prepared according to the protocol of Fujiwara *et al.* (2001) with some modifications. Briefly,
107 0.2 - 0.5 ml of blood was collected from an anesthetized fish by puncture of the *vena cava*
108 *caudalis* using a heparinized sterile syringe. The leucocyte-rich plasma was used to set up
109 primary cultures in 5 ml of the Medium 199 (SIGMA, St. Louis, MO, USA) supplemented
110 with 10% fetal calf serum (FBS Superior, Biochrom, Berlin, Germany), 1% Antibiotic
111 Antimycotic Solution (SIGMA), LPS from *E. coli* (0.5 mg per 5 ml of medium), PHA-P (90
112 µg per 5 ml of medium, (Remel, Lenexa, KS, USA), Kanamycin (0.3 mg per 5 ml of medium
113 (SIGMA) and 0.175 µl 10% mercaptoethanol (SIGMA). After 120 hours of incubation at
114 20°C, 5 ml of culture was harvested using standard colchicine (two drops of 0.1% colchicine

115 per 5 ml of media) and 0.075 ml KCl hypotonic (8 min.) treatments followed by fixation in a
116 freshly prepared fixative (methanol: acetic acid 3:1, v/v) three times.
117
118 *Karyological analysis*
119 Chromosomes were initially stained in buffered Giemsa (5%, 10 min) to visualize their
120 morphology. In total, 20 metaphases of each individual have been inspected in order to
121 confirm the chromosome morphology and composition of the karyotype. For localization of
122 nucleolar organizer regions (NORs, i.e. transcriptionally active major rDNA unit) we have
123 performed $AgNO_3$ impregnation (Ag-staining) as described by Howell and Black ('80).
124 Chromomycin A_3 (CMA_3, DNA dye specific for GC-rich regions) and DAPI (AT-specific)
125 fluorescent staining was performed to reveal GC genome composition as described by Sola et
126 al. ('92). A C-banding technique visualizing blocks of constitutive heterochromatin was
127 performed according to Sumner ('72) with slight modifications described in Pokorná et al.
128 (2014). The chromosomes after C-banding were counterstained by DAPI to enhance the
129 contrast and the microphotographs were taken in the fluorescent regime (not in the light
130 microscopy regime as in the Giemsa-stained C-banding) and inverted.
131
132 *Probes preparation, FISH*
133 Probes for FISH experiments were produced by PCR with the primers pairs and thermal
134 cycling conditions according Komiya and Takemura ('79) for 5S rDNA and White et al. ('90)

135 for 18S rDNA (primers NS1 and NS4). The PCR reactions were carried out in a final volume
136 of 25 µl consisting of 100 ng genomic DNA, 12.5 µl PPP master mix, 0.01 mM of each
137 primer and PCR water to complete the volume (all reagents from TopBio, Prague, Czech
138 Republic). Cycling conditions were as follows: (a) 18S: 2 min at 95 °C; 35 cycles of 1 min at
139 95 °C, 40 s at 55 °C and 2 min at 72 °C; 5 min at 72 °C ; (b) 5S rDNA: 5 min at 94 °C; 2
140 cycles of 1 min at 95 °C, 30 s at 61 °C and 45 s at 72 °C; 2 cycles of 1 min at 95 °C, 30 s at 59
141 °C and 45 s at 72 °C; 2 cycles of 1 min at 95 °C, 30 s at 57 °C and 45 s at 72 °C; 25 cycles of
142 1 min at 95 °C, 30 s at 61 °C and 45 s at 72 °C; 7 min at 72 °C. The amplified fragments were
143 sequenced at the ABI 3700 sequencer prior FISH experiments. Probes were indirectly labelled
144 with biotin-16-dUTP (Roche, Mannheim, Germany) and digoxigenin-11-dUTP (Roche)
145 through PCR re-amplification of previously sequenced PCR products. Re-amplification was
146 carried out under the same condition as previous PCR reaction. Labelled PCR products were
147 precipitated. A hybridization mixture was made consisting of hybridization buffer (Symonová
148 et al., 2015), sonicated salmon sperm blocking DNA (15 µg/slide; Sigma-Aldrich, St. Louis,
149 Mo., USA) and differently labelled PCR products of both genes.

150 The hybridization and detection procedure were carried out under conditions described
151 by Symonová et al. (2015). The biotin-dUTP labelled probes were detected by either the
152 Invitrogen Cy™3-Streptavidin (Invitrogen, San Diego, CA, USA; Cat. No. 43-4315) or by the
153 FITC-Streptavidin (Cat. No. 43-4311). The digoxigenin-dUTP labelled probes were detected
154 by either the Roche Anti-Digoxogenin-Fluorescein (Cat. No. 11207741910) or by the Anti-

Digoxigenin-Rhodamin (Cat. No. 11207750910). The chromosomes were counterstained with Vectashield/DAPI (1.5 mg/ml) (Vector, Burlingame, Calif., USA). Hybridization lasted for 1 day in a dark humid chamber at 37°C.

Image Analysis

Chromosomal preparations were examined by an Olympus Provis AX 70 epifluorescence microscope. Images of metaphase chromosomes were recorded with a cooled Olympus DP30BW CCD camera. The IKAROS and ISIS imaging programs (Metasystems, Altlussheim, Germany) were used to analyse grey-scale images. The captured digital images from FISH experiments were pseudocoloured (red for Anti-Digoxigenin-Rhodamin, green for Invitrogen FITC-Streptavidin) and superimposed using Microimage and Adobe Photoshop software, version CS5, respectively. In the case of CDD staining, the CMA_3 signal was inserted into the red and the DAPI signal into the green channel to enhance the contrast between these two types of signals. The chromosomal categories were classified according to Levan et al. ('64). Chromosomes were ordered within each group in decreasing size order.

Results

Karyological analysis, FISH

The diploid chromosome number in *A. calva* was 2n = 46, the fundamental number (i.e. number of chromosomal arms determined by scoring metacentrics-submetacentrics as bi-

175 armed and subtelocentrics-acrocentrics as uni-armed) was NF = 58. Results of karyological
176 analyses together with results of FISH experiments performed on *A. calva* chromosomes are
177 summarized in the ideogram (Fig 1a). Karyotype was composed of 1 pair of meta-, 5 pairs of
178 submeta- and 4 pairs of subtelo- and 13 pairs of acrocentric chromosomes (Figs 1a, 1b). The
179 submetacentric pair No. 5 (Figs 1a, 1b) possessed a polymorphic secondary constriction in the
180 terminal short *p* arm causing differences in the size of homologs. The CMA_3/DAPI
181 fluorescence demonstrated homogenously stained chromosomes, i.e. a balanced proportion of
182 AT/GC up to the extremely GC-rich i.e. CMA_3 positive NOR sites on the chromosome pair
183 No. 5 (Figs 1a, 1c). This position was also an Ag-NOR site (Figs 1a, 1d). C-banded
184 chromosomes with visualized constitutive heterochromatin showed signals in centromeres of
185 all chromosomes; in addition, strong signals, corresponding to 5S rDNA sites, have been
186 detected on chromosome pair No. 13 (Figs 1a, 1e).

187 The FISH experiments with 5S and 45S rDNA probes showed a nonsyntenic pattern,
188 i.e. signals on distinct chromosomal pairs have been detected in both individuals (Figs 1a, 1f).
189 While the 45S rDNA site (28S rDNA, 5.8S rDNA and 18S rDNA) was located at the
190 telomeric region on short (*p*) arms of the submetacentric pair No. 5, the 5S rDNA sites were
191 situated at the centromeric regions of the acrocentric pair No. 13 (Figs 1a, 1f).

192

193 **Discussion**

194 *Bowfin karyotype*

195 Our results confirmed the 2n = 46 in bowfin, as reported by previous studies (Ohno et al., '69;
196 Suzuki and Hirata, '91). Ohno et al. ('69) described its karyotype with 10 pairs of meta- and 13
197 pairs of acrocentric chromosomes. These authors used squash tissue protocol for chromosome
198 preparations and our inspection of published karyotype documents theirs low quality. Suzuki
199 and Hirata ('91), using direct kidney preparation protocol, reported its karyotype with one pair
200 of meta-, 12 pairs of submeta-subtelocentric and 10 pairs of acrocentric chromosomes. Both
201 studies examined bowfin karyotype using Giemsa-stained chromosomes. However Suzuki
202 and Hirata ('91) improved their study with C-banding technique in order to document the
203 distribution of constitutive heterochromatin. Their study revealed a middle-sized acrocentric
204 chromosome pair bearing secondary constriction in *p* arms and hypothesized also that only
205 metacentric pair in bowfin karyotype originated *via* centric fusion decreasing thus 2n from 48
206 to 46.

207 Our interpretation of the bowfin karyotype here based on leucocyte culture confirmed
208 the presence of one distinct pair of metacentric chromosomes and distinguished in more detail
209 five pairs of submeta-, four pairs of subtelo- and 14 pairs of subtelo- to acrocentric
210 chromosomes with some chromosomes, however, at borderline between chromosome
211 categories. We further identified a pair of middle-sized acrocentric chromosome pair with
212 secondary constriction in *p* arms as the major rDNA-bearing (45S rDNA), CMA_3-positive and
213 Ag-positive element. Since bowfin is the only extant survivor of one of the major
214 actinopterygian lineages, it is impossible to compare cytogenetic results with other species of

215 amiiform fishes. Instead, we can discuss possible cytotaxonomic interrelationships of bowfin
216 with other major actinopterygian groups based on available data.

217

218 *Cytotaxonomic comparison of bowfin with major lineages of Actinopterygii*

219 Actinopterygii includes five major lineages, four of which exhibit long-retained
220 ecomorphologies alongside low species counts, and are thus referred to as „living fossils"
221 (Amiiformes, Polypteriformes, Acipenseriformes, Lepisosteiformes) (Sallan 2014). The fifth
222 lineage, the teleosts, represents the vast majority of extant ray-finned fishes and about half of
223 all living vertebrates (Nelson, 2006; Sallan, 2014). Summary of genome and chromosome
224 organisation characteristics of major clades of Actinopterygii is given in Table 1.

225 In comparison with other actinopterygian lineages, Polypteriformes and
226 Acipenseriformes significantly differ in genomes, karyotypes and other chromosomal
227 characteristics. These differences are ascribed to separate evolutionary histories of these
228 lineages (Sallan, 2014). Polypterids are among the clades of ray-finned fishes that exhibit a
229 significantly low rate of lineage diversification (Near et al., 2014). Recent phylogenies shows
230 they diverged as the first branch of the actinopterygian stem at least 390 MYA and hence are
231 placed as sister group to all other lineages (Near et al., 2012; Betancur-R et al., 2013;
232 Broughton et al., 2013). Polypterids exhibits the lowest chromosomal counts among other
233 non-teleost actinopterygians and at the same time huge chromosome sizes. All analyzed
234 bichirs and ropefish (Morescalchi et al., 2011 and references therein) possess 2n = 36, except

235 *P. weeksii* where 2n = 38 was likely caused by a centric fission of one metacentric
236 chromosome and *P. dolloi* with 2n = 68, an apparent case of autopolyploidy without
237 noticeable morphological effects (Vervoort, '80). Except the last mentioned, their karyotypes
238 composed exclusively by bi-armed and extremely large chromosomes. The C-value of the
239 Polypteriformes (up to 7.25 pg; Table 1.; Gregory, 2015).

240 Acipenseriformes represent one of the oldest crown group lineages within ray-finned
241 fishes, dating back to at least the early Jurassic (some 200 - 175 MYA; Sallan, 2014). This
242 group, together with gars and bowfin, diverged from teleosts before the teleost specific whole
243 genome duplication (TGD) (Volff, 2005; Froschauer et al., 2006; Amores et al., 2011).
244 Molecular phylogenies of Acipenseriformes (Ludwig et al., 2001; Peng et al., 2007; Fontana,
245 2016; Havelka et al., 2016), and recently also genomic studies (Crow et al., 2012), have
246 documented several independent whole genome duplication (WGD) events. Unlike all other
247 actinopterygian genomes, acipenseriforms have karyotypes composed of macrochromosomes
248 and numerous dot-like microchromosomes of gradually decreasing size (Table 1).
249 Acipenseriformes represent lineage with large genome sizes which vary according to ploidy
250 level from 1,9 – 9,3 pg in *Acipenser* and 1,6 – 2,4 pg in *Polyodon* (Gregory, 2015).

251 The evolutionary history of the remaining ray-finned lineages, i.e. bowfin, gars and
252 teleosts is more complicated, causing the "gar – bowfin – teleost" problem. It is important to
253 note that extant species numbers of these lineages do not reflect their past diversity and all
254 molecular phylogenetic studies are based only on the subset of living species. All three

255 lineages diverged in the Triassic and were widely distributed during the Mesozoic era (Cavin,
256 2010; Grande, 2010; López-Arbarello, 2012). Whereas teleosts underwent their whole
257 genome duplication during the first 100 million years of the Mesozoic era, and have
258 subsequently speciated at great rates, the diversity of Amiids and Lespisosteids has been lost
259 over time (Grande, 2010). Nowadays teleosts represents vast majority of living ray-finned
260 fishes with more than 30 000 extant species, i.e. 96% of all extant fishes (Nelson, 2006), with
261 many new species being described each year (Eschmeyer and Fricke, 2015). However, extant
262 gars are represented by two genera, *Atractosteus* and *Lepisosteus*, and seven species (Nelson,
263 2006), and bowfin is the only survivors of Amiids (Cavin, 2010; Grande, 2010). Hence, a
264 detailed cytogenetic analysis of gars - bowfin - teleost lineages is a relevant and desirable
265 approach to address the Holostei-Halecostomi issue.

266 The bowfin 2n = 46 is comparable to the hypothesized ancestral teleost protokaryotype
267 after the TGD (2n = 48) (Jaillon et al., 2004; Kohn et al., 2006; Kasahara et al., 2007; but see
268 also Nakatani et al., 2007) which is retained in approximately 50% of examined teleost taxa
269 (Mank and Avise, 2006; Arai, 2011). However, the karyotype is known in less than 12% of
270 existing teleost diversity (Arai, 2011) and the available data document enormous genome
271 diversity with chromosome numbers spanning from 2n = 12 in deepwater Spark anglemouth
272 *Stigmops (Gonostoma) bathyphylum* (Gonostomatidae, Stomiiformes) (Post, '74) to 2n = ~
273 446 in schizothoracine species *Ptychobarbus dipogon,* (Cyprinidae, Cypriniformes) (Yu and
274 Yu, '90). Lepisosteiformes represent higher chromosomal counts than bowfin and teleosts.

275 The tropical gar (2n = 56) and the spotted gar (2n = 58) demonstrates difference in 2n by a
276 fusion/fission event of one chromosome pair Symonová et al. (*in this issue*). Since gars did
277 not undergo the TGD (Amores et al., 2011) their higher chromosomal counts related to teleost
278 were explained by chromosome fusions in the lineage leading to teleosts after divergence
279 from gar but before the TGD (Braasch et al., 2016). Although genome size of bowfin, gars
280 and teleosts are similar (Table 1), it is not a suitable marker to resolve their phylogenetic
281 relations because it can be affected e.g. by presence of repetitive elements or regional
282 duplications. Comparable C-value would suggest a possible independent WGD in Amiids but
283 this option have been already excluded, based on gene content analyses (e.g. Crow et al.,
284 2006; Mulley et al., 2006; Hurley et al., 2007; Braasch et al., 2016; Pasquier et al., 2016).
285 Another possibility is that chromosome fusions occurred in the lineage leading to bowfin but
286 additional analyses e.g. bowfin chromosome-level genome assembly will be necessary to test
287 this hypothesis.

288 Regarding morphology of chromosomes, bowfin and teleosts share the same type of
289 karyotypes while the gar karyotype consists of macrochromosomes together with very small
290 chromosomes. Considering the presence of very small chromosomes and conserved synteny
291 in chromosome content between gar and chicken as an ancestral vertebrate condition, their
292 absence in bowfin suggests that its karyotype is more derived compared to the gar lineage
293 (Braasch et al., 2016). Moreover, Symonová et al. (*in this issue*) demonstrated that gars and
294 bowfin differ in intrachromosomal organization. The application of cytogenetic approaches

295 and bioinformatics analyses revealed genome AT/GC heterogeneity in spotted gar genome
296 (Symonová *et al., in this issue*). On the other hand, both bowfin and teleosts demonstrate an
297 overall balanced proportion of AT/GC with the exception of extremely GC-rich NOR sites.

298 The teleost-resembling pattern of cytogenetic characteristics in bowfin may be
299 explained by an example of homoplasy, i.e. convergence between teleosts and *Amia*. Other
300 option, i.e. a sister group relationship between *Amia* and teleosts, i.e, the monophyletic
301 lineage Halecostomi as proposed from some morphological analyses and potentially obscured
302 by TGD in teleosts is in strong contrast with the molecular consensus now coalescing around
303 the nested Holostei–Teleostei topology (Sallan, 2014; Braasch et al., 2016).

304

305 **Conclusion**

306 In conclusion, our study partly confirmed cytogenetic results of previous analyses and
307 extended the description of bowfin chromosomes using available cytogenetic protocols. Our
308 cytogenetic data clearly document that chromosomal evolution of basal Actinopterygii led to
309 different genome organisation, genome size, and chromosome number among extant lineages.
310 Despite the fact that recent phylogenomic analyses support the monophyletic relationship of
311 the spotted gar and bowfin, cytogenetically the bowfin strongly resembles the teleost pattern
312 of genome organization, a phenomenon also observed for several morphological
313 characteristics.

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References

Amores A, Catchen J, Ferrara A, Fontenot Q, Postlethwait JH. 2011. Genome evolution and meiotic maps by massively parallel DNA sequencing: spotted gar, an outgroup for the teleost genome duplication. Genetics 188:799–808.

Arai R. 2011. Fish karyotypes: a check list. 2011 edition. Tokyo ; New York: Springer.

Betancur-R R, Broughton RE, Wiley EO, Carpenter K, López JA, Li C, Holcroft NI, Arcila D, Sanciangco M, Cureton Ii JC, Zhang F, Buser T, Campbell MA, Ballesteros JA, Roa-Varon A, Willis S, Borden WC, Rowley T, Reneau PC, Hough DJ, Lu G, Grande T, Arratia G, Ortí G. 2013. The tree of life and a new classification of bony fishes. PLoS Curr 5. ecurrents.tol.53ba26640df0ccaee75bb165c8c26288.

Braasch I, Gehrke AR, Smith JJ, Kawasaki K, Manousaki T, Pasquier J, Amores A, Desvignes T, Batzel P, Catchen J, Berlin AM, Campbell MS, Barrell D, Martin KJ, Mulley JF, Ravi V, Lee AP, Nakamura T, Chalopin D, Fan S, Wcisel D, Cañestro C, Sydes J, Beaudry FEG, Sun Y, Hertel J,

334 Beam MJ, Fasold M, Ishiyama M, Johnson J, Kehr S, Lara M, Letaw JH, Litman GW, Litman
335 RT, Mikami M, Ota T, Saha NR, Williams L, Stadler PF, Wang H, Taylor JS, Fontenot Q, Ferrara
336 A, Searle SMJ, Aken B, Yandell M, Schneider I, Yoder JA, Volff J-N, Meyer A, Amemiya CT,
337 Venkatesh B, Holland PWH, Guiguen Y, Bobe J, Shubin NH, Di Palma F, Alföldi J, Lindblad-
338 Toh K, Postlethwait JH. 2016. The spotted gar genome illuminates vertebrate evolution and
339 facilitates human-teleost comparisons. Nat Genet 48:427–437.

340 Broughton RE, Betancur-R. R, Li C, Arratia G, Ortí G. 2013. Multi-locus phylogenetic analysis
341 reveals the pattern and tempo of bony fish evolution. PLoS Curr 5.
342 10.1371/currents.tol.2ca8041495ffafd0c92756e75247483e.

343 Cavin L. 2010. Diversity of Mesozoic semionotiform fishes and the origin of gars (Lepisosteidae).
344 Naturwissenschaften 97:1035–1040.

345 Crête-Lafrenière A, Weir LK, Bernatchez L. 2012. Framing the Salmonidae family phylogenetic
346 portrait: a more complete picture from increased taxon sampling. PLoS ONE 7:e46662.

347 Crow KD, Smith CD, Cheng J-F, Wagner GP, Amemiya CT. 2012. An independent genome
348 duplication inferred from Hox paralogs in the American paddlefish - a representative basal ray-
349 finned fish and important comparative reference. Genome Biol Evol 4:937–953.

350 Crow KD, Stadler PF, Lynch VJ, Amemiya C, Wagner GP. 2006. The "fish-specific" Hox cluster
351 duplication is coincident with the origin of teleosts. Mol Biol Evol 23:121–136.

352 Eschmeyer WN, Fricke R. 2015. Catalog of fishes: Genera, species, references. Zootaxa 3882:1.

353 Faircloth BC, Sorenson L, Santini F, Alfaro ME. 2013. A phylogenomic perspective on the
354 radiation of ray-finned fishes based upon targeted sequencing of ultraconserved elements (UCEs).
355 PLoS ONE 8:e65923.

356 Fontana F. 2016. Geneweb. Available from: http://www.unife.it/dipartimento/biologia-
357 evoluzione/progetti/geneweb

358 Froschauer A, Braasch I, Volff J-N. 2006. Fish genomes, comparative genomics and vertebrate
359 evolution. Curr Genomics 7:43–57.

360 Fujiwara A, Nishida-Umehara C, Sakamoto T, Okamoto N, Nakayama I, Abe S. 2001. Improved
361 fish lymphocyte culture for chromosome preparation. Genetica 111:77–89.

362 Grande L. 2010. An empirical synthetic pattern study of gars (Lepisosteiformes) and closely
363 related species, based mostly on skeletal anatomy the resurrection of Holostei. Lawrence, Kan:
364 American Society of Ichthyologists and Herpetologists.

365 Grande L, Bemis WE. 1998. A Comprehensive Phylogenetic Study of Amiid fishes (Amiidae)
366 based on comparative skeletal anatomy. an empirical search for interconnected patterns of natural
367 history. J Vertebr Paleontol 18:1–696.

368 Gregory TR. 2015. Animal genome size database. Available from: http://www.genomesize.com

369 Havelka M, Bytyutskyy D, Symonová R, Ráb P, Flajšhans M. 2016. The second highest
370 chromosome count among vertebrates is observed in cultured sturgeon and is associated with
371 genome plasticity. Genet Sel Evol 48:12.

372 Howell WM, Black DA. 1980. Controlled silver-staining of nucleolus organizer regions with a
373 protective colloidal developer: a 1-step method. Experientia 36:1014–1015.

374 Hurley IA, Mueller RL, Dunn KA, Schmidt EJ, Friedman M, Ho RK, Prince VE, Yang Z,
375 Thomas MG, Coates MI. 2007. A new time-scale for ray-finned fish evolution. Proc R Soc Lond
376 B Biol Sci 274:489–498.

377 Inoue JG, Miya M, Tsukamoto K, Nishida M. 2003. Basal actinopterygian relationships: a
378 mitogenomic perspective on the phylogeny of the "ancient fish." Mol Phylogenet Evol 26:110–
379 120.

380 Jaillon O, Aury J-M, Brunet F, Petit J-L, Stange-Thomann N, Mauceli E, Bouneau L, Fischer C,
381 Ozouf-Costaz C, Bernot A, Nicaud S, Jaffe D, Fisher S, Lutfalla G, Dossat C, Segurens B,
382 Dasilva C, Salanoubat M, Levy M, Boudet N, Castellano S, Anthouard V, Jubin C, Castelli V,
383 Katinka M, Vacherie B, Biémont C, Skalli Z, Cattolico L, Poulain J, de Berardinis V, Cruaud C,
384 Duprat S, Brottier P, Coutanceau J-P, Gouzy J, Parra G, Lardier G, Chapple C, McKernan KJ,
385 McEwan P, Bosak S, Kellis M, Volff J-N, Guigó R, Zody MC, Mesirov J, Lindblad-Toh K,
386 Birren B, Nusbaum C, Kahn D, Robinson-Rechavi M, Laudet V, Schachter V, Quétier F, Saurin
387 W, Scarpelli C, Wincker P, Lander ES, Weissenbach J, Roest Crollius H. 2004. Genome
388 duplication in the teleost fish *Tetraodon nigroviridis* reveals the early vertebrate proto-karyotype.
389 Nature 431:946–957.

390 Kasahara M, Naruse K, Sasaki S, Nakatani Y, Qu W, Ahsan B, Yamada T, Nagayasu Y, Doi K,
391 Kasai Y, Jindo T, Kobayashi D, Shimada A, Toyoda A, Kuroki Y, Fujiyama A, Sasaki T, Shimizu

392 A, Asakawa S, Shimizu N, Hashimoto S-I, Yang J, Lee Y, Matsushima K, Sugano S, Sakaizumi
393 M, Narita T, Ohishi K, Haga S, Ohta F, Nomoto H, Nogata K, Morishita T, Endo T, Shin-I T,
394 Takeda H, Morishita S, Kohara Y. 2007. The medaka draft genome and insights into vertebrate
395 genome evolution. Nature 447:714–719.

396 Kohn M, Högel J, Vogel W, Minich P, Kehrer-Sawatzki H, Graves JAM, Hameister H. 2006.
397 Reconstruction of a 450-My-old ancestral vertebrate protokaryotype. Trends Genet TIG 22:203–
398 210.

399 Komiya H, Takemura S. 1979. Nucleotide sequence of 5S ribosomal RNA from rainbow trout
400 (*Salmo gairdnerii*) liver. J Biochem (Tokyo) 86:1067–1080.

401 Levan A, Fredga K, Sandberg AA. 1964. Nomenclature for centromeric position on
402 chromosomes. Hereditas 52:201–220.

403 López-Arbarello A. 2012. Phylogenetic interrelationships of ginglymodian fishes (Actinopterygii:
404 Neopterygii). PLoS ONE 7:e39370.

405 Ludwig A, Belfiore NM, Pitra C, Svirsky V, Jenneckens I. 2001. Genome duplication events and
406 functional reduction of ploidy levels in sturgeon (*Acipenser*, *Huso* and *Scaphirhynchus*). Genetics
407 158:1203–1215.

408 Macqueen DJ, Johnston IA. 2014. A well-constrained estimate for the timing of the salmonid
409 whole genome duplication reveals major decoupling from species diversification. Proc R Soc B-
410 Biol Sci 281:20132881.

411 Mank JE, Avise JC. 2006. Phylogenetic conservation of chromosome numbers in
412 Actinopterygiian fishes. Genetica 127:321–327.

413 Morescalchi MA, Stingo V, Capriglione T. 2011. Cytogenetic analysis in *Polypterus ornatipinnis*
414 (Actinopterygii, Cladistia, Polypteridae) and 5S rDNA. Mar Genomics 4:25–31.

415 Mulley JF, Chiu C, Holland PWH. 2006. Breakup of a homeobox cluster after genome duplication
416 in teleosts. Proc Natl Acad Sci 103:10369–10372.

417 Nakatani Y, Takeda H, Kohara Y, Morishita S. 2007. Reconstruction of the vertebrate ancestral
418 genome reveals dynamic genome reorganization in early vertebrates. Genome Res 17:1254–1265.

419 Near TJ, Dornburg A, Tokita M, Suzuki D, Brandley MC, Friedman M. 2014. Boom and bust:
420 ancient and recent diversification in bichirs (Polypteridae: Actinopterygii), a relictual lineage of
421 ray-finned fishes. Evolution 68:1014–1026.

422 Near TJ, Eytan RI, Dornburg A, Kuhn KL, Moore JA, Davis MP, Wainwright PC, Friedman M,
423 Smith WL. 2012. Resolution of ray-finned fish phylogeny and timing of diversification. Proc Natl
424 Acad Sci USA 109:13698–13703.

425 Nelson GJ. 1969. Origin and diversification of teleostean fishes. Ann N Y Acad Sci 167:18–30.

426 Nelson JS. 2006. Fishes of the world. Fourth edition. Nelson, Joseph S.; Department of Biological
427 Sciences, University of Alberta, Edmonton, Alberta T6G 2E9, Canada, Canada.: John Wiley &
428 Sons, Inc.

429 Ohno S, Muramoto J, Stenius C, Christian L, Kittrell WA, Atkin NB. 1969. Microchromosomes
430 in holocephalian, chondrostean and holostean fishes. Chromosoma 26:35–40.

431 Pasquier J, Cabau C, Nguyen T, Jouanno E, Severac D, Braasch I, Journot L, Pontarotti P, Klopp
432 C, Postlethwait JH, Guiguen Y, Bobe J. 2016. Gene evolution and gene expression after whole
433 genome duplication in fish: the PhyloFish database. BMC Genomics 17:368.

434 Peng Z, Ludwig A, Wang D, Diogo R, Wei Q, He S. 2007. Age and biogeography of major clades
435 in sturgeons and paddlefishes (Pisces: Acipenseriformes). Mol Phylogenet Evol 42:854–862.

436 Pessia E, Popa A, Mousset S, Rezvoy C, Duret L, Marais GAB. 2012. Evidence for Widespread
437 GC-biased Gene Conversion in Eukaryotes. Genome Biol Evol 4:787–794.

438 Phillips R, Ráb P. 2001. Chromosome evolution in the Salmonidae (Pisces): an update. Biol Rev
439 Camb Philos Soc 76:1–25.

440 Pokorná M, Rens W, Rovatsos M, Kratochvíl L. 2014. A ZZ/ZW sex chromosome system in the
441 thick-tailed Gecko (*Underwoodisaurus milii*; Squamata: Gekkota: Carphodactylidae), a member
442 of the ancient gecko lineage. Cytogenet Genome Res 142:190–196.

443 Post A. 1974. Chromosomes of tree species of family Gonostomatidae (Osteichthyes,
444 Stomiatoidei). In: Results of research cruises of FRV "Walther Hertwig" to South-America.
445 XXXIV. Vol. 25. . p 51–55.

446 Saitoh K, Chen W-J, Mayden RL. 2010. Extensive hybridization and tetrapolyploidy in spined
447 loach fish. Mol Phylogenet Evol 56:1001–1010.

448 Sallan LC. 2014. Major issues in the origins of ray-finned fish (Actinopterygii) biodiversity. Biol
449 Rev 89:950–971.

450 Šlechtová V, Bohlen J, Tan HH. 2007. Families of Cobitoidea (Teleostei; Cypriniformes) as
451 revealed from nuclear genetic data and the position of the mysterious genera *Barbucca*,
452 *Psilorhynchus*, *Serpenticobitis* and *Vaillantella*. Mol Phylogenet Evol 44:1358–1365.

453 Sola L, Rossi AR, Iaselli V, Rasch EM, Monaco PJ. 1992. Cytogenetics of bisexual/unisexual
454 species of Poecilia. II. Analysis of heterochromatin and nucleolar organizer regions in *Poecilia*
455 *mexicana mexicana* by C-banding and DAPI, quinacrine, chromomycin A3, and silver staining.
456 Cytogenet Cell Genet 60:229–235.

457 Soltis P, Soltis DE. 2012. Polyploidy and Genome Evolution. Springer Science & Business
458 Media.

459 Sumner AT. 1972. A simple technique for demonstrating centromeric heterochromatin. Exp Cell
460 Res 75:304–306.

461 Suzuki A, Hirata J. 1991. Chromosomes and DNA contents of *Amia calva*. Chromosome Inf Serv
462 50:34–37.

463 Symonová R, Sember A, Majtánová Z, Ráb P. 2015. Characterization of fish genomes by GISH
464 and CGH. In: Ozouf-Costaz C, Pisano E, Foresti F, de Almeida L, editors. Fish Cytogenetic
465 Techniques. CRC Press. p 118–131. Available from:
466 http://www.crcnetbase.com/doi/10.1201/b18534-17

467 Taylor MI, Alexandrou MA. 2012. Whole genome duplication accelerates speciation in
468 Corydoradinae catfishes. In: ICPHB 2012 – International Conference on Polyploidy,
469 Hybridization and Biodiversity. Průhonice, Czech Republic. p S1-2, 7–10.

470 Uyeno T, Smith G. 1972. Tetraploid origin of karyotype of Catostomid fishes. Science 175:644-.

471 Vervoort A. 1980. Karyotypes and nuclear-DNA contents of Polypteridae (Osteichthyes).
472 Experientia 36:646–647.

473 Volff JN. 2005. Genome evolution and biodiversity in teleost fish. Heredity 94:280–294.

474 White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal
475 ribosomal RNA genes for phylogenetics. In: Innis M, Gelfand J, Sninsky J, White T, editors. PCR
476 Protocols: a Guide to Methods and Applications. Orlando, Frorida: Academic Press. p 315–322.

477 Yang L, Sado T, Hirt MV, Pasco-Viel E, Arunachalam M, Li J, Wang X, Freyhof J, Saitoh K,
478 Simons AM, Miya M, He S, Mayden RL. 2015. Phylogeny and polyploidy: Resolving the
479 classification of cyprinine fishes (Teleostei: Cypriniformes). Mol Phylogenet Evol 85:97–116.

480 Yu X, Yu X. 1990. A schizothoracine fish species *Diptychus dipogon* with a very high number of
481 chromosomes. Chromosome Inf Serv 48:17–18.

482

Tables

Table 1. Summary of traits and factors involved in genome and chromosomes organisation of Actinopterygia

Group	2n chromosome counts (basic features)	Micro-chromosomes	CG heterogeneity	Whole genome duplication (WGD) after R1 and R2	C-value/haploid DNA content (pg) www.genomesize.com	Specific features in the genome history and chromosomal evolution
Acipenseriformes (sturgeons, paddlefish)	~ 120 – 240 – 360	YES ~ 50%	only NOR	multiple in sturgeons, one in paddlefish	*Acipenser* 1.8 – 9.3 *Polyodon* 1.6 – 2.4	multiple WGD, ploidy diversity
Lepisosteiformes (gars)	56 – 58	small sized chromosomes	in both extant genera	not observed	*Atractosteus* 1.2 *Lepisosteus* 1.4	regionally high recombination rate **
Amiiformes (bowfin)	46	NO	only NOR	not observed	*Amia calva* 1.2	convergent genome evolution with teleosts?
Polypteriformes (bichirs)	36 – 38 biarmed, extremelly large	NO	only NOR	not observed	*Erpetoichthis* 4.5 *Polypterus* 3.6 – 7.2	not investigated
Teleostei	~ 50 (exceptionally up to 100 – 150)	Micro B-chromosomes	only NOR	TGD and lineage specific WGDs	0.4 – ~ 1.0 for exceptions see *	from genome compaction to lineage specific WGD

* lineages with undergone WGD: Salmoniformes, Cypriniformes, Synbranchiformes, Siluriformes (Soltis and Soltis, 2012)

** Pessia et al. (2012)

Figure legends

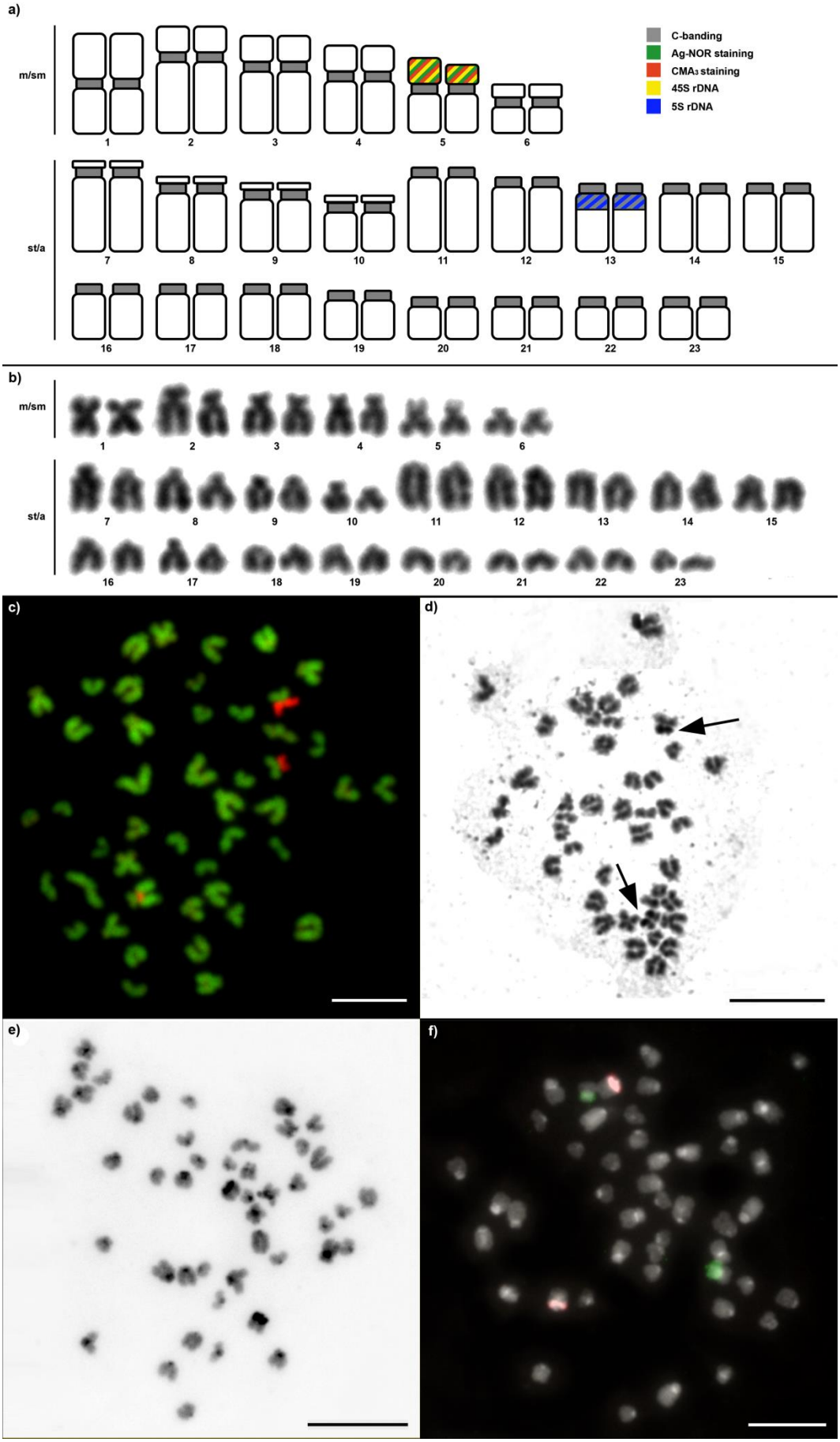
Fig. 1. Cytogenetics of *Amia calva*

(a) Schematic representations of chromosomes after cytogenetic experiments: C-banded chromosomal regions are marked by grey color; active NORs visualized by Ag-NOR staining are marked by green color; CMA3-stained regions are marked by red color; rDNA signals are marked by yellow (45S rDNA) and blue (5S rDNA). m/sm – metacentric-submetacentric, st/a

– subtelocentric-acrocentric chromosomes. (b) Representative karyotype arranged from Giemsa stained chromosomes. (c) CDD-stained chromosomes. The CMA3 signal was inserted from green into red and the DAPI signal from blue into the green channel to enhance the contrast between these signals. (d) Ag-NOR stained chromosomes; (e) C- banded chromosomes. The C-banding pattern was counterstained with DAPI and inverted; (f) Metaphase chromosomes after FISH with 45S rDNA (green signals) and 5S rDNA (red signals) probes. The DAPI-counterstaining signal was reverted from blue to grey to enhance the contrast. Particular images were adjusted separately before assembling the plate. Bars equal 5 µm.

Author Contributions

PR designed the study and co-drafted MS. RS and ZM designed the study, performed cytogenetic analyses and co-drafted the manuscript. LS provided the phylogenetic and paleobiological context and co-drafted the manuscript together with LAR. LAR participated on cytogenetic analyses and provided chromosome preparations. All co-authors revised the text and agreed the final version.


a)
m/sm
st/a
C-banding
Ag-NOR staining
CMA3 staining
45S rDNA
5S rDNA
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b)
m/sm
st/a
c)
d)
e)
f)

7.3. Publication III

Symonová R., **Majtánová Z.**, Arias-Rodriguez L., Mořkovský L., Kořínková T., Cavin L., Johnson Pokorná M., Doležálková M., Flajšhans M., Normandeau E., Ráb P., Meyer A., Bernatchez L. 2016. Unique genome organisation in gars (Lepisosteidae: Actinopterygii) shows more similarities to mammals than to any other ray-finned fishes. Journal of Experimental Zoology Part B: Molecular and Develomental Evolution. Invited manuscript to the special issue „Genome of the spotted gar". IF_{2015}: 2.083

Author contribution: chromosome preparation, karyotype analyses, C-banding, G-banding, CMA_3 staining, image processing, analysing of the data, co-drafting the manuscript

I declare that the contribution of Zuzana Majtánová to the publication Symonová et al. (2016), as stated here, is true.

25.10.2016
date

first author's signature

Genome Compositional Organization in Gars Shows More Similarities to Mammals than to Other Ray-Finned Fish

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This document contains: 4 text figures, 0 graphs, and 0 charts

Abbreviated title: Cytogenomics of gars

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Abstract

Genomic GC content can vary locally and GC-rich regions are usually associated with increased DNA thermostability in thermophilic prokaryotes and warm-blooded eukaryotes. Among vertebrates, fish and amphibians appeared to possess a distinctly less heterogeneous AT/GC organization in their genomes, whereas cytogenetically detectable GC heterogeneity has so far only been documented in mammals and birds. The subject of our study is the gar, an ancient "living fossil" of a basal ray-finned fish lineage, known from the Cretaceous period. We carried out cytogenomic analysis in two gar genera (*Atractosteus* and *Lepisosteus*) uncovering a GC chromosomal pattern uncharacteristic for fish. Bioinformatic analysis of the spotted gar (*Lepisosteus oculatus*) confirmed a GC compartmentalization on GC profiles of linkage groups. This indicates a rather mammalian mode of compositional organization on gar chromosomes. Gars are thus the only analysed extant ray-finned fishes with a GC compartmentalized genome. Since gars are cold-blooded anamniotes, our results contradict the generally accepted hypothesis that the phylogenomic onset of GC compartmentalization occurred near the origin of amniotes. Eco-physiological findings of other authors indicate a metabolic similarity of gars with mammals. We hypothesize that gars might have undergone convergent evolution with the tetrapod lineages leading to mammals on both metabolic and genomic levels. Their metabolic adaptations might have left footprints in their compositional genome evolution, as proposed by the metabolic rate hypothesis. The genome organization described here in gars sheds new light on the compositional genome evolution in vertebrates generally and contributes to better understanding of the complexities of the mechanisms involved in this process.

Key words: basal actinopterygians, genome evolution, compositional heterogeneity, *Lepisosteus*, *Atractosteus*.

Data archival location: https://github.com/libor-m/vertebrate-GC

66 **Introduction**

67 Genomic compositional architecture is a complex and non-random organization of DNA
68 bases (AT/GC). Vertebrate genomes can be described as mosaics of compositionally
69 homogenous DNA domains (sometimes called isochores) with a distinct GC content
70 (Bernardi, 2005). So called "light" domains with lower GC proportions represent the late-
71 replicating gene-poor genome "desert" and "heavy" domains with higher GC proportions
72 represent the early-replicating gene-rich genome "core" (Bernardi, 2005). Ray-finned fish
73 genomic architecture with a narrower range in GC proportions differs considerably from
74 mammalian architecture where a broad range of GC proportions occurs (Costantini et al,
75 2007). Genomes of hundreds of vertebrates, including about 300 fish species, were
76 investigated for genomic base composition (Bernardi and Bernardi, '90). However, as gars
77 were not covered in this work, we have therefore analysed the genome and chromosomes of
78 the spotted gar (*Lepisosteus oculatus*) and the chromosomes of the tropical gar (*Atractosteus*
79 *tropicus*).

80 Extant gars (Lepisosteiformes) are represented by only seven species divided into two
81 genera – *Atractosteus* and *Lepisosteus* (Nelson, 2006; Fig. 1) and are the only survivors of an
82 early radiation of ray-finned fishes highly diversified and widely distributed in the Mesozoic
83 (Cavin, 2010; López-Arbarello, 2012). Gars are believed to be sister to another ancient
84 lineage, Amiiformes, represented by a single extant species, the bowfin (*Amia calva*) (Grande,
85 2010). Together with other "ancient fishes" *sensu* Inoue et al. (2003), gars are sometimes
86 referred to as "living fossils" since they do not appear to have undergone many apparent
87 morphological changes during the last 100 million years (Wright et al, 2012; Sallan, *pers.*
88 *comm.*). Gars and bowfin did not go through any further whole genome duplications (WGD)
89 after the two basal rounds of vertebrate genome duplications (VGD1+2/R1+2) that followed
90 the origin of vertebrates (Fig. 1; Meyer and Malaga-Trillo, '99; Vandepoele et al, 2004). They

91 thus exemplify one of the basal "non-teleost" actinopterygian lineages that branched off the
92 stem lineage before the teleost specific WGD (TGD; Crow et al, 2006) and can therefore
93 serve as an outgroup for exploring evolutionary mechanisms associated with TGD (Amores et
94 al, 2011; Braasch et al, 2016). Eco-physiologically, gars embody a derived group highly
95 specialized to extreme conditions (low oxygen and high temperature environments) with a
96 metabolic organization that is unique among fishes in their heightened capacity to synthesize
97 and oxidise glucose, heightened capacity for anaerobic glycolysis and the low number of
98 oxidative enzymes in gar tissues (Frick et al, 2007). The metabolic organization of their air-
99 breathing organ resembles that of lungs more than that of the swim bladder of most teleosts
100 (Frick et al, 2007). The spotted gar gene expression pattern exhibits many similarities with
101 those of tetrapod genomes (Braasch et al, 2014) and has been utilized to link teleost models
102 influenced by the TGD with the human genome in disease research (Amores et al, 2011;
103 Braasch et al, 2014, 2016).

104 There has been only limited investigation into gar chromosomes so far, with
105 predominantly just basic karyotypic characteristics and GC-rich ribosomal DNA (rDNA) sites
106 being identified (Ráb et al, '99), along with the very recent research of Braasch et al. (2016)
107 which uncovered the striking conservation between chromosomes of the spotted gar and the
108 chicken. Braasch et al found that the karyotypes of these two species differed by merely 17
109 large fissions, fusions or translocations (Braasch et al, 2016).

110 Independently, rDNA sites in Eukaryotes are generally known as regions of substantial
111 GC enrichment in association with GC-biased gene conversion (Escobar et al, 2011). The
112 genome GC content was initially investigated using analytical centrifugation (Thiery et al,
113 '76). From these studies, later supplemented with genomics, a generally accepted concept
114 arose about the genomic GC compositional heterogeneity in birds and mammals and a
115 substantially less heterogeneous GC organization in fishes and amphibians, with transitional

116 states in reptiles (reviewed in Bernardi, 2005). A "Thermodynamic stability hypothesis" ~~as~~
117 proposed to explain this phenomenon states that the GC compositional heterogeneity was an
118 adaptation to homeothermy since higher GC levels stabilize coding DNAs along with their
119 RNAs and proteins (Costantini et al, 2009).

120 We have combined cytogenetics and bioinformatics to obtain cytogenomic insights into
121 the organization of the gar genome and analysed it further by comparing gars with other
122 vertebrates. In both gar genera, we found GC genome heterogeneity that is uncharacteristic
123 for cold-blooded vertebrates. This was detected bioinformatically and cytogenetically in *L.*
124 *oculatus* and cytogenetically in *A. tropicus*. This led us to re-assess the applicability of the
125 conventional cytogenetic method of G-banding in fishes, a technique routinely working so far
126 only in mammals and birds. G-banding visualizes the alteration of gene-rich, early-replicating
127 and gene-poor, late-replicating chromosome regions *sensu* Bernardi (2005). A comparable
128 banding pattern can be obtained with AT-/GC-specific fluorochromes and replication
129 banding, both of which are less prone to interference by artefacts (Sumner, '90). The
130 unavailability of G-banding in lower vertebrates represents a serious obstacle to the study of
131 their cytogenetics, as it makes it impossible to properly identify homologous chromosomes,
132 which is one of the basic prerequisites for all down-stream cytogenetic analyses. There are
133 reports showing attempts with "G-banding" in fishes and other lower vertebrates but these
134 concluded that the banding pattern is irreproducible and incomparable with patterns routinely
135 produced in mammals (e.g. Medrano et al, '88; Ueda & Naoi, '99; Romanenko et al, 2015; this
136 study). Genuine G-banding has been reported to work in eels (Coluccia et al, 2010), cyprinids
137 (Luo, '98), tonguefish (Zhuang et al, 2006) and even in one lancelet species (Wang et al,
138 2004). However, these results have never been confirmed with any fluorochrome or
139 replication banding. Therefore, since G-banding provides a reproducible pattern in both gar
140 genera, and genomic data of several basal vertebrates and one lancelet species are available,

we could resolve this issue. We analysed GC profiles in teleosts; zebrafish, representing a GC homogenized teleost genome (Costantini et al, 2007), stickleback and pufferfish, both representing more GC heterogeneous genomes as reported by Costantini et al, 2007. Analysis of GC profiles among linkage groups elucidated the relationship between the GC distribution at the sequence and the chromosomal level.

A link between environment, metabolic rate and genomic GC content has recently been established (e.g. Chaurasia et al, 2011, 2014; Tarallo et al, 2016). Gars, with their physiological adaptations to extreme temperatures and low oxygen conditions (Frick et al, 2007) combined with their peculiar genome organization reported here, represent an excellent model system to bring new insights into this long-lasting and multi-level effort. Namely, the major evolutionary forces driving the GC content variation within and among genomes and the compositional differences between cold- and warm-blooded vertebrates still remained unclear. The GC heterogeneity across the genome results from complex interactions and can be investigated through various techniques involving cytogenetics, eco-physiology, molecular evolution, (phylo)genomics, bioinformatics, regulation of gene expression, DNA methylation and spatial distribution of DNA in interphase nuclei (Mugal et al, 2015a; Varriale 2014).

Materials and Methods

Blood culturing and Chromosome Preparation

Details on specimen acquisition are listed in the Supporting Information.

Leucocytes from blood samples of *A. tropicus*, *L. oculatus*, *A. calva, Polypterus ornatipinnis, Polypterus lapradei* were cultivated and chromosome spreads were prepared according to the protocol of Fujiwara et al. (2001) with some modifications. Briefly, 0.2 – 0.5 ml of blood was collected from an anesthetized fish by puncturing the *vena cava caudalis* using a heparinized

165 syringe. The leucocyte-rich plasma was used to set up primary cultures in 5 ml Medium 199
166 (SIGMA, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FBS Superior,
167 Biochrom, Berlin, Germany),1% Antibiotic Antimycotic Solution (SIGMA), LPS from *E. coli*
168 (0.5 mg per 5 ml medium), PHA-P (90 µg per 5 ml medium, (Remel, Lenexa, KS, USA),
169 Kanamycin (0.3 mg per 5 ml medium (SIGMA) and 0.175 µl 10% mercaptoethanol
170 (SIGMA). After 120 hours of incubation at 20°C, 5 ml cultures were harvested using standard
171 colchicine (two drops of 0.1% colchicine per 5 ml media) and 0.075 ml KCl hypotonic (8
172 min.) treatments followed by fixation in a freshly prepared fixative (methanol: acetic acid 3:1,
173 v/v) three times. Cell suspensions of *Anolis carolinensis*, *Eremias velox* and *Gallus gallus*
174 were obtained by leucocyte cultivation *sensu* Sohn and Ryu ('99) and Pokorná et al. (2010)
175 with slight modifications. Chromosome preparations of *M. musculus* were obtained from bone
176 marrow *sensu* Ford and Hamerton ('56).

177 All national guidelines for the care and use of animals were followed. This study was
178 covered by the "Valid Animal Use Protocols" Nr. CZ00221 of the Laboratory of Fish
179 Genetics, IAPG, issued by the Czech Ministry of Agriculture; by the valid Mexican
180 permission SAGARPA/CONAPESCA, DGOPA 09004.04111.3088 and by the Animal
181 facilities accreditation of the Faculty of Science, Charles University in Prague, Czech
182 Republic (24773/2008-10001).

183

184 **CMA_3/DAPI (CDD) Staining**

185 Chromomycin A_3 (CMA_3, DNA dye specific for GC-rich regions) and DAPI (AT-specific)
186 fluorescent staining was performed as described by Sola et al. ('92) on metaphases of the
187 following species: *A. tropicus, L. oculatus, A. calva, A. stellatus, R. rutilus, A. anguilla, P.*
188 *ornatipinnis, P. lapradei, P. lessonae, A. carolinensis, E. velox, G. gallus* and *M. musculus*.

189

190 **G- and C-banding**

191 G-banding was performed *sensu* Seabright ('71, '72) on the same chromosome preparations
192 treated with CDD staining to directly compare banding patterns. During G-banding, we tested
193 15 s, 20 s, 30 s of trypsin treatment. G-banding was tested on chromosomes of *Amia calva*,
194 *Acipenser baeri, A. gueldenstaedtii, A. ruthenus and A. stellatus.*

195 C-banding was performed *sensu* Sumner ('90) with the slight modifications described
196 in Pokorná et al. (2014) to determine whether heterochromatin accumulations resemble the G-
197 banding pattern as described previously (Schmid and Guttenbach, '88; Graphodatsky, '89;
198 Holmquist and Ashley, 2006). Chromosomes were counterstained by DAPI to enhance
199 contrast. Microphotographs were taken in the fluorescent regime and inverted. Chromosomes
200 were classified *sensu* Levan et al. ('64). Further details on the molecular cytogenetic and
201 image analyses are listed in the Supporting Information.

202

203 **Bioinformatic Analysis**

204 We calculated GC profiles for each linkage group (LG, are expected to correspond to
205 chromosomes, Braasch et al, 2016) in *L. oculatus* and compared them with GC profiles of
206 *Danio rerio*, *Tetraodon nigroviridis,* and *Gasterosteus aculeatus*. For comparison with
207 representative mammalian genomes we calculated GC profiles of the house mouse and
208 human. The profiles were calculated with a sliding window of 10 kbp, striding the
209 chromosome in 10 kbp steps. Profile plots were made after combining the original data to 100
210 kbp windows. Similarly, a GC profile and a histogram of GC proportions in the lancelet
211 (*Branchiostoma floridae*) were calculated to assess the published G-banding functionality in
212 another lancelet species (Wang et al, 2004). Since no LGs are currently available for any
213 lancelet species, the GC profile was calculated from available genomic fragments. Genome
214 assemblies of analysed species were downloaded from www.ensembl.org (version published

215 in Flicek et al, 2014), with the exception of the lancelet (Nordberg et al, 2014). In addition for
216 the spotted gar genome, an analysis of GC content in genes versus intergenic regions was
217 conducted. This analysis was performed twice, once with all sequence data and once with
218 transposons and low-complexity regions excluded from the intergenic regions. In order to
219 identify the genomic regions, transposons and low-complexity regions, we relied on
220 annotations provided by ENSEMBL.

221 Genome assemblies were pre-processed using Python, in that A/C/G/T bases were
222 counted for each genome, and a GC histogram of lancelet was calculated. These datasets were
223 then subjected to a GC content analysis performed for each LG separately using R v. 3.0,
224 packages ggplot, dplyr, tidyr (R Development Core Team 2012). All custom generated Python
225 and R scripts are available on the codes repository GitHub (https://github.com/libor-
226 m/vertebrate-GC).

227

228 ## Results

229 ### Alternation of AT- and GC-rich Regions on Gar Chromosomes

230 In *A. tropicus,* the diploid chromosome number was 2n = 56, the chromosomal arms number
231 NF = 90 in both females and males (Fig. 2a). The karyotype was composed of 18 pairs of
232 macrochromosomes and 10 pairs of very small chromosomes (Fig. 2a, c, e, g; marked as
233 st/a*). The chromosomes consisted of 5 pairs of meta- (m), 11 pairs of submeta- (sm) and 12
234 pairs of subtelo- (st) to acrocentric (a) chromosomes (Fig. 2a, c, e, g). In metaphases with less
235 condensed chromosomes after CDD staining, 2 pairs of biarmed and 8 pairs of uniarmed very
236 small chromosomes could be distinguished among the st/a category. There were no detectable
237 differences between males and females.

238 In *L. oculatus*, 2n = 58 in unsexed animals, NF = 92, the karyotype composed of 19
239 pairs of macrochromosomes and 10 pairs of very small chromosomes (Fig. 2b, d, f, h; marked

240 as st/a*). Chromosomes consisted of 4 pairs of metacentrics, 10 pairs of submetacentrics, 14
241 pairs of subtelo- to acrocentric chromosomes (Fig. 2b, d, f, h). In less condensed metaphases
242 after CDD staining, 2 pairs of bi- and 8 pairs of uniarmed very small chromosomes could be
243 distinguished among the st/a category. There were no apparent differences between males and
244 females.

245 The base specific CDD (DAPI/CMA_3) staining of chromosomes of *A. tropicus* and *L.*
246 *oculatus* demonstrated a clear CMA_3^+ banding pattern (GC-rich regions) along with a $DAPI^+$
247 pattern (AT-rich regions). This alternation of AT/GC bands enabled the unambiguously
248 distinguishing between homologous and homeologous chromosomes, excluding the very
249 small (the ten smallest) chromosomes (Fig. 2a, b). Giemsa-stained karyotypes of *A. tropicus*
250 and *L. oculatus* are shown in Figure 2g, h. This banding pattern enabled us to generate
251 ideograms for both species (Fig. S1a, b). Fluorescence *in situ* hybridization (FISH) with
252 rDNA yielded two (*A. tropicus*) or three (*L. oculatus*) signals for 28S rDNA and two signals
253 for 5S rDNA were detected in both species (Fig. S2). Contrary to gars, all other ray-finned
254 fishes included in this analysis showed a "teleost pattern" with homogenously stained
255 chromosomes, i.e. a balanced proportion of AT/GC and GC-rich rDNA sites (Figs. 3c-g). The
256 same pattern was observed in amphibians represented by *P. lessonae* (Fig. 3h). In reptiles, we
257 identified a homogenous pattern in *Anolis carolinensis* (Fig. 3j) and indications of
258 compositional heterogeneity in *Eremias velox* (Fig. 3i). In the chicken, CDD staining yielded
259 mostly homogenously stained macrochromosomes (some with GC^+ regions terminally) and
260 GC-rich microchromosomes (Fig. 3k). In the mouse, the pattern of altering AT^+/GC^+ bands
261 occurred on all chromosomes (Fig. 3l; details are in the Supplementary File).

262

263 **Heterochromatic C-bands do not mimic G-bands**

264 G-banding in gars produced a pattern enabling the karyotyping of chromosomes (Fig. 2c-d).
265 The G-banding pattern corresponds to the CDD pattern - the positive G-bands (dark; *sensu*
266 Sumner, '90) match up with the AT-rich bands and the negative G-bands (pale; *sensu* Sumner
267 '90) match up with the GC-rich bands. No clear G-banding pattern was observed in sturgeons
268 and bowfin (not shown). Further details are in the Supplementary File.

269 C-banding, which is used to visualize constitutive heterochromatin, produced bright
270 signals in the GC-rich centromeres and weaker signals interstitially in *L. oculatus* and *A.*
271 *tropicus* (Fig. 2e-f).

272 Based on our results we can summarize the relationship between the constitutive
273 heterochromatin (the C-bands), G-bands and AT-/GC-rich regions in gars as follows: i) The
274 centromeric constitutive heterochromatin overlaps with the GC-rich centromeric regions but
275 does not overlap with G-bands that are mostly absent in centromeres; ii) The usually
276 terminally located GC-rich regions (Fig. 2, e.g. the 1. and 2. metacentric, the 3. and 4.
277 submetacentric chromosomes) are clearly C-negative as well as G-negative; iii) The
278 interstitial GC-rich bands appear to be present in the vicinity of the C-bands, however this
279 finding needs to be verified by specialized analysis; iv) The G-bands can be found in clearly
280 C-negative, C-positive and slightly C-positive regions; v) The very GC-rich, and also to a
281 large extent heterochromatinized, chromosomal pair is G-positive on the q arm. Details are
282 described in the Supplementary file. This indicates the euchromatic genome occupation of a
283 significant proportion of G-bands in gars similar to the situation in warm-blooded vertebrates
284 (*sensu* Bickmore and Craig, '96). By comparing the results with the CDD patterns we can rule
285 out that the C-bands mimic the G-banding pattern in gars.

286

287 **GC profiles in Genomes of Spotted Gar and other relevant species**

288 GC profiles of LGs of the spotted gar (*L. oculatus)* genome appear to correspond to our
289 cytogenetic results and demonstrate the AT/GC heterogeneity observed along gar
290 chromosomes at the sequence level (Fig. 4a). The GC profiles also enabled the quantification
291 of the qualitative cytogenetic data showing an alternation of GC-rich and GC-poor regions
292 along chromosomes. Bernardi (2005) defines "heavy" GC-rich DNA domains as having >
293 45% GC and "light" domains with 35 - 45% GC (Fig. 4a). The GC-rich regions in *L. oculatus*
294 frequently exceeded 50% GC over DNA regions of several Mbp e.g. in LG1-2, LG4-6, LG8-
295 12 and most of the small chromosomes. The GC-rich and AT-rich regions form discernible
296 compositionally homogenous domains. The AT-rich regions form flattened stretches. The
297 GC-rich regions occur as GC-rich peaks interrupted by sharp decreases in GC content. The
298 GC-rich regions are mostly telomerically but also interstitially situated and correspond to the
299 GC-rich bands after CDD staining. However, it was impossible at this stage to identify
300 exactly which linkage group of the *L. oculatus* genome represents which pair of
301 chromosomes, since there were no identification markers present to link both types of
302 datasets. GC profiles of LGs in the pufferfish with a compact teleost genome and a shift
303 towards a higher GC content show a pattern of very densely distributed fluctuations between
304 40 and 50% GC with terminal (telomeric) sharp narrow increases up to 55-60% of GC (Fig.
305 4b). GC profiles of the stickleback also show a slight shift towards a higher GC content,
306 compositional fluctuations between 40-50% GC, no prominent GC peaks and several regions
307 of profile flattened around 45% GC (Fig. 4c). GC profiles of the zebrafish (*Danio rerio*),
308 representing homogenized teleost genomes, are distinctly flattened between 35-40% GC.
309 Only very narrow peaks reaching up to 50% GC occur in the terminal locations in the
310 majority of LGs (Fig. 4d). In the house mouse and human, the same procedure shows
311 fluctuations in the range of 35 - 55% GC, with the exception of the Y chromosome with a

312 narrow compositional range of around 40 % GC (Figs. 4e-f, S5a-b). Comparable patterns
313 were obtained with window lengths of 10, 250, 500 and 1 000 kbp (not shown).

314 The analysis of GC content in genes versus intergenic regions of the spotted gar and
315 other tested actinopterygian genomes shows that gene-rich regions are more GC-rich in the
316 spotted gar as well as in pufferfish and stickleback, but not in zebrafish (Fig. S4a-d). In the
317 spotted gar, the gene-rich regions contain 25-75% GC, whereas the intergenic regions 0-55%
318 GC. Both the genic and intergenic regions show maximal density at approximately 38% GC
319 in the spotted gar (Fig. S4a). The GC-richness of gene-rich regions is even more pronounced
320 in stickleback and pufferfish (Fig. S4b-c). In both of these species, the maximal density in
321 genic regions shows a shift towards higher GC content when compared with the intergenic
322 regions (Fig. S4b-c).
323 Similar analyses of two lancelet species demonstrated a flattened GC profile (details are
324 shown in Supplementary file and Fig. S5).
325

326 ## Discussion

327 ### Cytogenetically detectable AT/GC Compartmentalization in Gars

328 CDD staining is a versatile and well-understood tool visualizing the global AT/GC
329 distribution in plant and animal chromosomes (Sumner, '90). This is not the case for G-
330 banding where uncertainties about exact interactions between the dye and DNA persist
331 (Sumner, '90). In gars, we have combined both these stainings and excluded that constitutive
332 heterochromatin mimics G-bands by C-banding in both gar species (Fig. 2e-f). This was
333 further supported by bioinformatic analyses showing that GC-rich regions are more gene-rich
334 (Fig. S4). So far, eels were the only exception among fishes with repeatedly reported
335 functioning G-bands (Wiberg, '83; Sola et al, '84). However, our CDD staining in the eel
336 yielded the "typical teleost pattern" i.e. without any GC heterogeneity along chromosomes up

337 to two GC^+ rDNA sites (Fig. 3f). Equilibrium centrifugation in eels also did not show any
338 pattern similar to the GC-heavy isochores (Bernardi, 2005) characteristic for birds and
339 mammals. *A. anguilla* exhibits an intermolecular compositional heterogeneity (Medrano et al,
340 '88; Bernardi and Bernardi, '90) similar to what was found in *A. rostrata* and explained by the
341 presence of GC-rich satellites (Hudson et al, '80). Medrano et al. ('88) admitted that the G-
342 bands they had produced in eels were not of the same quality as the results produced by the
343 same technique in mammals. Moreover, CDD staining in several anguilliform fishes
344 demonstrated that rDNA sites are the only GC-rich regions on otherwise homogenously
345 stained chromosomes (Salvadori et al, 2009; Coluccia et al, 2010). Therefore, no reliable
346 report has yet been published on the successful application of G-banding in fishes as also
347 noted by Sharma et al. (2002).

348 For anurans, Bernardi (2005) summarized equilibrium centrifugation results in *Xenopus,*
349 *Leptodactylus, Bufo* and *Rana* and one representative of the Caudata (*Pleurodeles waltlii*) and
350 concluded that the genome compositional properties of Amphibians are similar to those
351 described for fishes (Bernardi, 2005). Gazoni et al. (2012) performed CDD staining in eight
352 species of the *Leptodactylus* frog and showed a pattern similar to the ones we observed in *P.*
353 *lessonae* (Fig. S1) and teleosts in this study. Reproducible G-banding was never successful in
354 anurans, whereas replication banding was routinely possible (Schmid and Steinlein 2015).

355 The comparison of gars with homeotherm vertebrates showed a comparable pattern of
356 chromosomal compositional heterogeneity in gars and the house mouse with similar counts of
357 discernible AT/GC bands per chromosome (Fig. 4l). There was a difference in the
358 centromeres however, with them being clearly AT-rich in the mouse but distinctly GC-rich in
359 gars. The GC pattern in the chicken (Fig. 4k) is influenced by GC-rich microchromosomes
360 and mostly homogenous macrochromosomes.

361 The G-bands previously reported in teleosts should be considered "G-like" bands
362 unrelated to the genome GC compartmentalization observed in homeotherm vertebrates. We
363 ascribe these G-like bands to a specific phase of DNA spiralization (less condensed), when
364 some chromosomal regions are more susceptible to Giemsa staining and/or trypsin digestion.
365 We present bioinformatic support for such G-like bands unrelated to GC
366 compartmentalization in two lancelet species (Fig. S5). Whereas Wang et al. (2004) reported
367 G-bands in the lancelet, our bioinformatic analysis indicates a GC homogenous genome
368 without any substantial fluctuation in GC content (Fig. S5, details in Supplementary File).
369 Nevertheless, the alternation of early and late replicating DNA regions exists also in
370 cold-blooded vertebrates as demonstrated by replication banding in fish and amphibians
371 (Vinas et al, '96; Jankun et al, '98; Boroń, 2003; Schmid and Steinlein, 2015). The structural
372 role of gene-rich and gene-poor regions *sensu* Cremer and Cremer (2001) appears to be
373 universal across all vertebrates (Federico et al, 2006). Hence, the genome organization in
374 cold-blooded vertebrates is not accompanied with such prominent GC segregation as in
375 warm-blooded genomes. Even in species with more GC heterogeneous genomes (pufferfish,
376 stickleback) the base distribution appears more shuffled and without accumulation in any
377 distinctly GC homogenous (GC-rich or GC-poor) domains. This may be the reason why it is
378 impossible to visualize any bands in fish chromosomes (with the exception of gars).
379 Therefore, it is necessary to distinguish between the ranges of GC values represented in fish
380 genomes globally and between the AT/GC distributions along chromosomes. In anamniotes,
381 another important factor can be the fact that non-coding sequences with various GC content
382 may blur the higher GC content of coding sequences (Fortes et al, 2007). This is particularly
383 apparent on Fig. 4S a-d, where stickleback and pufferfish represent genomes with reduce
384 genome size (for comparison of genome sizes Fig. 4a-d) and a clear shift towards GC-

385 richness. This shift is however not accompanied by such pronounced GC heterogeneity as in
386 gars and amniotes.
387
388 **How did the GC Heterogeneity in Gar Arise?**
389 DNA nucleotides are not randomly distributed within genomes (Straalen and Roelofs, 2012).
390 Currently, six mutually non-exclusive hypotheses, with different weight, have been proposed
391 to explain the variation in AT/GC organization between cold- and warm-blooded vertebrates:
392 (1) the thermodynamic stability hypothesis, (2) selection for GC content, (3) AT-biased
393 mutations, (4) GC-biased gene conversion (gBGC), (5) DNA methylation (Mugal et al,
394 2015a) and (6) the footprint of metabolism (Berná et al, 2012). With our cytogenomic data
395 and literature sources we can assess three of these hypotheses: (1), (4), and (6).
396 The "Thermostability Hypothesis" (Bernardi, 2005 for review) explains the higher GC
397 heterogeneity of warm-blooded genomes as an adaptation to increased body temperature,
398 where higher GC levels contribute to the stabilization of coding DNA fractions, RNAs and
399 proteins (Costantini et al, 2009). Specifically, the stabilization of GC-rich and gene-rich open
400 chromatin structures with their high transcriptional activity associated with an increased DNA
401 bendability and decreased nucleosome formation potential (Bernardi, 2005; Vinogradov,
402 2005). It has not been explicitly tested whether gars are cold- or warm-blooded (Miller et al,
403 2006; Nelson, 2006). However, since they are actinopterygians (Cavin, 2010; Near et al,
404 2012) they are definitely anamniotes which rules out the possibility that GC heterogenization
405 occurred at the anamniotes – amniotes transition. This also opposes the discussed
406 "Thermostability Hypothesis" linking GC heterogenization with the homeothermy of
407 amniotes (Costantini et al, 2009; Uliano et al, 2010; Tarallo et al, 2016). Based on the report
408 of Farrell (2007), we assume that gars are cold-blooded. The tropical gar in Mexico is able to
409 tolerate the extremely high temperatures and very low oxygen conditions of tropical swamps

410 (Burleson et al, '98; Miller et al, 2006; Arias-Rodriguez own observations). However, gars
411 from the temperate regions of North America, including the spotted gar analysed in this study,
412 thrive also in low temperatures. Further investigations should be carried out to study their
413 thermoregulation adaptations e.g. in elasmobranchs and tuna fish (Block and Finnerty, '94).
414
415 GC-biased gene conversion (gBGC) belongs to widely evidenced hypotheses and states
416 that regions with higher recombination rates contain higher local GC content (Lartillot 2012;
417 Mugal et al, 2013, 2015b). In gars, the centromeres, the majority of very small chromosomes,
418 and some short chromosomal arms (all anticipated high-recombining regions), together with a
419 number of other regions, are distinctly GC-rich (Fig. 2a-b; Fig. S3a-b). This supports gBGC.
420 High GC levels also occur in high-recombining 45S rDNA regions in gars, which is consistent
421 with the gBGC model demonstrated by Escobar et al. (2011). Consequently, low
422 recombination rates are associated with decreased GC proportions e.g. in the short-tail
423 opossum where the low number of large chromosomes was proven to be linked with the low
424 recombination rate in autosomes and an overall low GC content (Mikkelsen et al, 2007). We
425 observed similar situations in the pool frog and the anole, where the large chromosomes were
426 homogenously CDD-stained (Fig 3). The same correlation between chromosome length and
427 GC distribution has been reported in birds and mammals (Romiguier et al, 2010; Weber et al,
428 2014). The gBGC appears to be an important factor greatly influencing the regional GC
429 content in pufferfish, stickleback and also in zebrafish. The mostly terminally occurring GC
430 enrichments corresponds to the regionally (terminally) increased recombination rates
431 described in the stickleback (Roesti et al., 2013). In the compact genome of fugu (Takifugu
432 rubripes) belonging to tetraodontids together with the pufferfish, a high recombination rate
433 was revealed, which stands out especially in comparison with other vertebrates (Kai et al.,
434 2011). Nonetheless, even high quality DAPI-stained fugu chromosomes presented by Kai et

435 al., 2011 show no AT banding pattern. This means that there must be another factor operating
436 on the GC content and distribution (compartmentalization) in teleost fishes.
437
438 **Footprint of gars´ unique metabolism in the organization of their genomes**
439 A growing number of studies (Uliano et al, 2010; Berná et al, 2012; Chaurasia et al, 2011,
440 2014; Tarallo et al. 2016) supports the hypothesis that environment temperature, oxygen
441 content and lifestyle are factors correlating with the genome GC composition and that
442 purifying selection is the mechanism responsible for variation in the genomic GC.
443 Specifically, an increment in metabolic rate, known to be higher in mammals and birds,
444 affects transcription activity and chromatin structure (discussed by Berná et al, 2012).
445 In the Florida gar, the following adaptations for living in high-temperature and low-
446 oxygen environments were identified: i) a heightened capacity to synthesize and oxidise
447 glucose, which sets this species apart from most other fishes; ii) a heightened capacity for
448 anaerobic glycolysis; iii) a low number of oxidative enzymes in gar tissues, and iv) the
449 evolution of their modified swim bladder (Frick et al, 2007). The "swim bladder" in gars
450 resembles the lungs of tetrapods in form and function to such an extent that several authors
451 use the term "lung" in place of the often used "air-breathing organ" (Echelle and Grande,
452 2014 and references therein). Gars are bimodal in respiration – they use their gills for water
453 breathing and their lung(s) for air breathing. This gives them a greater respiratory efficiency
454 in stressful and exhaustive situations allowing them to continue normal activity under
455 conditions that would incapacitate virtually every other fish (Echelle and Grande, 2014).
456 Chaurasia et al, 2014 proposed a correlation between temperature-corrected metabolic
457 rate (MR), entire genomic GC content, and the length and GC content of introns in teleosts.
458 This might also apply to gars. Introns have previously been shown to be shorter in GC-rich
459 genes and devoid of repetitive sequences and/or transposable elements (Duret et al, '95).

460 Hence, the overall proportion of introns and their GC content may indirectly influence the GC
461 content of the entire genome (e.g. in compact genomes). This implies selection for
462 transcription efficiency/economy (Chaurasia et al, 2014) that may result in a different
463 genomic ratio of AT/GC. The MR thus appears to be one of the important factors driving
464 genome architecture evolution (Chaurasia et al, 2014). In this regard, gars may differ from
465 other tropical fish in their modified metabolism and/or increased MR (Uliano et al, 2010).
466 Therefore the temperature component might be involved in the compositional genome
467 evolution as suggested by Bernardi (2005). However, the final modulation might instead be
468 influenced by metabolism-driven traits (Uliano et al, 2010; Chaurasia et al, 2011). These
469 correlations and potential causes are yet to be properly analysed, particularly in gars and
470 bowfin. Differences in physiology between gars and the bowfin indicate specific metabolic
471 traits in gars resembling those of mammals (Frick et al, 2007). This might help to explain why
472 gars possess cytogenetically and bioinformatically detectable GC heterogeneity whereas their
473 putative sister lineage represented by the bowfin exhibit the typical "teleost pattern" on its
474 chromosomes.

475 Gars are considered to have evolved slowly and to show low rates of speciation
476 (Rabosky et al, 2013; Braasch et al, 2016). However, these analyses are based only on the
477 living subset of vertebrate lineages, overlooking the great diversity seen in the fossil record,
478 more so than that found in Polypteriformes, Chondrostea and Amiidae (Cavin, 2010; López-
479 Arbarello 2012; Sallan, *pers.com.*). The palaeobiology of gars and the bowfin based on solid
480 fossil evidence (Sallan, *pers.com.*) indicates a 50 million year long period of accelerated
481 evolution that occurred in gars but not in amiids with an otherwise very similar evolutionary-
482 ecological history (Grande, 2010). This period of accelerated evolution might have also been
483 accompanied by dynamic physiological/metabolic adaptations in gars, which may have left a
484 footprint in their genome in the form of GC compartmentalization. (More details are

485 presented in the Supplementary Discussion). Moreover, based on the fossil evidence, gars and
486 early tetrapods could have faced similar selective pressures and converged on similar
487 solutions (Sallan, *pers.com.*). On the other hand, at this stage, we also have to take into
488 account that gars could have retained the ancestral genome structure of Gnathostomata.

489 The compositional genome heterogeneity among vertebrates cannot be attributed merely
490 to the transition from anamniotes to amniotes as generally accepted before this study. The
491 genomic DNA base architecture in vertebrates appears to rather result from a interplay of
492 multiple opposing forces e.g. AT-biased mutations and GC-biased gene conversions (e.g.
493 Weller et al, 2014), genome and chromosome sizes (e.g. Romiguier et al. 2010), selection for
494 GC and drift on the one side (Duret & Arndt, 2008) and by diverse cellular, metabolic
495 (Chaurasia et al, 2014) and environmental (e.g. Uliano et al, 2010; Chaurasia et al, 2011)
496 factors influenced by physical and chemical properties of AT/GC and their role in the
497 regulation of gene expression (Vinogradov, 2005). In gars, such interplay should be explored
498 with regards to their metabolic adaptations (*sensu* Uliano et al, 2010; Chaurasia et al, 2011;
499 Chaurasia et al, 2014). The situation presented here in gars, in the light of the known genomic
500 traits of their closest extant relatives, implies important consequences for the phylogenomic
501 evolution not only of basal vertebrates but also for amniotes.

502

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511

512 Data archiving

513 All custom generated Python and R scripts produced for this study are available at the codes
514 repository GitHub https://github.com/libor-m/vertebrate-GC
515

516 Competing interests

517 There are no competing interests associated with the publication of this manuscript.
518

519 Author contributions

520 RS designed the study. RS and ZM performed cytogenetic analyses and co-drafted the
521 manuscript together with PR, AM, EN and LB. LM performed bioinformatic analyses. LC
522 provided the paleobiological context and co-drafted the manuscript together with MJP and
523 ZM. TK and LAR participated on cytogenetic analyses. LAR, MJP, MF and MD provided
524 chromosome preparations. All co-authors revised the text and agreed to the final version.
525

526 References

527 Amores A, Catchen J, Ferrara A, Fontenot Q, Postlethwait JH. 2011. Genome evolution and
528 meiotic maps by massively parallel DNA sequencing: spotted gar, an outgroup for the
529 teleost genome duplication. Genetics 188:799–808.
530 Berná L, Chaurasia A, Angelini C, Federico C, Saccone S, D'Onofrio G. 2012. The footprint
531 of metabolism in the organization of mammalian genomes. BMC Genomics 13:174.

532 Bernardi G. 2005. Structural and evolutionary genomics: natural selection in genome
533 evolution. Elsevier.
534 Bernardi G, Bernardi G. 1990. Compositional patterns in the nuclear genome of cold-blooded
535 vertebrates. J Mol Evol 31:265–281.
536 Bickmore WA, Craig J. 1996. Chromosome Bands: Patterns in the Genome. 1 edition. New
537 York; Austin, Tex.: Springer.
538 Block BA, Finnerty JR. 1994. Endothermy in fishes: a phylogenetic analysis of constraints,
539 predispositions, and selection pressures. Environ Biol Fishes 40:283–302.
540 Boroń A. 2003. Replication banding patterns in the spined loach, *Cobitis taenia* L. (Pisces,
541 Cobitidae). Genetica 119:51–55.
542 Braasch I, Gehrke AR, Smith JJ, Kawasaki K, Manousaki T, Pasquier J, Amores A,
543 Desvignes T, Batzel P, Catchen J, Berlin AM, Campbell MS, Barrell D, Martin KJ, Mulley
544 JF, Ravi V, Lee AP, Nakamura T, Chalopin D, Fan S, Wcisel D, Cañestro C, Sydes J,
545 Beaudry FEG, Sun Y, Hertel J, Beam MJ, Fasold M, Ishiyama M, Johnson J, Kehr S, Lara
546 M, Letaw JH, Litman GW, Litman RT, Mikami M, Ota T, Saha NR, Williams L, Stadler
547 PF, Wang H, Taylor JS, Fontenot Q, Ferrara A, Searle SMJ, Aken B, Yandell M,
548 Schneider I, Yoder JA, Volff J-N, Meyer A, Amemiya CT, Venkatesh B, Holland PWH,
549 Guiguen Y, Bobe J, Shubin NH, Di Palma F, Alföldi J, Lindblad-Toh K, Postlethwait JH.
550 2016. The spotted gar genome illuminates vertebrate evolution and facilitates human-
551 teleost comparisons. Nat Genet 48:427–437.
552 Braasch I, Guiguen Y, Loker R, Letaw JH, Ferrara A, Bobe J, Postlethwait JH. 2014.
553 Connectivity of vertebrate genomes: Paired-related homeobox (Prrx) genes in spotted gar,
554 basal teleosts, and tetrapods. Comp Biochem Physiol Toxicol Pharmacol CBP 163:24–36.
555 Burleson M, Shipman B, Smatresk N. 1998. Ventilation and acid-base recovery following
556 exhausting activity in an air-breathing fish. J Exp Biol 201:1359–1368.

557 Cavin L. 2010. Diversity of Mesozoic semionotiform fishes and the origin of gars
558 (Lepisosteidae). Naturwissenschaften 97:1035–1040.
559 Chaurasia A, Tarallo A, Bernà L, Yagi M, Agnisola C, D'Onofrio G. 2014. Length and GC
560 content variability of introns among teleostean genomes in the light of the metabolic rate
561 hypothesis. PloS One 9:e103889.
562 Chaurasia A, Uliano E, Berná L, Agnisola C, D'Onofrio G. 2011. Does Habitat Affect the
563 Genomic GC Content? A lesson from teleostean fish: a mini review. In: Fish Ecology. Inc.
564 Hauppauge NY (USA): Nova Science Publishers. p 61–80.
565 Cohen N, Dagan T, Stone L, Graur D. 2005. GC composition of the human genome: in search
566 of isochores. Mol Biol Evol 22:1260–1272.
567 Coluccia E, Deiana AM, Libertini A, Salvadori S. 2010. Cytogenetic characterization of the
568 moray eel *Gymnothorax tile* and chromosomal banding comparison in Muraenidae
569 (Anguilliformes). Mar Biol Res 6:106–111.
570 Costantini M, Auletta F, Bernardi G. 2007. Isochore patterns and gene distributions in fish
571 genomes. Genomics 90:364–371.
572 Costantini M, Cammarano R, Bernardi G. 2009. The evolution of isochore patterns in
573 vertebrate genomes. BMC Genomics 10:146.
574 Cremer T, Cremer C. 2001. Chromosome territories, nuclear architecture and gene regulation
575 in mammalian cells. Nat Rev Genet 2:292–301.
576 Crow KD, Smith CD, Cheng J-F, Wagner GP, Amemiya CT. 2012. An independent genome
577 duplication inferred from Hox paralogs in the American paddlefish--a representative basal
578 ray-finned fish and important comparative reference. Genome Biol Evol 4:937–953.
579 Crow KD, Stadler PF, Lynch VJ, Amemiya C, Wagner GP. 2006. The "fish-specific" Hox
580 cluster duplication is coincident with the origin of teleosts. Mol Biol Evol 23:121–136.

581 Duret L, Arndt PF. 2008. The impact of recombination on nucleotide substitutions in the
582 human genome. PLoS Genet 4:e1000071.
583 Duret L, Mouchiroud D, Gautier C. 1995. Statistical analysis of vertebrate sequences reveals
584 that long genes are scarce in GC-rich isochores. J Mol Evol 40:308–317.
585 Echelle AA, Grande L. 2014. Lepisosteidae: Gars. In: Warren ML, Burr BM, editors.
586 Freshwater fishes of North America: Volume 1: Petromyzontidae to Catostomidae. JHU
587 Press. p 243–278.
588 Escobar JS, Glémin S, Galtier N. 2011. GC-biased gene conversion impacts ribosomal DNA
589 evolution in vertebrates, angiosperms, and other eukaryotes. Mol Biol Evol 28:2561–2575.
590 Farrell AP. 2007. Cardiovascular systems in primitive fishes. In: David J. McKenzie APF and
591 CJB, editor. Fish Physiology. Vol. Volume 26. Primitive Fishes. Academic Press. p 53–
592 120.
593 Federico C, Scavo C, Cantarella CD, Motta S, Saccone S, Bernardi G. 2006. Gene-rich and
594 gene-poor chromosomal regions have different locations in the interphase nuclei of cold-
595 blooded vertebrates. Chromosoma 115:123–128.
596 Flicek P, Amode MR, Barrell D, Beal K, Billis K, Brent S, Carvalho-Silva D, Clapham P,
597 Coates G, Fitzgerald S, Gil L, Girón CG, Gordon L, Hourlier T, Hunt S, Johnson N,
598 Juettemann T, Kähäri AK, Keenan S, Kulesha E, Martin FJ, Maurel T, McLaren WM,
599 Murphy DN, Nag R, Overduin B, Pignatelli M, Pritchard B, Pritchard E, Riat HS, Ruffier
600 M, Sheppard D, Taylor K, Thormann A, Trevanion SJ, Vullo A, Wilder SP, Wilson M,
601 Zadissa A, Aken BL, Birney E, Cunningham F, Harrow J, Herrero J, Hubbard TJP,
602 Kinsella R, Muffato M, Parker A, Spudich G, Yates A, Zerbino DR, Searle SMJ. 2014.
603 Ensembl 2014. Nucleic Acids Res 42:D749-755.
604 Ford CE, Hamerton JL. 1956. A colchicine, hypotonic citrate, squash sequence for
605 mammalian chromosomes. Stain Technol 31:247–251.

606 Fortes GG, Bouza C, Martínez P, Sánchez L. 2007. Diversity in isochore structure among
607 cold-blooded vertebrates based on GC content of coding and non-coding sequences.
608 Genetica 129:281–289.
609 Frick NT, Bystriansky JS, Ballantyne JS. 2007. The metabolic organization of a primitive air-
610 breathing fish, the Florida gar (*Lepisosteus platyrhincus*). J Exp Zool Part Ecol Genet
611 Physiol 307:7–17.
612 Fujita MK, Edwards SV, Ponting CP. 2011. The *Anolis* lizard genome: an amniote genome
613 without isochores. Genome Biol Evol 3:974–984.
614 Fujiwara A, Nishida-Umehara C, Sakamoto T, Okamoto N, Nakayama I, Abe S. 2001.
615 Improved fish lymphocyte culture for chromosome preparation. Genetica 111:77–89.
616 Gazoni T, Gruber SL, Silva APZ, Araújo OGS, Narimatsu H, Strüssmann C, Haddad CFB,
617 Kasahara S. 2012. Cytogenetic analyses of eight species in the genus *Leptodactylus*
618 Fitzinger, 1843 (Amphibia, Anura, Leptodactylidae), including a new diploid number and
619 a karyotype with multiple translocations. BMC Genet 13:109.
620 Grande L. 2010. An empirical synthetic pattern study of gars (Lepisosteiformes) and closely
621 related species, based mostly on skeletal anatomy the resurrection of Holostei. Lawrence,
622 Kan: American Society of Ichthyologists and Herpetologists.
623 Graphodatsky AS. 1989. Conserved and variable elements of mammalian chromosomes. In:
624 Halnan CRE, editor. Cytogenetics of animals. Oxon, UK: CAB International Press. p 95–
625 124.
626 Holmquist GP. 1989. Evolution of chromosome bands: molecular ecology of noncoding
627 DNA. J Mol Evol 28:469–486.
628 Holmquist GP, Ashley T. 2006. Chromosome organization and chromatin modification:
629 influence on genome function and evolution. Cytogenet Genome Res 114:96–125.

630 Hudson AP, Cuny G, Cortadas J, Haschemeyer AE, Bernardi G. 1980. An analysis of fish
631 genomes by density gradient centrifugation. Eur J Biochem FEBS 112:203–210.
632 Inoue JG, Miya M, Tsukamoto K, Nishida M. 2003. Basal actinopterygian relationships: a
633 mitogenomic perspective on the phylogeny of the "ancient fish." Mol Phylogenet Evol
634 26:110–120.
635 Jankun M, Ocalewicz K, Woznicki P. 1998. Replication, C- and fluorescent chromosome
636 banding patterns in european whitefish, *Coregonus lavavetus* L. Hereditas 128:195–199.
637 Kai W, Kikuchi K, Tohari S, Chew AK, Tay A, Fujiwara A, Hosoya S, Suetake H, Naruse K,
638 Brenner S, Suzuki Y, Venkatesh B. 2011. Integration of the genetic map and genome
639 assembly of fugu facilitates insights into distinct features of genome evolution in teleosts
640 and mammals. Genome Biol Evol. 3:424-442
641 Lartillot N. 2012. Phylogenetic patterns of GC-biased gene conversion in placental mammals,
642 and the evolutionary dynamics of recombination landscapes. Mol Biol Evol:mss239.
643 Levan A, Fredga K, Sandberg AA. 1964. Nomenclature for centromeric position on
644 chromosomes. Hereditas 52:201–220.
645 López-Arbarello A. 2012. Phylogenetic Interrelationships of ginglymodian fishes
646 (Actinopterygii: Neopterygii). PLoS ONE 7:e39370.
647 Ludwig A, Belfiore NM, Pitra C, Svirsky V, Jenneckens I. 2001. Genome duplication events
648 and functional reduction of ploidy levels in sturgeon (*Acipenser*, *Huso* and
649 *Scaphirhynchus*). Genetics 158:1203–1215.
650 Luo C. 1998. Multiple chromosomal banding in grass carp, *Ctenopharyngodon idellus*.
651 Heredity 81:481–485.
652 Medrano L, Bernardi G, Couturier J, Dutrillaux B, Bernardi G. 1988. Chromosome banding
653 and genome compartmentalization in fishes. Chromosoma 96:178–183.

654 Meyer A, Málaga-Trillo E. 1999. Vertebrate genomics: More fishy tales about Hox genes.
655 Curr Biol CB 9:R210-213.
656 Mikkelsen TS, Wakefield MJ, Aken B, Amemiya CT, Chang JL, Duke S, Garber M, Gentles
657 AJ, Goodstadt L, Heger A, Jurka J, Kamal M, Mauceli E, Searle SMJ, Sharpe T, Baker
658 ML, Batzer MA, Benos PV, Belov K, Clamp M, Cook A, Cuff J, Das R, Davidow L,
659 Deakin JE, Fazzari MJ, Glass JL, Grabherr M, Greally JM, Gu W, Hore TA, Huttley GA,
660 Kleber M, Jirtle RL, Koina E, Lee JT, Mahony S, Marra MA, Miller RD, Nicholls RD,
661 Oda M, Papenfuss AT, Parra ZE, Pollock DD, Ray DA, Schein JE, Speed TP, Thompson
662 K, VandeBerg JL, Wade CM, Walker JA, Waters PD, Webber C, Weidman JR, Xie X,
663 Zody MC, Broad Institute Genome Sequencing Platform, Broad Institute Whole Genome
664 Assembly Team, Graves JAM, Ponting CP, Breen M, Samollow PB, Lander ES, Lindblad-
665 Toh K. 2007. Genome of the marsupial *Monodelphis domestica* reveals innovation in non-
666 coding sequences. Nature 447:167–177.
667 Miller RR, Minckley WL, Norris SM. 2006. Freshwater fishes of México. Chicago:
668 University of Chicago Press.
669 Mugal CF, Arndt PF, Ellegren H. 2013. Twisted signatures of GC-biased gene conversion
670 embedded in an evolutionary stable karyotype. Mol Biol Evol 30:1700–1712.
671 Mugal CF, Arndt PF, Holm L, Ellegren H. 2015a. Evolutionary consequences of DNA
672 methylation on the GC content in vertebrate genomes. G3 Bethesda Md 5:441–447.
673 Mugal CF, Weber CC, Ellegren H. 2015b. GC-biased gene conversion links the
674 recombination landscape and demography to genomic base composition. Bioessays
675 37:1317-1326
676 Near TJ, Eytan RI, Dornburg A, Kuhn KL, Moore JA, Davis MP, Wainwright PC, Friedman
677 M, Smith WL. 2012. Resolution of ray-finned fish phylogeny and timing of diversification.
678 Proc Natl Acad Sci U S A 109:13698–13703.

679 Nelson JS. 2006. Fishes of the world. Fourth edition. Nelson, Joseph S.; Department of
680 Biological Sciences, University of Alberta, Edmonton, Alberta T6G 2E9, Canada, Canada.:
681 John Wiley & Sons, Inc.
682 Neusser M. 2004. Karyotypevolution, Genomorganisation und Zellkernarchitektur der
683 Neuweltaffen.
684 Nordberg H, Cantor M, Dusheyko S, Hua S, Poliakov A, Shabalov I, Smirnova T, Grigoriev
685 IV, Dubchak I. 2014. The genome portal of the Department of Energy Joint Genome
686 Institute: 2014 updates. Nucleic Acids Res 42:D26-31.
687 Pokorná M, Rábová M, Ráb P, Ferguson-Smith MA, Rens W, Kratochvíl L. 2010.
688 Differentiation of sex chromosomes and karyotypic evolution in the eye-lid geckos
689 (Squamata: Gekkota: Eublepharidae), a group with different modes of sex determination.
690 Chromosome Res Int J Mol Supramol Evol Asp Chromosome Biol 18:809–820.
691 Pokorná M, Rens W, Rovatsos M, Kratochvíl L. 2014. A ZZ/ZW sex chromosome system in
692 the thick-tailed Gecko (*Underwoodisaurus milii*; Squamata: Gekkota: Carphodactylidae), a
693 member of the ancient gecko lineage. Cytogenet Genome Res 142:190–196.
694 Ráb P, Rábová M, Reed KM, Phillips RB. 1999. Chromosomal characteristics of ribosomal
695 DNA in the primitive semionotiform fish, longnose gar *Lepisosteus osseus*. Chromosome
696 Res Int J Mol Supramol Evol Asp Chromosome Biol 7:475–480.
697 Rabosky DL, Santini F, Eastman J, Smith SA, Sidlauskas B, Chang J, Alfaro ME. 2013. Rates
698 of speciation and morphological evolution are correlated across the largest vertebrate
699 radiation. Nat Commun 4:1958.
700 R Development Core Team. 2012. R: a language and environment for statistical computing.
701 Vienna, Austria. Available from: http://www.R-project.org/
702 Roesti M, Moser D, Berner D. 2013. Recombination in the threespine stickleback genome-
703 patterns and consequences. Mol Ecol 22(11):3014-27

704 Romanenko SA, Biltueva LS, Serdyukova NA, Kulemzina AI, Beklemisheva VR, Gladkikh
705 OL, Lemskaya NA, Interesova EA, Korentovich MA, Vorobieva NV, Graphodatsky AS,
706 Trifonov VA. 2015. Segmental paleotetraploidy revealed in sterlet (*Acipenser ruthenus*)
707 genome by chromosome painting. Mol Cytogenet 8:90.
708 Romiguier J, Ranwez V, Douzery EJP, Galtier N. 2010. Contrasting GC-content dynamics
709 across 33 mammalian genomes: relationship with life-history traits and chromosome sizes.
710 Genome Res 20:1001–1009.
711 Sallan LC. 2014. Major issues in the origins of ray-finned fish (Actinopterygii) biodiversity.
712 Biol Rev 89:950–971.
713 Salvadori S, Coluccia E, Cannas R, Cau A, Deiana AM. 2009. A ZZ-ZW sex chromosome
714 system in the finless eel *Dalophis imberbis* (Anguilliformes, Ophichtidae). Genetica
715 135:283–288.
716 Schmid M, Guttenbach M. 1988. Evolutionary diversity of reverse (R) fluorescent
717 chromosome bands in vertebrates. Chromosoma 97:101–114.
718 Schmid M, Steinlein C. 2015. Chromosome Banding in Amphibia. XXXII. The Genus
719 *Xenopus* (Anura, Pipidae). Cytogenet Genome Res 145:201–217.
720 Seabright M. 1971. A rapid banding technique for human chromosomes. Lancet 2:971–972.
721 Seabright M. 1972. The use of proteolytic enzymes for the mapping of structural
722 rearrangements in the chromosomes of man. Chromosoma 36:204–210.
723 Sharma OP, Tripathi NK, Sharma KK. 2002. A review of chromosome banding in fishes. In:
724 Sobit RC, Obe G, Athwal RS, editors. Some aspects of chromosome structure and
725 functions. Springer Netherlands. p 109–122.
726 Sohn SH, Ryu EK. 1999. Chromosome preparation from chick leucocyte culture using ficoll
727 treatment. J AgricTechnol Res 12:131–140.

728 Sola L, Camerini B, Cataudella S. 1984. Cytogenetics of Atlantic eels: C- and G-banding,
729 nucleolus organizer regions, and DNA content. Cytogenet Genome Res 38:206–210.
730 Sola L, Rossi AR, Iaselli V, Rasch EM, Monaco PJ. 1992. Cytogenetics of bisexual/unisexual
731 species of *Poecilia*. II. Analysis of heterochromatin and nucleolar organizer regions in
732 *Poecilia mexicana mexicana* by C-banding and DAPI, quinacrine, chromomycin A3, and
733 silver staining. Cytogenet Cell Genet 60:229–235.
734 Straalen NM van, Roelofs D. 2012. An introduction to ecological genomics. Oxford
735 University Press.
736 Sumner AT. 1990. Chromosome banding. Unwin Hyman.
737 Tarallo A, Angelini C, Sanges R, Yagi M, Agnisola C, D'Onofrio G. 2016. On the genome
738 base composition of teleosts: the effect of environment and lifestyle. BMC Genomics.
739 17:173.
740 Thiery J, Macaya G, Bernardi G. 1976. Analysis of eukaryotic genomes by density gradient
741 centrifugation. J Mol Biol 108:219–235.
742 Ueda T, Naoi H. 2011. BrdU-4Na-EDTA-Giemsa band karyotypes of 3 small freshwater fish,
743 *Danio rerio, Oryzias latipes,* and *Rhodeus ocellatus*. Genome 3:531–535.
744 Uliano E, Chaurasia A, Bernà L, Agnisola C, D'Onofrio G. 2010. Metabolic rate and genomic
745 GC. What we can learn from teleost fish. Mar Genomics 3:29–34.
746 Vandepoele K, De Vos W, Taylor JS, Meyer A, Van de Peer Y. 2004. Major events in the
747 genome evolution of vertebrates: paranome age and size differ considerably between ray-
748 finned fishes and land vertebrates. Proc Natl Acad Sci U S A 101:1638–1643.
749 Varriale A. 2014. DNA Methylation, epigenetics, and evolution in vertebrates: facts and
750 challenges. Int J Evol Biol 2014:e475981.
751 Vinas A, Gómez C, Martínez P, Sánchez L. 1996. Replication banding in the chromosomes of
752 the European eel (*Anguilla anguilla*). Genetica 98:107–110.

753 Vinogradov AE. 2005. Noncoding DNA, isochores and gene expression: nucleosome
754 formation potential. Nucleic Acids Res 33:559–563.
755 Wang C, Zhang S, Chu J. 2004. G-banding patterns of the chromosomes of amphioxus
756 *Branchiostoma belcheri tsingtauense*. Hereditas 141:2–7.
757 Weber CC, Boussau B, Romiguier J, Jarvis ED, Ellegren H. 2014. Evidence for GG-biased
758 gene conversion as a driver of between-lineage differences in avian base composition.
759 BMC Genome Biology, 15:549.
760 Weller AM, Rödelsperger C, Eberhardt G, Molnar RI, Sommer RJ. 2014. Opposing forces of
761 A/T-biased mutations and G/C-biased gene conversions shape the genome of the nematode
762 *Pristionchus pacificus*. Genetics 196:1145–1152.
763 Wiberg UH. 1983. Sex determination in the European eel (*Anguilla anguilla*, L.). A
764 hypothesis based on cytogenetic results, correlated with the findings of skewed sex ratios
765 in eel culture ponds. Cytogenet Cell Genet 36:589–598.
766 Wright JJ, David SR, Near TJ. 2012. Gene trees, species trees, and morphology converge on a
767 similar phylogeny of living gars (Actinopterygii: Holostei: Lepisosteidae), an ancient clade
768 of ray-finned fishes. Mol Phylogenet Evol 63:848–856.
769 Zhuang ZM, Wu D, Zhang SC, Pang QX, Wang CL, Wan RJ. 2006. G-banding patterns of the
770 chromosomes of tonguefish *Cynoglossus semilaevis* Günther, 1873. J Appl Ichthyol
771 22:437–440.

772
773 **Figure Captions**

774 **Fig. 1** Time-scaled consensus tree of osteichthyes focused on the non-teleost actinopterygians
775 and tetrapod clades in sarcopterygians. Relevant genomic and eco-physiological adaptations
776 are plotted on the tree: vertebrate genome duplications (VGD1/2), following whole genome
777 duplication (WGD), genome base compartmentalization, air-breathing and terrestrialization.

778 The genomic base compartmentalization is shown to be isolated in gars within the group
779 Actinopterygii. In this phylogeny, we favoured a double appearance of heterogeneity within
780 the amniotes (i.e. warm-blooded mammals and birds), although we cannot exclude the
781 possibility that heterogeneity occurred at the amniotes node, then disappeared in several
782 'reptilian' lineages.

783

784 **Fig. 2** Karyotypes of tropical gar (a) CDD-stained; (c) G-banded; (e) C-banded; (g) Giemsa
785 stained and of spotted gar (b) CDD-stained; (d) G-banded; (f) C-banded; (h) Giemsa stained.
786 The CMA_3 signal was inserted from green into red and the DAPI signal from blue into the
787 green channel. The C-banding pattern was counterstained with DAPI and inverted. m –
788 metacentric, sm – submetacentric, st/a – subtelocentric-acrocentric chromosomes, st/a* –
789 subtelocentric-acrocentric very small chromosomes. Images were adjusted separately before
790 being incorporated into the plate.

791

792 **Fig. 3** CDD-stained chromosomes of different vertebrate representatives: (a) tropical gar
793 *Atractosteus tropicus;* (b) spotted gar *Lepisosteus oculatus;* (c) bowfin *Amia calva;* (d) ornate
794 bichir *Polypterus ornatipinnis;* (e) starry sturgeon *Acipenser stellatus;* (f) European eel
795 *Anguilla anguilla; (*g) roach *Rutilus rutilus;* (h) pool frog *Pelophylax lessonae;* (i) rapid
796 racerunner *Eremias velox;* (j) green anole *Anolis carolinensis;* (k) chicken *Gallus gallus* and
797 (l) house mouse *Mus musculus.* The CMA_3 signal was inserted from green into red and the
798 DAPI signal from blue into the green channel to enhance the contrast between these signals.
799 Images were adjusted separately before being incorporated into the plate. Bars equal 5 μm.

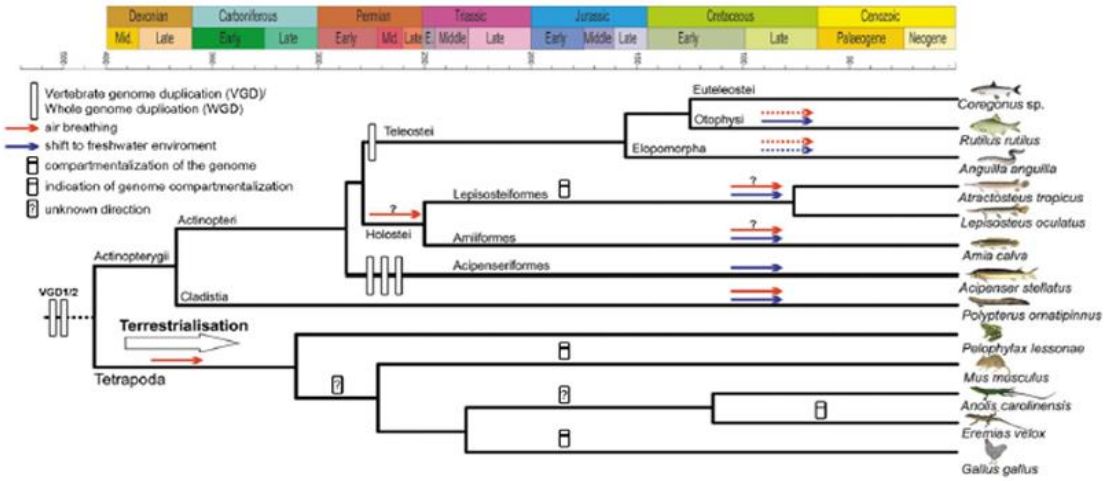
800 **Fig. 4** GC profiles along linkage groups (LGs) in (a) spotted gar, (b) pufferfish, (c)
801 stickleback and (d) zebrafish. For comparison, chicken (e) and a partial human (f) GC profile

802 are provided (for full human GC profile, see Fig. S3a). GC profiling was performed with
803 uniform scaling and differing genome sizes are reflected in sizes of profiles. LGs are arranged
804 according to their numbers in the Ensembl database and separated by vertical lines above
805 profiles and by the alteration of grey and black colours (x axis – genome position, y axis –
806 percentage of GC).

807

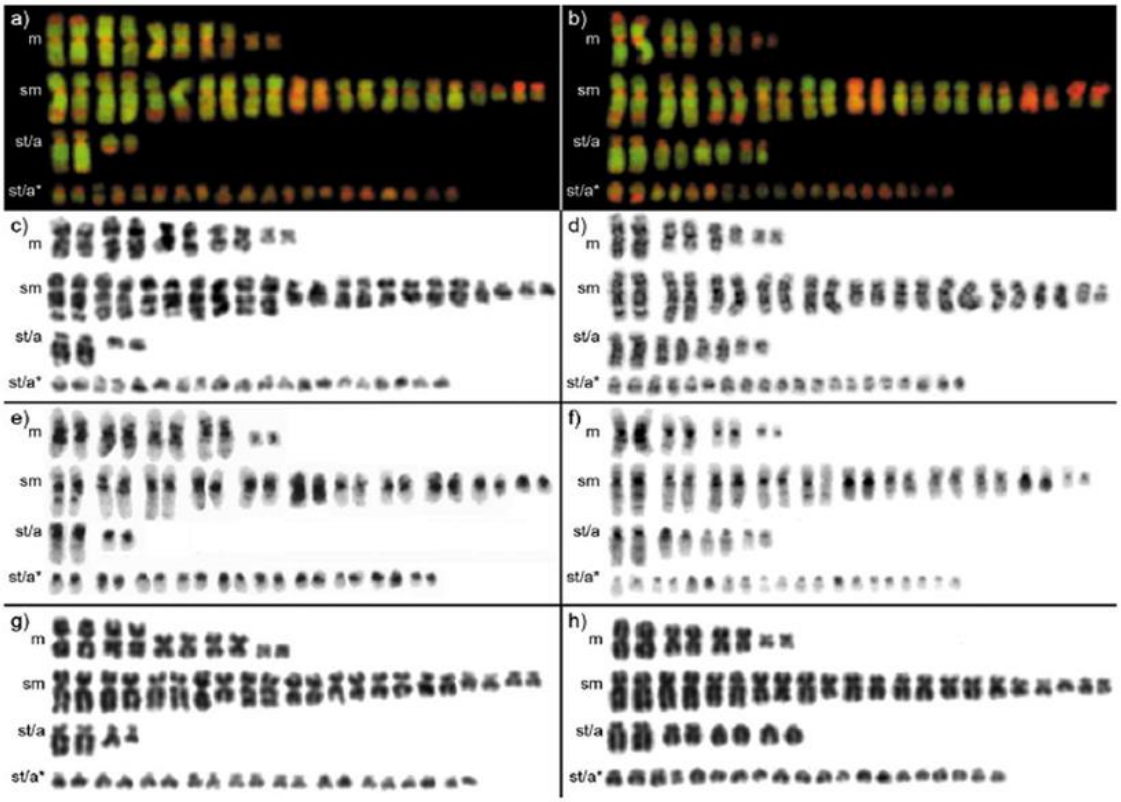
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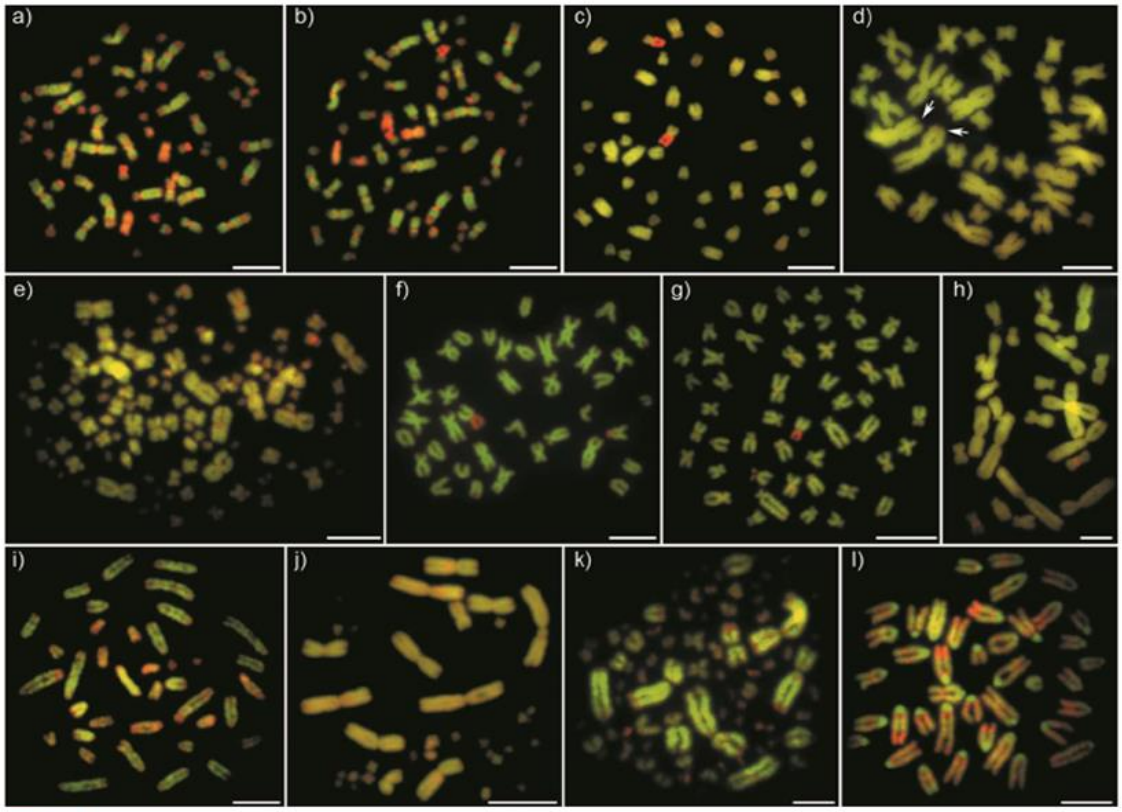
811 **Fig. 1**



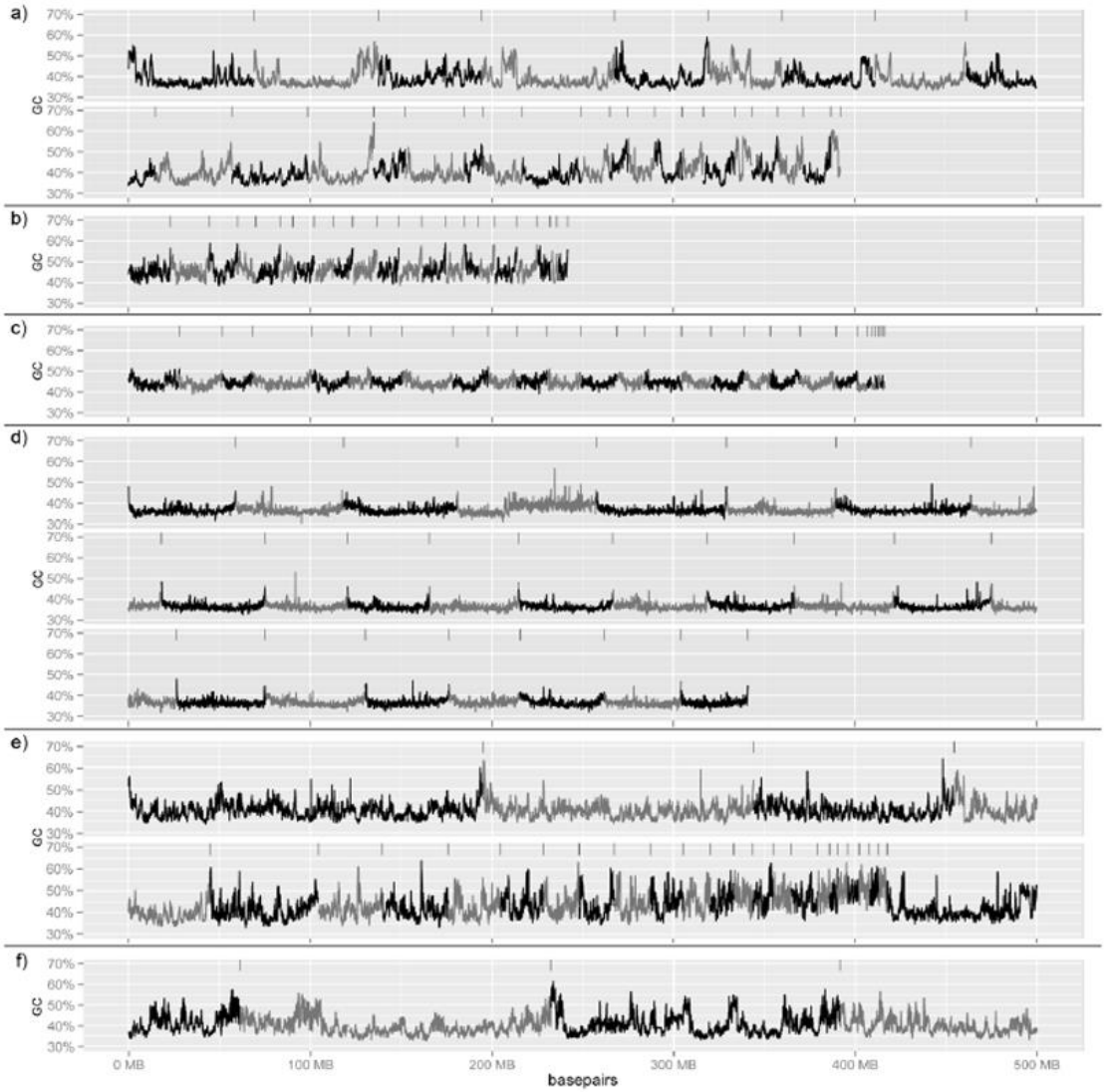
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813 **Fig. 2**

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816 **Fig. 3**


a)
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818 **Fig. 4**

7.4. Publication IV

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Author contribution: chromosome preparation, probe preparation, FISH experiments with rDNA and retrotransposon *Rex1* probes, co-drafting the manuscript

I declare that the contribution of Zuzana Majtánová to the publication Symonová et al. (2013), as stated here, is true.

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BMC Evolutionary Biology

RESEARCH ARTICLE Open Access

Genome differentiation in a species pair of coregonine fishes: an extremely rapid speciation driven by stress-activated retrotransposons mediating extensive ribosomal DNA multiplications

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Abstract

Background: Sympatric species pairs are particularly common in freshwater fishes associated with postglacial lakes in northern temperate environments. The nature of divergences between co-occurring sympatric species, factors contributing to reproductive isolation and modes of genome evolution is a much debated topic in evolutionary biology addressed by various experimental tools. To the best of our knowledge, nobody approached this field using molecular cytogenetics. We examined chromosomes and genomes of one postglacial species pair, sympatric European winter-spawning *Coregonus albula* and the local endemic dwarf-sized spring-spawning *C. fontanae*, both originating in Lake Stechlin. We have employed molecular cytogenetic tools to identify the genomic differences between the two species of the sympatric pair on the sub-chromosomal level of resolution.

Results: Fluorescence *in situ* hybridization (FISH) experiments consistently revealed a distinct variation in the copy number of loci of the major ribosomal DNA (the 45S unit) between *C. albula* and *C. fontanae* genomes. In *C. fontanae*, up to 40 chromosomes were identified to bear a part of the major ribosomal DNA, while in *C. albula* only 8–10 chromosomes possessed these genes. To determine mechanisms how such extensive genome alternation might have arisen, a PCR screening for retrotransposons from genomic DNA of both species was performed. The amplified retrotransposon *Rex1* was used as a probe for FISH mapping onto chromosomes of both species. These experiments showed a clear co-localization of the ribosomal DNA and the retrotransposon *Rex1* in a pericentromeric region of one or two acrocentric chromosomes in both species.

Conclusion: We demonstrated genomic consequences of a rapid ecological speciation on the level undetectable by neither sequence nor karyotype analysis. We provide indirect evidence that ribosomal DNA probably utilized the spreading mechanism of retrotransposons subsequently affecting recombination rates in both genomes, thus, leading to a rapid genome divergence. We attribute these extensive genome re-arrangements associated with speciation event to stress-induced retrotransposons (re)activation. Such causal interplay between genome differentiation, retrotransposons (re)activation and environmental conditions may become a topic to be explored in a broader genomic context in future evolutionary studies.

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Background

Intra-lacustrine fish speciation as an example of ecological speciation is a much debated topic in evolutionary biology addressed by various experimental tools, mostly in complex systems with a number of species, in particular in ancient freshwater lakes [1]. In Europe, with its comparatively depauperate fish fauna, issues of adaptive radiation and ecological speciation in fishes are highly relevant in temperate postglacial lakes (originating after the last glaciation i.e. 12–15 kyrs BP). To assess potential modes of speciation in fishes, numerous model systems are available [2], among which one of the best groups with a robust knowledge on adaptive speciation and complex speciation patterns in postglacial lakes are coregonine fishes (Coregoninae, [3]) [4-6]. Within coregonines, their numerous sympatric species pairs and recent species flocks [7-9] are of particular importance [10]. In *Coregonus*, based on extensive genetic and population genetic [11], phylogenetic, biogeographic, morphological and eco-physiological data, six potential modes of speciation have been proposed [12]. However, none of these approaches utilized cytogenetic data despite salmonid fishes, to which coregonines belong, being one of the best karyologically studied fish groups in terms of the number of species, populations, individuals and material (adults and embryos) examined. Available cytogenetic data demonstrate that salmonids include two basic karyotypes – the high chromosome number 2n ~ 80 (type A and its derivatives) and the low chromosome number 2n ~ 60 (type B and its derivatives) – co-occurring in all recognized salmonid phylogenetic lineages (except graylings, Thymallinae), including whitefish, ciscoes and innconu (Coregoninae). Species with the type B karyotypes have in common either prominent anadromous behaviour and/or are found in lacustrine environments and are likely products of intra-lacustrine speciation (for review [13]). Such apparent parallelism might be explained by specific life history strategies leading in both types of environments to small effective population sizes, thus, enabling increased probability of fixation of genic or chromosomal mutations. Observed evolution of chromosome number in salmonids is likely affected by selection for increased or decreased genetic recombination rate as proposed by Quimseyh [14], explaining high variability in chromosome numbers in mammals based on fundamental numbers (NF, chromosome arms number).

In this study, we examined chromosomes and genomes of the sympatric species *Coregonus albula* and *C. fontanae* in the dimictic Lake Stechlin, northern Germany to test whether the above outlined parallelism on karyotype differentiation in intralacustrine species pairs can also be observed in incipient speciation processes in young postglacial lakes. Both species are pelagic zooplanktivores, but they differ considerably in their size, spawning time [15] and temperature-dependent metabolic physiological adaptations [16]. Up to now, *C. fontanae* has not yet been subjected to any cytogenetic analysis as opposed to *C. albula* (see [17] and references therein). The level of genetic differentiation between *C. albula* and *C. fontanea* tested by combined analyses of mitochondrial DNA and microsatellite loci showed a weak differentiation (F_{ST} = 0–0.008) between these two species when compared with another sympatric species pair *C. albula* and *C. lucinensis* [18]. Further population genetic analyses based on 1244 polymorphic AFLP loci demonstrated a lower differentiation between allopatric than sympatric populations of the *C. albula* complex and suggested a rather complex colonization history than simple sympatric speciation [6]. Therefore, we have employed a novel approach in this field to explore the up to now neglected aspects of genome evolution in this species pair and used different parts of ribosomal DNA of the 45S rDNA unit as cytotaxonomic markers.

At the first stage of this study, we have employed conventional methods of karyotype analysis (Giemsa and Ag staining, CMA_3 and DAPI fluorescence). At the second stage, we have performed molecular cytogenetic analyses (CGH and FISH with various rDNA fragments and non-LTR retrotransposons as probes) to identify any differences between chromosomal complements of these two species on the sub-chromosomal level of resolution since the karyotype analyses showed no significant differences. At the third stage, we performed molecular biological analyses of the 45S ribosomal RNA genes and the *Rex1* non-LTR retrotransposon. Furthermore, we discuss these results in the context of populations of small effective sizes under extreme stress conditions under which retrotransposons (re)activation could have contributed to accelerated speciation.The major cluster of ribosomal RNA genes is expressed as the 45S transcriptional unit (Figure 1). This unit consists of 18S, 5.8S and 28S rDNA genes, separated by internal transcribed spacers (ITS1, ITS2) and surrounded by external transcribed spacers (ETS). The 45S rDNA units are arranged in tandem repetitions with high copy numbers [19,20] therefore, they represent a useful cytotaxonomic marker. The individual units are separated by intergenic spacers (IGS) [21,22]. The structure and the order of genes within the unit are highly conserved among Eukaryota [23]. Different parts of the 45S transcriptional unit display different mutational rates. The most conserved region is the 18S rRNA gene and the most variable are ITSs [23].

Results

Karyotyping and comparative cytogenetics

Karyotypes of both examined ciscoes were very similar (2n = 80 in both species) and both belong to the

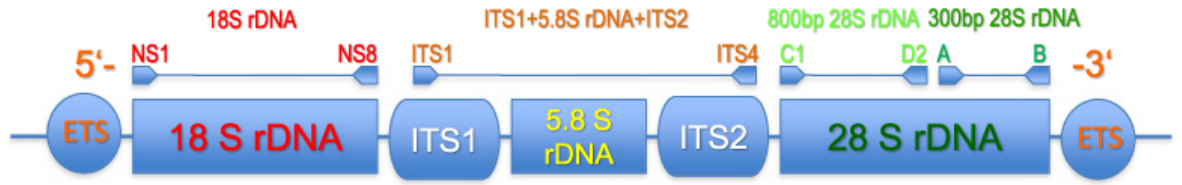


Figure 1 Schematic representation of the 45S rDNA unit and FISH probes construction including primers nesting sites. (not to scale).

karyotype category A *sensu* [13]. They both had 8 pairs of meta- (m) to submetacentric (sm) and 32 pairs of acrocentric (a) chromosomes (both sexes in *C. fontanae*, only males in *C. albula* were available), The NF was 96 in both species (Figure 2a-d). The sequential Chromomycin A_3 (CMA_3, particularly specific for CG rich regions) and DAPI (specific for AT rich regions) stainings revealed in both species a varying number of 6–8 sites with $CMA3^+/DAPI^-$ signals. The signals occurred at telomeric regions of 3–4 metacentric chromosomes and at pericentromeric regions of 3–4 acrocentric/submetacentric chromosomes (Figure 3a, b). In some nuclei, several other weakly CMA_3^+ regions not corresponding to $DAPI^-$ signals mostly with pericentromeric locations were observed (Figure 3a). This variability occurs on the inter-individual as well as on the intra-individual level.

Cytogenetic mapping of ribosomal DNA and comparative genomic hybridization (CGH)

Fluorescence *in situ* hybridization (FISH) with 28S ribosomal DNA (rDNA) probes derived from two non-overlapping regions of the 28S rRNA gene of both species (an 800 bp region adjacent towards the 5′-end of the 28S rDNA gene and a 300 bp region adjacent towards the 3′-end) showed strikingly different results. FISH using the shorter fragment as a probe revealed the presence of 6–10 chromosomes in both *C. albula* and *C. fontanae* bearing such sequences distributed similarly as the $CMA_3^-/DAPI^-$ (Figure 3c for *C. albula* only). FISH with the longer fragment revealed bright signals on 6–10 chromosomes in *C. albula* (shown in co-localization with *Rex1* retrotransposon, Figure 4c) but up to 40 signals (varying numbers) on chromosomes in *C. fontanae* (Figure 3d). Most of the signals of the 800 bp probe of the 28S rDNA in *C. fontanae* were localized in the AT rich (i.e. $DAPI^+$) centromeric or pericentromeric regions of acrocentric chromosomes. Two signals of the 800 bp 28S rDNA probe corresponded to the major NOR sites evidenced also by the 300 bp rDNA and the CMA_3/DAPI staining that were localized in telomeric regions of two large metacentric chromosomes (Figure 3d).

To verify these striking differences between *C. albula* and *C. fontanae*, we carried out a set of reciprocal comparative genomic hybridization (CGH) experiments. A mixture of the whole genome DNA (gDNA) of both *C. albula* and *C. fontanae* was hybridized simultaneously to both *C. fontanae* and *C. albula* chromosomes. This resulted in nearly no significant differences on *C. albula* chromosomes, i.e. a balanced hybridization of both gDNA probes was observed (Figure 3e). While signal of the *C. fontanae* gDNA when *in situ* compared with the

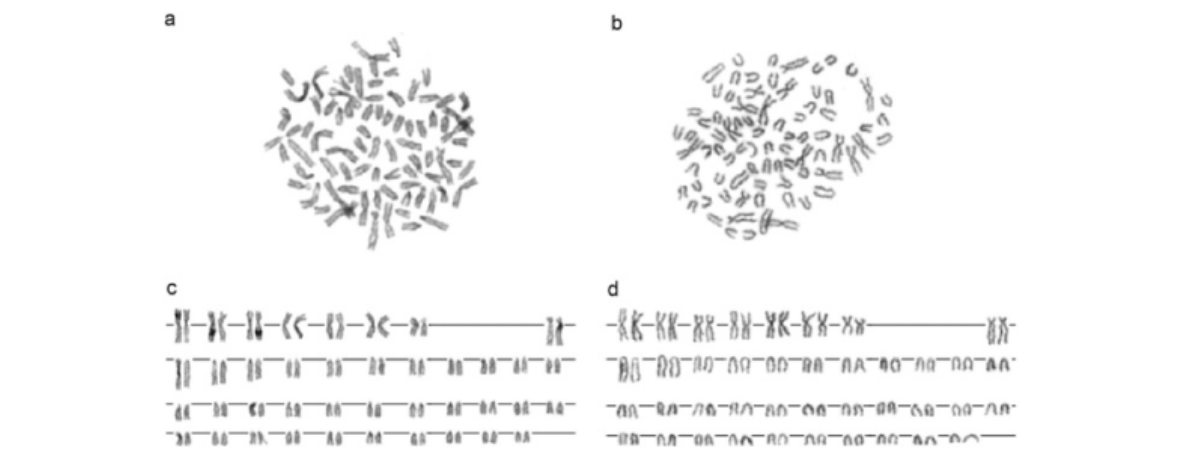


Figure 2 Giemsa-stained metaphase plates and corresponding karyogram of *C. albula* (a, c) and *C. fontanae* (b, d). Bar = 5 μm.

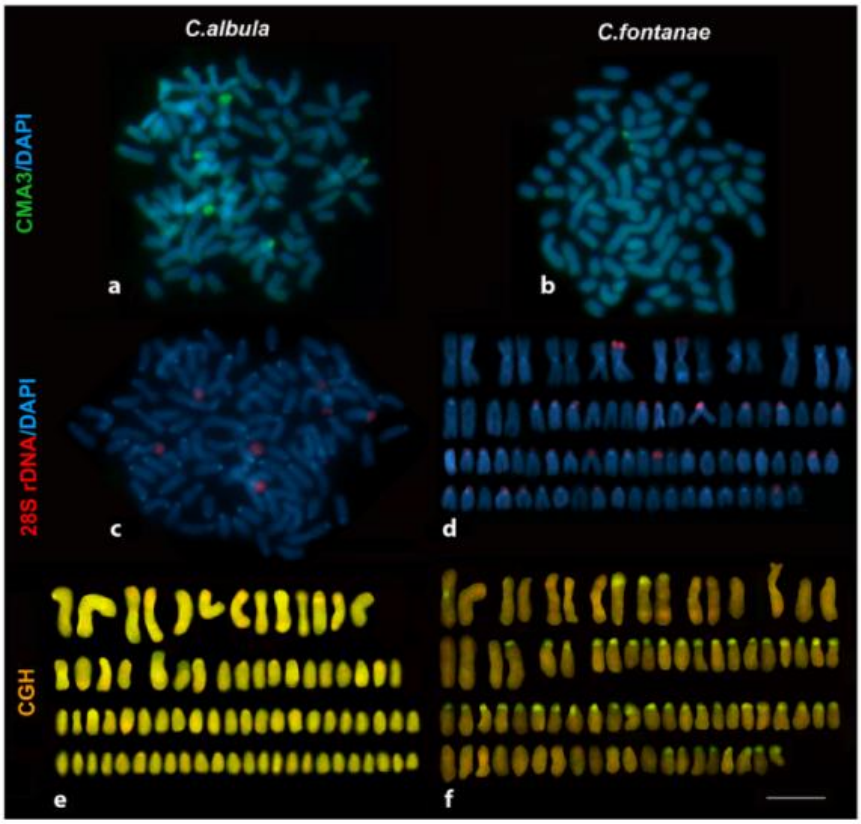


Figure 3 Metaphase plates and karyograms of *C. albula* and *C. fontanae* showing Chromomycin A_3/DAPI staining, FISH and CGH experiments. Chromomycin A_3 (CMA_3) fluorescent staining (green) and DAPI (blue) staining in *C. albula* (**a**) and *C. fontanae* (**b**). FISH with the 28S rDNA (300 bp probe) (red), DAPI counterstaining (blue) in *C. albula* (**c**). FISH with the 28S rDNA (800 bp probe) (red), DAPI counterstaining (blue) in *C. fontanae* (**d**). A set of reciprocal comparative genomic hybridization (CGH) experiments to *C. albula* chromosomes (**e**) and *C. fontanae* chromosomes (**f**). In both (**e**, **f**), the *C. albula* genomic DNA was labelled in red and the *C. fontanae* genomic DNA in green. Bar = 5 μm.

C. albula gDNA onto *C. fontanae* chromosomes was distinctly overrepresented in mostly pericentromeric regions of about 40 chromosomes (green signals in Figure 3f). This pattern corresponded to results of the FISH experiment with 800 bp 28S rDNA to *C. fontanae* chromosomes.

To assess quantitative differences in the distribution of the whole 45S rDNA unit in both species, a further set of comparative FISH experiments with a cocktail of the 18S rDNA and ITS1-ITS2 (including 5.8S rDNA) as probes amplified from both of the genomes were performed to *C. albula* and *C. fontanae* chromosomes. In the genome of *C. albula*, both the ITS1 and ITS2 were present in 6–12 signals with a varying number of signals (Figure 4a). In the genome of *C. fontanae*, both the ITS1 and ITS2 were multiplied to the same extent as the 800 bp 28S rDNA part, i.e. a varying number of approximately 40 signals (Figure 4b). The subsequent FISH experiment with the 18S rDNA in both species showed the number of 6–10 signals (Figure 4d for *C. fontanae* only). The typical chromosomes bearing rDNA signals in the unamplified condition (i.e. ITS1-ITS2 and 28S rDNA in *C. albula* and 18S rDNA in *C. fontanae*) are shown in Figures 4e-f. There is a reproducible difference in location of one of the 28S rDNA in *C. fontanae* (when compared with *C. albula*) related to a distinct $DAPI^+$ band on a large metacentric chromosome pair (Figure 4f). In *C. albula*, the rDNA signal was always located on the opposite arm than the $DAPI^+$ band occurred (Figure 4e). In *C. fontanae*, one signal is located on the same arm and one signal is on the opposite one (Figure 4f). The construction of the FISH probes used in this study is visualized in Figure 1.

Molecular characterization of multiplied rDNA sites

To determine mechanisms how such extensive multiplication of parts of rRNA genes in *C. fontanae* might have arisen, a PCR screening for non-LTR retrotransposons in genomic DNA of both species was performed. Retrotransposons of the *Rex* family are known to have

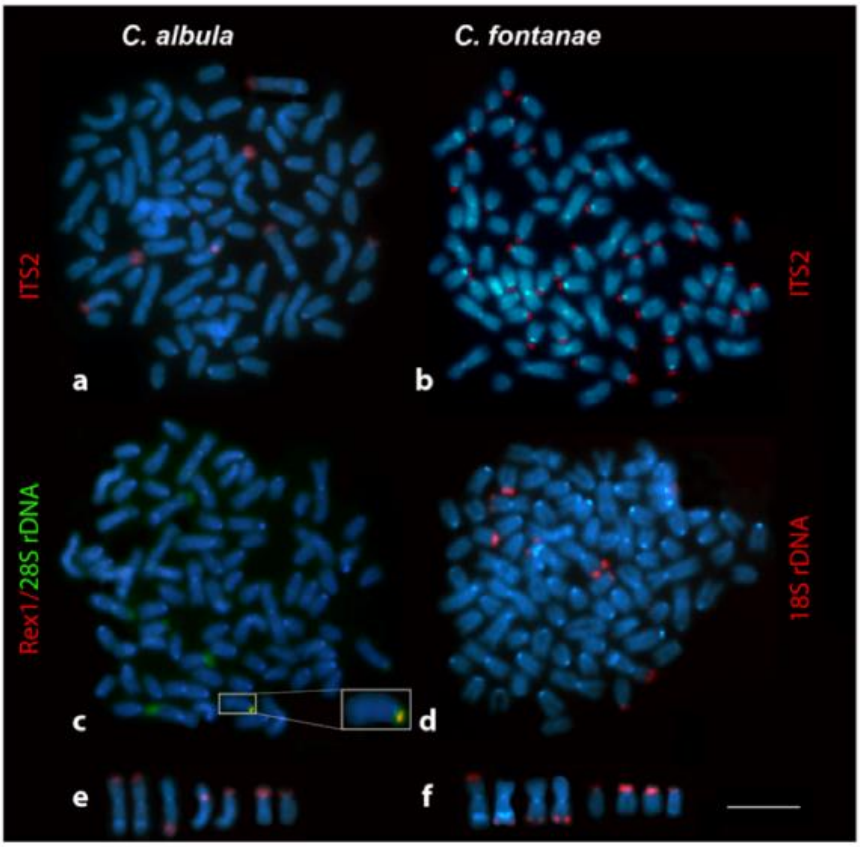


Figure 4 Metaphase plates and selected chromosomes of *C. albula* and *C. fontanae* showing FISH experiments. FISH with ITS2 (red) as probe hybridized to *C. albula* (**a**) and to *C. fontanae* (**b**), counterstained with DAPI. Double-FISH analysis with the *Rex1* retrotransposon (red) and the 800 bp 28S rDNA (green) to *C. albula* (**c**), detail of the chromosome with *Rex1* and 28S rDNA co-localization in inset. FISH with the 18S rDNA (red) to *C. fontanae* chromosomes (**d**) counterstained with DAPI. Chromosomes bearing the ITS2 (red) signal in *C. albula* (**e**). Chromosomes bearing the 18S rDNA signals (red) in *C. fontanae* (**f**). Chromosomes on (**f**) represent the hypothetically ancestral condition of rDNA distribution prior to its multiplication in *C. fontanae*. Bar = 5 μm.

invaded fish genomes in multiple lineages [24] and to also insert into rDNA, particularly in fishes [25]. Therefore, the retroelements *Rex1*, *Rex3* and *Rex6* were tested in this study. FISH with the *Rex3* and *Rex6* retroelements yielded inconclusive results. The *Rex1* element, as a probe hybridized to chromosomes of *C. albula* and *C. fontanae*, typically showed a dispersed pattern of signals on all chromosomes with a distinct accumulation in a pericentromeric region of one single acrocentric chromosome. Co-hybridization of the *Rex1* element with the 800 bp 28S rDNA probe in a double-FISH experiment showed co-localization of these two probes typically on one (exceptionally two to several), mostly acrocentric chromosomes in both *C. albula* and *C. fontanae* (Figure 4c for *C. albula* only, detail of the co-localization in inset). The *Rex1* signal with a distinctly weaker intensity occurred dispersed also on other sites corresponding to the NOR loci in both genomes. The sequences of the *Rex1* derived from the *C. albula* and *C. fontanae* genome were deposited in GenBank under the accession numbers JQ731754 and JQ731760, respectively.

Sequencing of the 18S and 28S rDNA, as well as ITS1 and ITS2 (deposited in GenBank under accession numbers JQ731749-JQ731753 and JQ731755-JQ731759) yielded no significant differences in these genes between *C. albula* and *C. fontanae*.

Discussion

Our findings of extensive genomic re-arrangements of a substantial fraction of the 45S rDNA unit in the *C. fontanae* genome when compared with the situation in *C. albula* are in strong contrast with previously reported low genetic differentiation between these two species [6,18].

Our results indicate that in the genome of *C. fontanae* next to the complete 6–10 NOR loci corresponding to similar number of NOR-bearing chromosomes in *C. albula*,

up to 30 supernumerary and incomplete NOR loci occur. This is supported by results of the sequential fluorescent staining (CMA_3 and DAPI), showing about 6–8 signals in karyotypes of both species, although on chromosomes of *C. fontanae* the signals were slightly weaker. However, these supernumerary sites in *C. fontanae* were not represented by repeating of the complete 45S rDNA unit (i.e. 18S rDNA, ITS1, 5.8S rDNA, ITS2, 28S rDNA)$_n$ but only by a part including probably complete ITS1 and ITS2 and a part of the 28S rDNA adjacent to the ITS2, i.e. the 5′ end of the 28S rDNA gene (the region of 5.8S rDNA was not investigated separately).

Most of the supernumerary signals of the 45S rDNA in chromosomes of *C. fontanae* were localized in the AT rich pericentromeric regions as well as the major accumulation of the *Rex1* retrotransposon on both *C. albula* and *C. fontanae* chromosomes. This is in accordance with findings of other authors describing accumulations of transposable elements in centromeric heterochromatin e.g. in genome of humans [26] and in a cichlid fish *Cichla kelberi* [27,28]. TEs in fishes generally tend to insert to heterochromatic areas of chromosomes ([29]; reviewed by [30]). There are also records of specific integration of some non-LTR retrotransposons at the rRNA genes found in most animal phyla (summarized by [31]), in insects *Drosophila melanogaster* and *Bombyx mori* [32] or in the fish *Erythrinus erythrinus*, where *Rex3* retrotransposons were found in the 5S rRNA genes [25].

In the above-mentioned *E. erythrinus* fish, a similar multiplication of rRNA genes was described [25]. In that case, four karyomorphs of *E. erythrinus* differ in their chromosomal number, karyotype, presence or absence of heteromorphic sex chromosomes and numbers of 5S rDNA loci. The karyomorph A in *E. erythrinus* showed only two 5S rDNA loci, while in the karyomorph D, 21–22 5S rDNA loci could be observed. All 5S rDNA sites co-localized with the *Rex3* retrotransposon. On the other hand, no changes in the heterochromatin and 18S rDNA patterns were found between these two karyomorphs [25]. Such two karyomorphs within a single species *E. erythrinus* may be seen as an incipient stage of a speciation event. This situation can thus represent an initial stage, later resulting in the condition observed in morphologically [15], ecologically and physiologically [33,34] diverged species pair *C. fontanae* and *C. albula* described in this study. A similar observation of extremely multiplied NOR sites (46 and 49 countable FISH signals), however, without any further detailed analysis, were reported in brook char *Salvelinus fontinalis* (Salmonidae) [35].

In salmonid fishes, TEs have been studied intensively [30,36,37]. Microarray studies showed that transcription of rainbow trout transposons is activated by external stimuli, such as toxicity, stress and bacterial antigens [38]. In the oligotrophic Lake Stechlin, the food availability for coregonines was extremely limited and the size at maturity and the maximal size of *C. albula* are far behind the other populations of this species in adjacent lakes in northern Germany [39]. *C. fontanae* is the smallest species of the genus *Coregonus* in Europe [8]. Raising both species in the laboratory demonstrated that both grew much larger if supported with unlimited food (unpublished obs., Freyhof). Therefore, it can be speculated that both species, especially *C. fontanae*, live in an extreme permanent starvation in the Lake Stechlin. It can be also hypothesized that the spring-spawning habit of *C. fontanae* might have originated simply by the shift of sexual maturity in the part of the population that has not been able to attain sexual maturity in autumn due to the lower food intake and hence environmental starvation stress.

Link between environmental stress and chromatin modification/regulation

Effects of stress on the genome can result in important perturbations creating new combinations better compatible with survival (summarized by [40]; more recently reviewed by [41]). After the discovery of transposable elements (TE) more than 50 years ago, their mutagenic effect had been increasingly viewed in association with rapid genome reorganizations by the creation of new regulation patterns and chromosome restructuring during last years [41]. Stress activated mobilization of these elements by failure of epigenetic silencing (the host defence model of repressing the movement of mobile elements; [42,43]) can lead to (re)activation of mobile elements and consequently to major and rapid genome alterations [40,41,44,45].

Barbara McClintock [46] already considered TE as a source of hypermutagenicity creating viable and fertile individuals from a stressed population under risk of extinction. Moreover, she originally named TE "controlling elements" due to their ability to alter gene activity and genome structure [47].

TE-mediated genome rearrangements as a factor in speciation

With growing evidence for the importance of TEs in the genome evolution, the role of TE-mediated genome changes in the speciation by their possible contribution to pre- and post-mating reproductive isolation formation has been increasingly taken into account and discussed generally in eukaryotes [48,49], *Drosophila* [50], fishes [51], mammals [52], and plants [53]. However, lack of experimental data makes it difficult to prove this possibility (reviewed by [51,54,55]). On the other hand, [41] provides an overview of TE transposition bursts

concomitant with radiation periods in seven cases. The same authors also discuss TE-induced rapid speciation associated with the ability of TEs to induce chromosomal rearrangements. Therefore, the sympatric species pair *C. albula* and *C. fontanae* in the context of other congeneric coregonine species and their variable evolutionary history in the Eurasian post-glacial lakes appears to be a suitable model system for exploring mechanisms of genomic differentiation and speciation with or without TE contribution.

In a very similar, but North American study system (lake whitefish species pairs, *Coregonus* spp.), [56] next generation sequencing (NGS) showed that TEs appeared to be highly expressed in hybrids between two recently diverged species. This may be potentially the mechanism responsible for post-zygotic reproductive isolation. Moreover, NGS can be viewed as a useful tool complementary with molecular cytogenetic approach presented in this study enabling confirmation of here documented results and search for other candidate groups of TEs involved in the genome re-arrangements and accelerated speciation.

Conclusion

In the sympatric species pair *C. albula* and *C. fontanae*, we encounter a complex situation involving several evolutionary phenomena and factors. Firstly, a rapid ecological speciation event with an unclear sympatric scenario, i.e. the derived species *C. fontanae* fully differentiated from *C. albula* physiologically, ecologically and morphologically within about 12 – 14 kyrs in the newly colonized Stechlin Lake after the last glacier retreated [15]. Secondly, genetic differentiation of these two species remained weak as the combined analyses of mtDNA and microsatellite loci [18] showed, as well as major karyotypic and chromosomal markers presented in this study. This is in contrast with extensive genome re-arrangements in a large proportion of the 45S rDNA cassette in *C. fontanae* when compared with its most likely ancestral species – *C. albula*. The genome re-arrangements are exhibited as a distinct loci number differences and relocation of variable number (about 30) AT rich pericentromeric regions in *C. fontanae*. The molecular mechanism behind these re-arrangements might be a retrotransposition of a part of the 45S rDNA unit mediated by retrotransposons. Retrotransposonal activity can be mobilized under certain conditions (stress, environmental changes) and cause rapid and extensive structural changes to the host genome. These structural genomic differences in *C. fontanae* accumulated to pericentromeric heterochromatin in almost half of the chromosome complement. This might then have been acting as a partial but permanent reproductive barrier by hampering recombination, thus, enabling and accelerating the morphological, ecological and physiological differentiation of *C. fontanae*. Moreover, interspecific hybridization between the old and the newly arising species might have activated retrotransposonal activity in hybrids resulting in hybrid sterility or unviability as reviewed by [51]. The population genetic parameters of this speciation event, favouring fixation of the re-arranged genomes, remain to be elucidated in detail, but small effective population size is a good hypothesis to be tested.

Methods

Materials

For this study, we had 12 individuals of *Coregonus albula* (Linnaeus, 1758) and 16 individuals of *C. fontanae* [15], both from Lake Stechlin (northern Germany, Brandenburg, 53° 10' N; 13° 02' E). All fish were raised in the laboratory under identical conditions as described by [33,34]. In *C. albula*, 3 individuals (samples alb 1, 2 and 5, males only) yielded metaphases usable for down-stream FISH and CGH experiments. In *C. fontanae*, 3 individuals also (samples font 2, 5 and 7, both males and females) yielded usable chromosome preparations. Of all studied individuals, we isolated genomic DNA from fin clips and muscles. All tissue and DNA samples, including cell suspensions and chromosome preparations, are deposited in the Laboratory of Fish Genetics of the Institute of Animal Physiology and Genetics (IAPG). This study was covered by the "Valid Animal Use Protocols" Nr. CZ 00221 at the IAPG issued by the Czech Ministry of Agriculture on 10 June 2009.

Chromosome preparations

Metaphases were prepared according to [57] with slight modifications. Briefly, the fish were injected with 0.1% colchicine solution (w/v, SIGMA), 1 ml/100 g body weight, for 45 minutes then sacrificed by overdose of anaesthetic 0.5% Phenoxyethanol (v/v, SIGMA). Kidneys were removed, dissected in 0.075 M KCl and the cell suspension free of tissue fragments was hypotonized for 8 min in 0.075 M KCl, fixed in methanol: acetic acid 3:1 (v/v) fixative, washed twice in fixative, and finally spread onto slides (Superfrost quality). Mitotic activity was not stimulated because these fish showed extremely high sensitivity to agents increasing mitotic rate. Simultaneously, the blood (around 0.5 ml) was collected from all analysed individuals by fine heparinized syringe for leukocyte culture according to the protocol of [58]. Briefly, partly washed leukocytes were cultivated in 5 ml of a complete medium composed of TC 199 (SIGMA, St. Louis, MO, USA), 10% FBS Superior (Biochrom, Berlin, Germany), 0.5% Antibiotic Antimycotic Solution (SIGMA), 1% Kanamycin monosulfate (SIGMA), 1% LPS (SIGMA), 0.2% PHA H15 (Remel, Lenexa, KS, USA) and 0.175μl Mercaptoethanol (SIGMA) at 19.5°C for 6–7 days,

then 2 drops of the 0.1% colchicine were added for 45 minutes at RT and cells harvested as for the direct preparation described above.

Fluorescence *in situ* hybridization (FISH) and comparative genomic hybridization (CGH)

Probes for *in situ* hybridization experiments were produced either by PCR (FISH probes) or directly from the genomic DNA (CGH probes). Probes were indirectly labelled with haptens (biotin and digoxigenin) by means of nick translation (whole genomic DNA and FISH probe longer than 600 bp) using the Roche Nick Translation Mix (Roche, Mannheim, Germany; Cat.No. 11745808910) according to the manufacturer's instructions. Shorter DNA fragments were labelled by PCR using the Roche PCR DIG Labeling Mix (Cat.No. 11585550910). The biotin-dUTP labelled probes (Roche, Cat. No. 11093070910) were detected by either the Invitrogen Cy™3-Streptavidin (Invitrogen, San Diego, CA, USA; Cat.No. 43–4315) or by the FITC-Streptavidin (Cat.No. 43–4311). The digoxigenin-dUTP labelled probes (Roche, Cat.No. 11093088910) were detected by either the Roche Anti-Digoxogenin-Fluorescein (Cat.No. 11207741910) or by the Anti-Digoxigenin-Rhodamin (Cat.No. 11207750910). An unlabelled DNA competitor for suppression of nonspecific hybridization of fragment size of 100–200 bp was added with 20-fold the concentration of the DNA probe in CGH experiments. The CGH DNA probe concentration was 1 ug per reaction for both genomes compared. An aging of chromosome preparations at 37°C for 3 hours was carried out prior to each of the hybridization experiment. Pepsinization, hybridization and detection were carried out under conditions as described by [59].

All rDNA FISH probes were constructed using published, mostly generally used PCR primer sets of the 45S rDNA unit to cover its major regions and to map them physically onto chromosomes.

Table 1 PCR primers used in this study

Name	Region of DNA/FISH probe	Primer sequence (5'to3')	Ref.
28S A	3' end of the 28S rDNA involving the regions A and B, F primer	AAA CTC TGG TGG AGG TCC GT	[61]
28S B	Internally nested in the regions A and B of the 28S rDNA, R primer	CTT ACC AAA AGT GGC CCA CTA	[61]
28S C1	5' end of the 28S rDNA adjacent to the ITS2, F primer	ACC CGC TGA ATT TAA GCA T	[62]
28S D2	Internally nested in the region C3 involving D2, C2, D1, C1, R primer	TCC GTG TTT CAA GAC GGG	[63]
ITS1	3' end of the 18S rDNA adjacent to the ITS1, F primer	TCC GTA GGT GAA CCT GCG G	[64]
ITS2	3' end of the 5.8S rDNA adjacent to the ITS2, R primer	GCT GCG TTC TTC ATC GAT GC	[64]
ITS3	5' end of the 5.8S rDNA adjacent to the ITS1, F primer	GCA TCG ATG AAG AAC GCA GC	[64]
ITS4	5' end of the 28S rDNA adjacent to the ITS2, R primer	TCC TCC GCT TAT TGA TAT GC	[64]
NS1	5' end of the 18S rDNA F primer	GTA GTC ATA TGC TTG TCT	[64]
NS2	18 S rDNA R primer	GGC TGC TGG CAC CAG ACT TGC	[64]
NS3	18 S rDNA F primer	GCA AGT CTG GTG CCA GCA GCC	[64]
NS4	18 S rDNA R primer	CTT CCG TCA ATT CCT TTA AG	[64]
NS5	18 S rDNA F primer	AAC TTA AAG GAA TTG ACG GAA G	[64]
NS6	18 S rDNA R primer	GCA TCA CAG ACC TGT TAT TGC CTC	[64]
NS7	18 S rDNA F primer	GAG GCA ATA ACA GGT CTG TGA TGC	[64]
NS8	3' end of the 18S rDNA R primer	TCC GCA GGT TCA CCT ACG GA	[64]
RTX1F1	Rex1 F primer	TTC TCC AGT GCC TTC AAC ACC	[28]
RTX1R3	Rex1 R primer	TCC CTC AGC AGA AAG AGT CTG CTC	[28]
RTX3F1	Rex3 F primer	TAC GGA GAA AAC CCA TTT CG	[65]
RTX3F2	Rex3 F primer	AAC ACC TTG GCT GCG CCT AG	[65]
RTX3F3	Rex3 F primer	CGG TGA YAA AGG GCA GCC CTG	[28]
RTX3R1	Rex3 R primer	AAA GTT CCT CGG TGG CAA GG	[65]
RTX3R2	Rex3 R primer	CCR GGG GTG GAT GAR RTC CGC CC	[65]
RTX3R3	Rex3 R primer	TGG CAG ACN GGG GTG GTG GT	[28]
RTX6F	Rex6 F primer	TAA AGC ATA CAT GGA GCG CCA C	[28]
RTX6R	Rex6 R primer	GGT CCT CTA CCA GAG GCC TGG G	[28]

PCR amplification of FISH probes and the analysis of the 45S rDNA unit

All primer sets used in this study are summarized in Table 1. Primers nesting within the 45S rDNA unit relevant for this study are shown in Figure 1. Thermal profiles were used according to references given in Table 1. FISH probes were constructed from PCR conducted on the respective species as they were later hybridized. All sequences used in this study as FISH probe or in the molecular-biological analyses of the 45S rDNA unit were deposited in the GenBank [60] under accession numbers JQ731749 - JQ731760.

Cloning, sequencing and sequences analysis

PCR products were cloned using the QIAGEN PCR Cloning Kit and QIAGEN EZ Competent Cells (Qiagen, Hilden, Germany); the plasmids were isolated from the cells with Qia PREP Spin Miniprep Kit according to the manufacturer's instructions. The primary PCR products were first sequenced on the ABI 3130 Genetic Analyzer (Applied Biosystems, Hitachi, Foster City, CA, USA) using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). Furthermore, cloned DNA fragments that were later applied as FISH probes were commercially sequenced by Macrogen (Seoul, South Korea). The commercially obtained sequences were subjected to online megablast or discontiguous megablast [66] searches at the National Center for Biotechnology Information (NCBI) [67], where their similarity to the sequences deposited in the GenBank databases was checked.

Microscopy and image processing

Chromosome preparations were analysed with the Provis AX70 Olympus microscope equipped with standard fluorescence filter sets. Gray-scale hybridization signals on chromosomes and/or DAPI counterstained chromosomes were captured by the CCD camera (DP30W Olympus). Using the Olympus Acquisition Software, black and white images were pseudo-coloured and superimposed with the software MicroImage. The colour images have been analyzed and processed with Adobe Photoshop, Version CS5. The chromosomes were classified using the nomenclature proposed by [68]. Karyotypes based on the Giemsa-stained chromosomes were produced using the IKAROS (Metasystems) software. Chromosomal formulas were formed according to [69].

Abbreviations

AFLP: Amplified fragment length polymorphism; CGH: Comparative genomic hybridization; CMA_3: Chromomycin A_3; DAPI: 4', 6-diamidino-2-phenylindole; DAPI/$CMA_3^{+/-}$: DAPI/CMA_3 positive/negative signals; FISH: Fluorescence *in situ* hybridization; IAPG: Institute of animal physiology and genetics; kyrs BP: Thousand years before presence; NF: "Nombre fundamental" chromosome arm number; NGS: Next generation sequencing; NOR: Nucleolar organizer DNA; rDNA: Ribosomal DNA; TE: Transposable elements; 2n: Diploid chromosome number.

Competing interests

There are no competing interests to declare.

Authors' contributions

RS designed and performed experiments FISH and CGH and co-drafted the manuscript, ZM and AS performed FISH experiments, ZM performed PCRs and sequenced PCR products and co-drafted the manuscript, GS and JF collected and raised material, JB and RS prepared chromosome preparations, MR partly contributed to digital processing of images, PR designed the study and co-drafted the manuscript. All authors read and approved the final manuscript.

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References

1. Cristescu ME, Adamowicz SJ, Vaillant JJ, Haffner DG: **Ancient lakes revisited: from the ecology to the genetics of speciation.** *Mol Ecol* 2010, **19:**4837–4851.
2. Schluter D: **Ecological speciation in postglacial fishes.** *Philos T Roy Soc B* 1996, **351:**807–814.
3. Nelson JS: *Fishes of the world*. 4th edition. Hoboken, NJ: John Wiley & Sons; 2006.
4. Bodaly RA, Vuorinen J, Ward RD, Luczynski M, Reist JD: **Genetic comparison of new and old world coregonid fishes.** *J Fish Biol* 1991, **38:**37–51.
5. Hudson AG, Vonlanthen P, Seehausen O: **Rapid parallel adaptive radiations from a single hybridogenetic ancestral population.** *P Roy Soc B-Biol* 2011, **278:**58–66.
6. Mehner T, Pohlmann K, Elkin C, Monaghan MT, Nitz B, Freyhof J: **Genetic population structure of sympatric and allopatric populations of Baltic ciscoes (*coregonus albula* complex, teleostei, coregonidae).** *BMC Evol Biol* 2010, **10:**85.
7. Douglas MR, Brunner PC, Bernatchez L: **Do assemblages of *coregonus* (teleostei: salmoniformes) in the central alpine region of Europe represent species flocks?** *Mol Ecol* 1999, **8:**589–603.
8. Kottelat M, Jörg F: *Handbook of European Freshwater Fishes*. Cornol, Switzerland: Publications Kottelat; 2007.
9. Vonlanthen P, Roy D, Hudson AG, Largiader CR, Bittner D, Seehausen O: **Divergence along a steep ecological gradient in lake whitefish (*coregonus* sp.).** *J Evolution Biol* 2009, **22:**498–514.
10. Taylor EB: **Species pairs of north temperate freshwater fishes: evolution, taxonomy, and conservation.** *Rev Fish Biol Fisher* 1999, **9:**299–324.
11. Vuorinen J, Himberg M, Lankinen P: **Genetic differentiation in *coregonus albula* (salmonidae) populations in Finland.** *Hereditas* 1981, **94:**113–121.
12. Hudson AG, Vonlanthen P, Müller R, Seehausen O: **Review: the geography of speciation and adaptive radiation in coregonines.** *Adv Limnol* 2007, **60:**111–146.
13. Phillips R, Räb P: **Chromosome evolution in the salmonidae (pisces): an update.** *Biol Rev* 2001, **76:**1–25.
14. Quimseyh MB: **Evolution of number and morphology of mammalian chromosomes.** *J Hered* 1994, **85:**455–465.
15. Schulz M, Freyhof J: ***Coregonus fontanae*, a new spring-spawning Cisco from lake stechlin, northern Germany (salmoniformes: coregonidae).** *Ichthyol Explor Fresh* 2003, **14:**209–216.
16. Ohlberger J, Mehner T, Staaks G, Hoelke F: **Is ecological segregation in a pair of sympatric coregonines supported by divergent feeding efficiencies?** *Can J Fish Aquat Sci* 2008, **65:**2105–2113.

17. Jankun M, Martinez P, Pardo BG, Kirtiklis L, Rab P, Rabova M, Sanchez L: **Ribosomal genes in coregonid fishes (*coregonus lavaretus, C. Albula* and *C. Peled*) (salmonidae): single and multiple nucleolus organizer regions.** *Heredity* 2001, **87:**672–679.
18. Schulz M, Freyhof J, Saint-Laurent R, Østbye K, Mehner T, Bernatchez L: **Evidence for independent origin of two spring-spawning ciscoes (Salmoniformes: Coregonidae) in Germany.** *J Fish Biol* 2006, **68:**119–135.
19. Swarzacher HG, Wachtler F: **The nucleolus.** *Anat Embryol* 1993, **188:**515–536.
20. Wachtler F, Stahl A: **The nucleolus: a structural and functional interpretation.** *Micron* 1993, **24:**473–505.
21. Moss T, Stefanovsky VY: **Promotion and regulation of ribosomal transcription in eukaryotes by RNA polymerase I.** *Prog Nucleic Acids Res Mol Biol* 1994, **50:**25–66.
22. Wang S, Zhao M, Li T: **Complete sequence of the 10.3 kb silkworm attacus ricini rDNA repeat,determination of the transcriptional initiation site and functional analysis of the intergenic spacer.** *DNA Seq* 2003, **14:**95–101.
23. Hillis DM, Dixon MT: **Ribosomal DNA: molecular evolution and phylogenetic inference.** *Q Rev Biol* 1991, **66:**411–453.
24. Volff J-N, Körting C, Schartl M: **Multiple lineages of the non-LTR retrotransposon *Rex1* with varying success in invading fish genomes.** *Mol Biol Evol* 2000, **17:**1673–1684.
25. Cioffi MB, Martins C, Bertollo LA: **Chromosome spreading of associated transposable elements and ribosomal DNA in the fish *Erythrinus erythrinus*. Implications for genome change and karyoevolution in fish.** *BMC Evol Biol* 2010, **10:**271.
26. Wong LH, Andy Choo KH: **Evolutionary dynamics of transposable elements at the centromere.** *Trends Genet* 2004, **20:**611–616.
27. Teixeira WG, Ferreira IA, Cabral-de-Mello DC, Mazzuchelli J, Valente GT, Pinhal D, Poletto AB, Martins C: **Organization of repeated DNA elements in the genome of the cichlid fish *cichla kelberi* and its contribution to the knowledge of fish genomes.** *Cytogenet Genome Res* 2009, **125:**224–234.
28. Valente GT, Mazzuchelli J, Ferreira IA, Poletto AB, Fantinatti BEA: **Cytogenetic mapping of the retroelements *Rex1*, *Rex3* and *Rex6* among cichlid fish: new insights on the chromosomal distribution of transposable elements.** *Cytogenet Genome Res* 2011, **133:**34–42.
29. DaSilva C, Hadji H, Ozouf-Costaz C, Nicaud S, Jaillon O, Weissenbach J, Roest Crollius H: **Remarkable compartmentalization of transposable elemetns and pseudogenes in the heterochromatin of the *tetraodon nigroviridis* genome.** *Proc Natl Acad Sci U S A* 2002, **99:**1636–1641.
30. Koop BF, Davidson WS: **Genomics and the Genome Duplication in Salmonids**. In *Fisheries for Global Welfare and Environment*. Edited by Tsukamoto K, Kawamura T, Takeuchi T, Beard TD Jr, Kaiser MJ. Tokyo: Terrapub; 2008:77–86.
31. Kurzynska-Kokorniak A, Jamburuthugoda VK, Bibillo A, Eickbush TH: **DNA-directed DNA polymerase and strand displacement activity of the reverse transcriptase encoded by the R2 retrotransposon.** *J Mol Biol* 2007, **374:**322–333.
32. Jakubczak JL, Xiong Y, Eickbush TH: **Type I (RI) and type II (R2) ribosomal DNA insertions of *drosophila melanogaster* are retrotransposable elements close11 related to those of bombyx Mori.** *J Mol Biol* 1990, **212:**37–52.
33. Ohlberger J, Mehner T, Staaks G, Hölker F: **Temperature-related physiological adaptations promote ecological divergence in a sympatric species pair of temperate freshwater fish. Coregonus spp.** *Funct Ecol* 2008, **22:**501–508.
34. Ohlberger J, Staaks G, Petzoldt T, Mehner T, Hölker F: **Physiological specialization by thermal adaptation drives ecological divergence in a sympatric fish species pair.** *Evol Ecol Res* 2008, **10:**1173–1185.
35. Fujiwara A, Abe S, Yamaha E, Yamazaki F, Yoshida MC: **Chromosomal localization and heterochromatin association of ribosomal RNA gene loci and silver-stained nucleolar organizer regions in salmonid fishes.** *Chromosome Res* 1998, **6:**463–471.
36. Matveev V, Okadaa N: **Retroposons of salmonoid fishes (actinopterygii: salmonoidei) and their evolution.** *Gene* 2008, **434:**16–28.
37. De Boer JG, Yazawa R, Davidson WS, Koop BF: **Burst and horizontal evolution of DNA transposons in the speciation of pseudotetraploid salmonids.** *BMC Genomics* 2007, **8:**422.
38. Krasnov A, Koskinen H, Afanasyev A, Mölsä H: **Transcribed Tcl-like transposons in salmonid fish.** *BMC Genomics* 2005, **6:**107.
39. Helland IP, Harrod C, Freyhof J, Mehner T: **Co-existence of a pair of pelagic planktivorous coregonid fishes.** *Evol Ecol Res* 2008, **10:**373–390.
40. Arnault C, Dufournel I: **Genome and stresses: reactions against aggressions, behaviour of transposable elements.** *Genetica* 1994, **93:**149–160.
41. Rebollo R, Horard B, Hubert B, Vieira C: **Jumping genes and epigenetics: towards new species.** *Gene* 2010, **454:**1–7.
42. Bestor TH, Tycko B: **Creation of genomic methylation patterns.** *Nat Genet* 1996, **12:**363–367.
43. Yoder JA, Walsh CP, Bestor TH: **Cytosine methylation and the ecology of intragenomic parasites.** *Trends Genet* 1997, **13:**335–340.
44. Wichman HA, Van Den Bussche RA, Hamilton MJ, Baker RJ: **Transposable elements and the evolution of genome organization in mammals.** *Genetica* 1992, **86:**287–293.
45. Biémont C: **A brief history of the status of transposable elements: from junk DNA to major players in evolution.** *Genetics* 2010, **186:**1085–1093.
46. McClintock B: **The significance of responses of the genome to challenge.** *Science* 1984, **226:**792–801.
47. McClintock B: **Mutable loci in maize.** *Carnegie Inst Wash Yrbk* 1949, **48:**142–154.
48. Hurst GDD, Werren JH: **The role of selfish elements in eukaryotic evolution.** *Nat Rev Genet* 2001, **2:**597–606.
49. Hua-Van A, Le Rouzic A, Boutin TS, Filée J, Capy P: **The struggle for life of the genome's selfish architects.** *Biol Direct* 2011, **6:**19.
50. Vieira C, Lepetit D, Dumont S, Biémont C: **Wake up of transposable elements following *drosophila simulans* worldwide colonization.** *Mol Biol Evol* 1999, **16:**1251–1255.
51. Volff JN: **Genome evolution and biodiversity in teleost fish.** *Heredity* 2005, **94:**280–294.
52. Böhne A, Brunet F, Galiana-Arnoux D, Schultheis C, Volff J-N: **Transposable elements as drivers of genomic and biological diversity in vertebrates.** *Chromosome Res* 2008, **16:**203–215.
53. Noor MAF, Chang AS: **Evolutionary genetics: jumping into a New species.** *Curr Biol* 2006, **16:**R890–R892.
54. Hurst GDD, Schilthuizen M: **Selfish genetic elements and speciation.** *Heredity* 1998, **80:**2–8.
55. Kazazian HH Jr: **Mobile elements: drivers of genome evolution.** *Science* 2004, **303:**1626–1632.
56. Renaut S, Nolte AW, Bernatchez L: **Mining transcriptome sequences towards identifying adaptive single nucleotide polymorphisms in lake whitefish species pairs (*coregonus* spp. Salmonidae).** *Mol Ecol* 2010, **19**(Suppl.1):115–131.
57. Ráb P, Roth P: **Cold-blooded vertebrates**. In *Methods of chromosome analysis*. Edited by Balicek P, Forejt J, Rubes J. Brno: Cytogenet Sect Cs Biol Soc Publishers; 1988:115–124.
58. Fujiwara A, Nishida-Umehara C, Sakamoto T, Okamoto N, Nakayama I, Abe S: **Improved fish lymphocyte culture for chromosome preparation.** *Genetica* 2001, **111:**77–89.
59. Cremer M, Grasser F, Lanctôt C, Müller S, Neusser M, Zinner R, Solovei I, Cremer T: **Multicolor 3D Fluorescence *In Situ* Hybridization for Imaging Interphase Chromosomes**. In *The Nucleus: Volume I: Nuclei and Subnuclear Components, Methods in Molecular Biology™*, Volume Chapter 15, Volume 463. Edited by Hancock R.: Humana Press; 2008:205–239. Springer Protocols.
60. *Database GenBank*. http://www.ncbi.nlm.nih.gov/genbank/.
61. Zhang Q, Cooper RK, Tiersch TR: **Chromosomal location of the 28S ribosomal RNA gene of channel catfish by in situ polymerase chain reaction.** *J Fish Biol* 2000, **56:**388–397.
62. Dayrat B, Tillier A, Lecointre G, Tillier S: **New clades of euthyneuran gastropods (mollusca) from 28S rRNA sequences.** *Mol Phylogenet Evol* 2001, **19:**225–235.
63. Chombard C, Boury-Esnault N, Tillier S: **Reassessment of homology of morphological characters in tetractinellid sponges based on molecular data.** *Syst Biol* 1998, **47:**351–366.
64. White TJ, Bruns T, Lee S, Taylor JW: **Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics**. In *PCR Protocols: A Guide to Methods and Applications*. Edited by Innis MA, Gelfand DH, Sninsky JJ, White TJ. New York: Academic Press Inc; 1990:315–322.

65. Volff JN, Körting C, Meyer A, Schartl M: **Evolution and discontinuous distribution of Rex3 retrotransposons in fish.** *Mol Biol Evol* 2001, **18**:427–431.
66. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ: **Basic local alignment search tool.** *J Mol Biol* 1990, **215**:403–410.
67. *National Center for Biotechnology Information.* http://www.ncbi.nlm.nih.gov/blast.
68. Levan AK, Fredga K, Sandberg AA: **Nomenclature for centromeric position on chromosomes.** *Hereditas* 1964, **52**:201–220.
69. Matthey R: **L'evolution de la formule chromosomale chez les vertébrés.** *Experientia* 1945, **1**:50–56.

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7.5. Publication V

Ráb P., Yano C., Lavoué S., Jegede O., Bertollo L., Ezaz T., **Majtánová Z.**, Oliveira E., Cioffi M. 2016. Karyotype and repetitive DNAs mapping in the African butterfly fish *Pantodon buchholzi*, the sole species of the family Pantodontidae. Cytogenet. Genome Res. DOI: 10.1159/000450534. IF_{2015}: 1.638

Author contribution: amplification, purification, sequencing and analysing of *MLL* gene, preparation of online suppl. Fig. 1, co-drafting the manuscript

I declare that the contribution of Zuzana Majtánová to the publication Ráb et al. (2016), as stated here, is true.

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Karyotype and Mapping of Repetitive DNAs in the African Butterfly Fish *Pantodon buchholzi*, the Sole Species of the Family Pantodontidae

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Key Words
African butterfly fish · Conventional staining techniques · Fluorescence in situ hybridization · Karyotype · Osteoglossiformes · Repetitive DNAs

Abstract
The monophyletic order Osteoglossiformes represents one of the most ancestral groups of teleosts and has at least 1 representative in all continents of the southern hemisphere, with the exception of Antarctica. However, despite its phylogenetic and biogeographical importance, cytogenetic data in Osteoglossiformes are scarce. Here, karyotype and chromosomal characteristics of the lower Niger River population of the African butterfly fish *Pantodon buchholzi*, the sole species of the family Pantodontidae (Osteoglossiformes), were examined using conventional and molecular cytogenetic approaches. All specimens examined had 2n = 46 chromosomes, with a karyotype composed of 5 pairs of metacentric, 5 pairs of submetacentric, and 13 pairs of acrocentric chromosomes in both sexes. No morphologically differentiated sex chromosomes were identified. C-bands were located in the centromeric/pericentromeric region of all chromosomes and were associated with the single AgNOR site. FISH with ribosomal DNA probes revealed that both 5S and 18S rDNA were present in only 1 pair of chromosomes each, but did not colocalize. CMA_3^+ bands were observed near the telomeres in several chromosome pairs and also at the 18S rDNA sites. The mapping of di- and trinucleotide repeat motifs, *Rex6* transposable element, and U2 snRNA showed a scattered distribution over most of the chromosomes, but for some microsatellites and the U2 snRNA also a preferential accumulation at telomeric regions. This study presents the first detailed cytogenetic analysis in the African butterfly fish by both conventional and molecular cytogenetic protocols. This is the first of a series of further cytogenetic and cytogenomic studies on osteoglossiforms, aiming to comprehensively examine the chromosomal evolution in this phylogenetically important fish order.
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The monophyletic order Osteoglossiformes is an ancient group of fishes that are restricted to freshwater [Bănărescu, 1990; Greenwood and Wilson, 1998; Hilton, 2003]. These fishes belong to the Osteoglossomorpha, one of the 3 monophyletic lineages making up Teleostei [Near et al., 2012], defined by their possession of bony tongues

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and intestine that passes posteriorly left to the stomach [Nelson, 2006]. They are currently distributed in tropical South America, Africa, Australia, and Southeast Asia [Nelson, 2006; Berra, 2007] where they adapted to a large range of ecological conditions and diversified into more than 220 described species. The Osteoglossiformes comprise 6 families, namely: Osteoglossidae, Arapaimidae, and Pantodontidae (these 3 families are sometimes considered as subfamilies of a more inclusive family Osteoglossidae), and Notopteridae, Gymnarchidae, and Mormyridae [Greenwood et al., 1966; Taverne, 1998; Hilton, 2003].

Among osteoglossiforms, the African freshwater butterfly fish *Pantodon buchholzi* is the sole species of the family Pantodontidae. This species is easily identifiable because of, among other characters, its distinctive head morphology and its enlarged pectoral fins [Greenwood and Thomson, 1960; Kershaw, 1970]. *P. buchholzi* is a small (usually less than 10 cm) and obligatory air breathing species and a surface swimmer that presents the peculiar ability to jump out of the water surface to escape predators or catch prey [Saidel et al., 2004]. The geographic distribution of *Pantodon* comprises 2 main regions in Africa, the lower Niger and Congo River basins [Lavoué et al., 2011] along with a potential relict population from the Jong River in Sierra Leone [Teugels, 2003]. Despite the absence of external morphological differentiation between the 2 allopatric populations (lower Niger vs. Congo River), their divergence was surprisingly estimated to more than 50 Mya using molecular data [Lavoué et al., 2011].

The phylogeny of the extant osteoglossiforms is relatively well-known, with morphological and molecular characteristics supporting the same topology, with the exception of the position of *Pantodon* [e.g., Hilton, 2003; Lavoué et al., 2011]. Morphology supports the hypothesis that *Pantodon* is closely related to Osteoglossidae and Arapaimidae [e.g., Li and Wilson, 1996; Taverne, 1998; Hilton, 2003; Wilson and Murray, 2008], whereas molecular data support an alternative hypothesis in which *Pantodon* is the sister group of the rest of Osteoglossiformes [Lavoué and Sullivan, 2004; Inoue et al., 2009; Lavoué et al., 2011; Mu et al., 2012; Lavoué, 2016]. The reasons for this 'genetics-morphology' incongruence are still poorly understood, and independent evidence, such as cytogenetic data, is relevant to evaluate which phylogenetic hypothesis should be favored.

Despite the importance to gather several lines of evidence from *P. buchholzi* for understanding the evolution of osteoglossiforms [Berra, 2007; Sallan, 2014 and references therein], the cytogenetic data of this species are restricted to only 1 study, published more than 40 years ago [Uyeno, 1973]. Due to the former technology, high resolution preparations were unavailable, and the results of Uyeno [1973] were mostly inconclusive. Precisely, this study reported an ambiguous chromosome number (2n = 46 or 48) and karyotype composition based on only few metaphase plates obtained by squashing of the gill epithelium. The only 2 *P. buchholzi* individuals examined by Uyeno [1973] were of unknown geographic origin, furthermore restraining the conclusions of this study.

To clarify the chromosomal features of *P. buchholzi* and to gain insight into its evolution within Osteoglossiformes, we performed a detailed analysis of the karyotypes applying standard and advanced molecular cytogenetic techniques to the lower Niger River population of this species [Lavoué et al., 2011]. In addition to conventional chromosomal methods, 9 classes of repetitive DNA sequences, including the multigene families 5S and 18S rDNA and U2 snRNA, di- and trinucleotide microsatellites, and the retrotransposable element *Rex6*, were mapped by FISH on the chromosomes. Our data unambiguously established a diploid chromosome number of 2n = 46 for *P. buchholzi* from the lower Niger River basin and represent the first reliable chromosomal data for *P. buchholzi* from individuals of known origin (i.e., from the Niger River basin) by both conventional and molecular cytogenetic protocols. This study is the first one of a series of cytogenetic and cytogenomic studies focusing on osteoglossiforms.

Materials and Methods

Materials, DNA Samples, Specimens and Identification

Ten individuals of *P. buchholzi* (6 males and 4 females) were obtained from the Oluwa River, Okitipupa, lower Niger River basin, Ondo State, Nigeria (6°51

Mitotic Chromosome Preparation and Banding

Mitotic chromosomes were obtained from cell suspensions of the anterior kidney and spleen, using the conventional air-drying [Bertollo et al., 2015] and the caudal fin regeneration methods [Völker and Ráb, 2015]. Conventional staining was done using 5% Giemsa solution in phosphate buffer pH 6.8 for 10 min. The C-banding method was used to demonstrate C-positive heterochromatin [Sumner, 1972], silver staining to detect AgNORs [Howell and Black, 1980], and chromomycin A_3 (CMA_3) staining to show GC-rich chromosome regions [Schmid, 1980].

Molecular Probes and FISH Experiments

Four tandemly arrayed DNA sequences were obtained via PCR from the nuclear DNA of *P. buchholzi*. These include a 5S rDNA repeat copy comprising 120 bp of the 5S rRNA gene and 200 bp of the non-transcribed spacer, produced according to Pendás et al. [1994]. The second probe contained an 18S rDNA repeat consisting of a 1,400-bp copy of the 18S rRNA gene, obtained according to Cioffi et al. [2009]. Both rDNA probes were cloned into plasmid vectors and propagated in DH5α *Escherichia coli* competent cells (Invitrogen, San Diego, Calif., USA). The retrotransposable element *Rex6* was obtained using primers described in Volff et al. [1999]. The U2 snDNA sequence was produced by PCR according to Úbeda-Manzanaro et al. [2010] using primers derived from the U2 coding sequence of several model organisms available in GenBank [Cross et al., 2006]. The 5S and 18S rDNA, U2 snDNA and *Rex6* probes were directly labeled with Spectrum Orange-dUTP by nick translation according to the manufacturer's recommendations (Roche, Mannheim, Germany).

FISH was performed under high stringency conditions on chromosome spreads as described in Pinkel et al. [1986]. The chromosome slides were incubated with RNAse (40 µg/ml) for 1.5 h at 37°C. After denaturation of the chromosomal DNA in 70% formamide/2× SSC at 70°C, spreads were incubated in 2× SSC, pH 7.0, for 4 min at 70°C. The hybridization mixture (2.5 ng/µl probes, 2 µg/µl salmon sperm DNA, 50% deionized formamide, 10% dextran sulfate) was dropped on the slides, and hybridization was performed for 14 h at 37°C in a moist chamber containing 2× SSC. The post-hybridization wash was carried out with 1× SSC for 5 min at 65°C. A final wash was performed at room temperature in 4× SSCT for 5 min. Finally, the chromosomes were counterstained with DAPI (1.2 µg/ml) and mounted in antifade solution (Vector, Burlingame, Calif., USA).

FISH experiments with the microsatellite probes $(CA)_{15}$, $(GC)_{15}$, $(TA)_{15}$, $(GAA)_{10}$, and $(CAG)_{10}$ were performed as described in Kubát et al. [2008], with slight modifications. These sequences were directly labeled with Cy3 at the 5′ termini during synthesis by Sigma (St. Louis, Mo., USA). The chromosomes were counterstained with DAPI (1.2 µg/ml) and mounted in antifade solution.

Image Processing

At least 30 metaphase spreads per individual were analyzed to confirm the diploid chromosome number, karyotype structure and FISH results. Images were captured using an Olympus BX50 microscope (Olympus Corporation, Ishikawa, Japan) with CoolSNAP and processed using Image Pro Plus 4.1 software (Media Cybernetics, Silver Spring, Md., USA). Chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st), or acrocentric (a) according to their arm ratios [Levan et al., 1964].

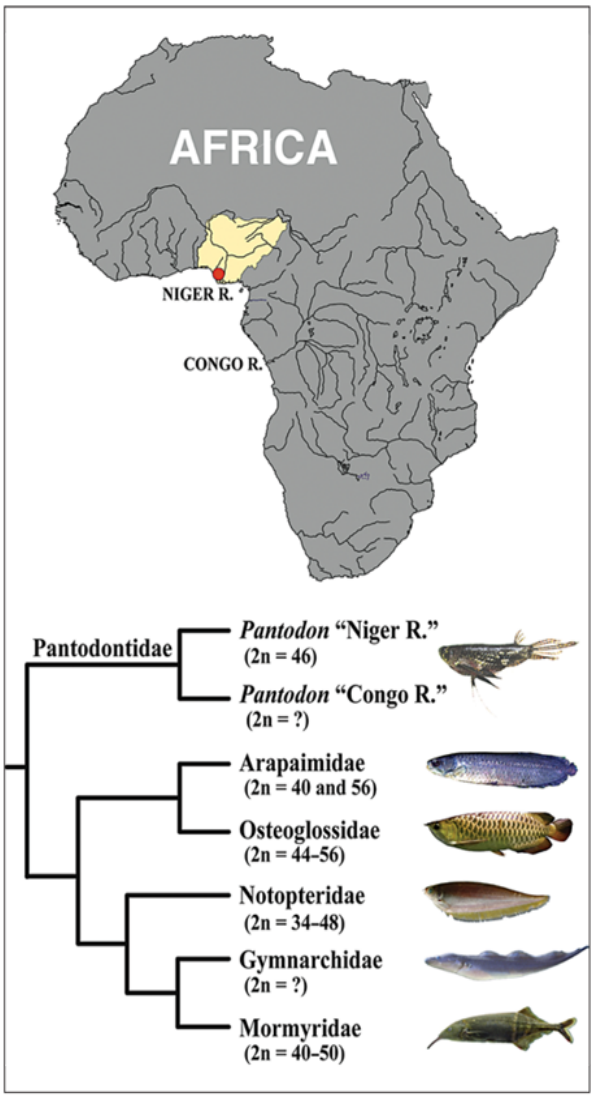


Fig. 1. Top: Map of Africa. The collection locality (Oluwa River, lower Niger River basin) in Nigeria of the *P. buchholzi* specimens examined in this study is indicated (red dot). Nigeria is highlighted in yellow. Bottom: Phylogenetic tree of osteoglossiform families showing their known 2n values.

Results

All *P. buchholzi* individuals had 2n = 46, with a karyotype composed of 10 metacentric, 10 submetacentric, and 26 acrocentric chromosomes and a fundamental number of 66 in both sexes, without morphologically differentiated sex chromosomes. C-positive heterochromatic bands were located in the centromeric/pericentromeric

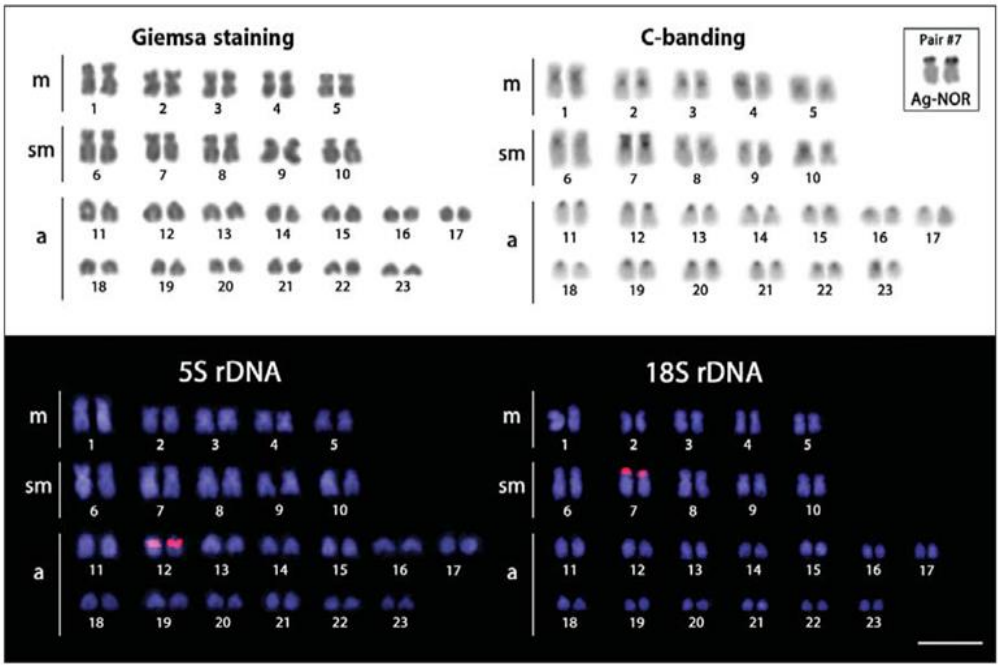


Fig. 2. Karyotypes of *P. buchholzi* arranged from Giemsa-stained (documenting 2n = 46, 10m+10sm+26a) and C-banded chromosomes (showing centromeric C-positive heterochromatin, with a conspicuous mark on the short arms of pair 7, corresponding to the AgNOR location – also in the detail) and after FISH with rDNAs. 5S and 18S rDNA sequences are located in pairs 12 and 7, respectively. Bar = 5 µm.

regions of all chromosomes, as well as at the single NOR site (fig. 2). FISH with the 5S rDNA probe showed a signal in the pericentromeric region of pair No. 12, while 18S rDNA was located in the distal part of the short arms of the submetacentric pair No. 7, corresponding to the Ag-NOR site. Multiple CMA_3^+ bands were observed, present in the telomeric region of several chromosome pairs and also at the 18S rDNA site (fig. 3).

The microsatellite repeat motifs were located within the heterochromatic regions where a significant fraction of repetitive DNA is present. Here, microsatellites $(CA)_{15}$ and $(GC)_{15}$ showed distinct accumulation in the subtelomeric regions of all chromosomes, while $(TA)_{15}$, $(GAA)_{10}$, and $(CAG)_{10}$ exhibited a dispersed pattern with signals broadly distributed over the chromosomes; $(GAA)_{10}$ also showed slight accumulation in the telomeric regions. The retrotransposon *Rex6* presented a similar hybridization pattern with dispersed signals across the whole chromosome complement, including euchromatic and heterochromatic regions (fig. 3). This same distribution pattern was also observed for the U2 snRNA, but with some accumulation in the terminal region of several chromosomes (fig. 3).

Discussion

Cytogenetic data in Osteoglossiformes are sporadic and usually restricted to the description of the diploid chromosome number, the karyotype composition, and some conventional banding and staining features, such as C-positive heterochromatin and AgNOR distribution patterns (see table 1). Regarding *P. buchholzi*, due to methodological limitations, Uyeno [1973] was not able to properly define the 2n (46 or 48) and karyotype structure for this species. Our data unambiguously established 2n = 46 for *P. buchholzi* from the lower Niger River basin. Furthermore, we demonstrated that 10 metacentric, 10 submetacentric, and 26 acrocentric chromosomes (10m+10sm+26a) compose its karyotype which is different to that reported by Uyeno [1973] (i.e., 12m+12sm/st+24a). Such differences can be the consequence of the progress in cytogenetic methods overcoming previous technical limitations. Since the locality of the *Pantodon* specimens examined by Uyeno [1973] is not specified, we cannot exclude the possibility that these variations may also be due to the different populations (lower Niger River vs. Congo River region).

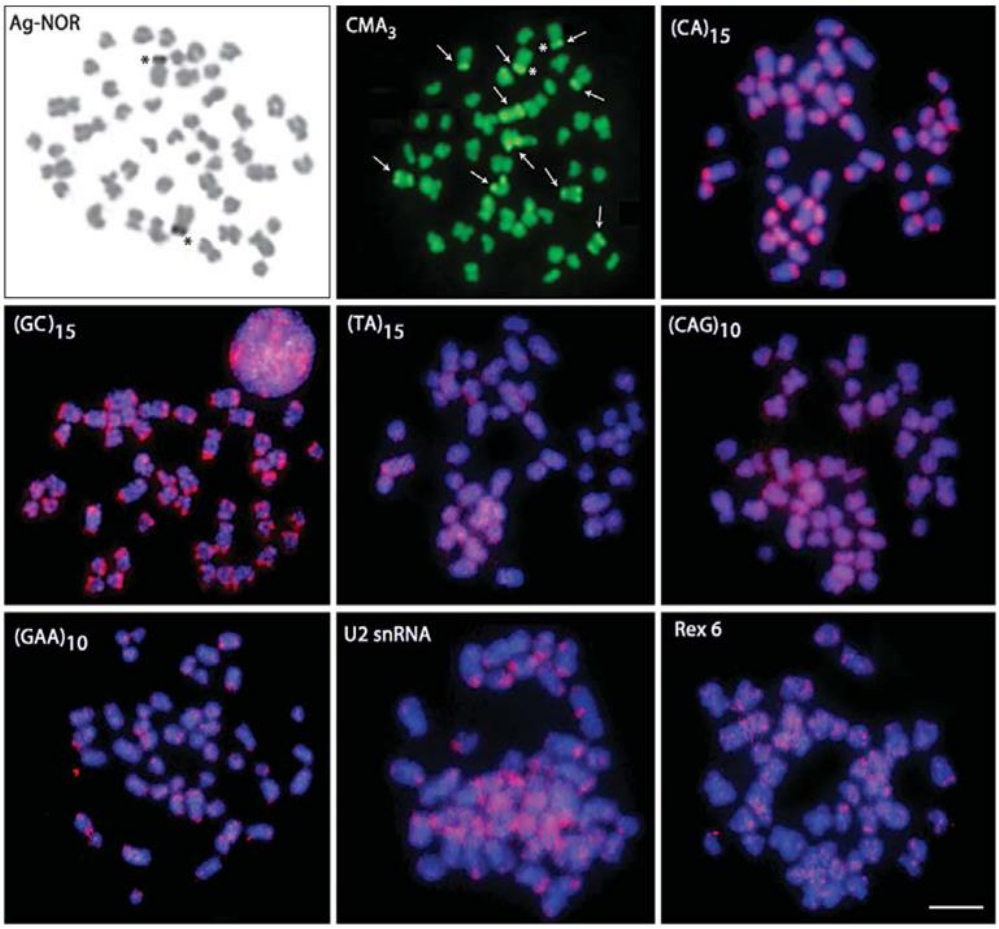


Fig. 3. Metaphase plates of *P. buchholzi* after AgNOR and CMA_3 staining and FISH with di- and trinucleotide microsatellite sequences, U2 snRNA and *Rex6* probes. The arrows indicate the CMA_3^+ sites, and the asterisks show the AgNOR pair. Bar = 5 μm.

Lavoué et al. [2011] showed that the 2 allopatric populations of *P. buchholzi*, from Niger and Congo River basins respectively, are genetically well differentiated from each other. Their divergence time was estimated to be at least ~57 Mya. The discovery of such intraspecific genetic differentiation between these 2 *Pantodon* populations was surprising because it is nearly as great as that between South American arowana *(Osteoglossum bicirrhosum)* and Southeast Asian arowana *(Scleropages formosus),* whereas there is no significant morphological differentiation within *Pantodon*. In this context, cytogenetic data from each of the 2 populations of *Pantodon* are necessary to further characterize their differentiation and to infer the degree of their reproductive incompatibility. Given the large genetic differentiation between the African populations of *Pantodon*, it is expected that the 2 populations present markedly different karyotypes.

Cytogenetic data from other osteoglossiform species are crucial to interpret the data from *Pantodon* from an evolutionary context. These data are reviewed in table 1 and hereafter. Among Osteoglossiformes, the diploid chromosome number ranges from 34 in *Papyrocranus afer* (Notopteridae) to 56 in *O. bicirrhosum* (Osteoglossidae) and *Arapaima gigas* (Arapaimidae) (see fig. 1). Within Osteoglossidae, the 2n is known for 5 species and ranges from 44 *(Scleropages leichardti)* to 56 *(O. bicirrhosum)*.

In this family, the karyotypes are dominated by monoarmed chromosomes, although in some species such as *S.*

Table 1. Review of available data on diploid chromosome numbers, karyotype compositions, and fundamental numbers in the order Osteoglossiformes

Family/Species	2n	Karyotype	FN	Locality	Reference
Arapaimidae					
Arapaima gigas	56	4m+12sm/st+40a	72	South America	Urushido et al., 1975
	56	28m/sm+28st/a	84	Brazil, Araguaia River	Marques et al., 2006
	56	28m/sm+28st/a	84	Brazil, Jamari River	da Rosa et al., 2009
Heterotis niloticus	40	26m+10sm+4a	76	Western Africa	Arai, 2011
Osteoglossidae					
Osteoglossum bicirrhosum	56	1sm+1st+54a	58	not known	Uyeno, 1973
	56	56st/a (3st+53a)	56	aquarium trade	Suzuki et al., 1982
	56	1sm+1st+54a	57/58	South America	Arai, 2011: U-68, H13, H40
	56	3st+53a	56/59	South America	Arai, 2011: S-125
Osteoglossum ferreirai	54	2m+4sm+14st+34a	60	aquarium trade	Suzuki et al., 1982
Scleropages formosus	50	10st+4sm+11a	39	Southeastern Asia	Urushido et al., 1975
	50	4m/sm+10st+36a	54/64	Southeastern Asia	Arai, 2011
	48	18sm+30a	64	Singapore, Qian Hu fish farm	Shen et al., 2014
Scleropages jardinii	48	16m+6sm+26a	70	New Guinea	Arai, 2011
	48	16m+6sm+26a	70	Australia	Hirata and Urushido, 2000
Scleropages leichardti	44	16m+8sm+6st+14a	74	Australia	Hirata and Urushido, 2000
	44	16m+8sm+6st+14a	68/74	Australia	Arai, 2011: H14
Pantodontidae					
Pantodon buchholzi	48	12m+12sm/st+24a	72	Africa	Uyeno, 1973
	46	10m+10sm+26a	66	Nigeria, Oluwa River	present work
	48	12m+12sm/st+24a	72	Western Africa	Arai, 2011
Notopteridae					
Xenomystus nigri	42	42a	42	Africa	Uyeno, 1973
Chitala chitala	48	12m+36a	60	India, Delhi, River Jumna	Nayyar, 1965
	42	42a	42	not known	Uyeno, 1973
	42	42a	42	aquarium trade	Takai and Ojima, 1998
Notopterus notopterus	42	42a	42	India, Kurukshetra	Rishi and Singh, 1983
	42	42a	42	Thailand	Srivastava, 1964
	48	12m+36a	60	India, Delhi, River Jumna	Nayyar, 1965
	42	42a	42	not known	Urushido et al., 1975
	42	42a	42	Thailand	Donsakul and Magtoon, 1990
	42	42a	42	Thailand, Chi Basin	Silawong et al., 2012
Chitala ornata	42	42a	42	Thailand, Chi Basin	Donsakul and Magtoon, 1990
	42	42a	42	Thailand, Chi Basin	Supiwong et al., 2012
Chitala blanci	42	42a	42	Thailand, Chi Basin	Donsakul and Magtoon, 1990
Papyrocranus afer	34	4sm+30a	38	Africa	Uyeno, 1973
Mormyridae					
Gnathonemus petersii	48	10m+6sm+32a	64	Africa	Uyeno, 1973
	48	18m+2sm+28a	68	aquarium trade	Ozouf-Costaz et al., 2015
Brienomyrus brachyistius	48	1m+4sm+2st+41a	53	Africa	Uyeno, 1973
Marcusenius moorii	50	4sm+46a	54	Cameroon, Ntem River	Ozouf-Costaz et al., 2015
Ivindomyrus opdenboshi	50	10m+2sm+38a	62	Cameroon, Ntem River	Ozouf-Costaz et al., 2015
Paramormyrops sp.7	50	2m+6sm+42a	56	Cameroon, Woleu River	Ozouf-Costaz et al., 2015
Stomatorhinus walkeri	50	2m+48a	52	Gabon, Ogooué River	Ozouf-Costaz et al., 2015
Petrocephalus microphthalmus	50	2m+48a	52	Gabon, Ogooué River	Ozouf-Costaz et al., 2015
Pollimyrus cf. nigricans	40	2m+38a	42	Côte d'Ivoire, White Nile basin	Krysanov and Golubtsov, 2014

formosus, the proportion of other chromosome types is significant. Within Arapaimidae, 40 chromosomes were determined for *Heterotis niloticus* [Arai, 2011] and 56 for *A. gigas* [Urushido et al., 1975; Marques et al., 2006; da Rosa et al., 2009] before cryptic diversity was discovered in the latter genus [Stewart, 2013a, b]. Data for Notopteridae indicate a lower 2n for this family compared to other osteoglossiform families, ranging from 34 in *P. afer* to 42 in *Chitala blanci, C. ornata, Notopterus notopterus*, and *Xenomystus nigri*. The notopterid karyotypes are generally composed of only acrocentric chromosomes. In addition, intraspecific variation was found in 1 notop-

terid species having different diploid numbers [Nayyar, 1965]. Concerning the African electric fishes (Gymnarchidae and Mormyridae), 40–50 chromosomes compose the karyotypes of the few mormyrids analyzed up to now, and most of these chromosomes are acrocentric (table 1). No cytogenetic data are available for *Gymnarchus niloticus*, the sole species of the family Gymnarchidae.

As reviewed above and in table 1, the cytogenetic data from Osteoglossiformes are still sporadic and far from complete. This hampers the evolutionary comparison of the *Pantodon* karyotype and the determination of the cytogenetic evolutionary trends in Osteoglossiformes. We note, for example, that all South American *(Osteoglossum)* and Southeast Asian/Australian *(Scleropages)* osteoglossids have distinct features related to their 2n and karyotype organizations. Similarly, the chromosomal data of South American and African arapaimid species significantly differ, as well as those of African and South Asian notopterids. Considering what it is known about the cytogenetics of Osteoglossiformes, the karyotype of *Pantodon*, with 2n = 46, represents a unique feature inside this order, supporting the phylogenetic hypothesis based on molecular analyses and its inclusion into a separate family.

GC-specific fluorochromes, such as CMA_3 combined with enhancing counterstains like DAPI or methyl green, have shown that teleostean fishes tend to contain only 1 pair of chromosomes bearing CMA_3^+ sites which are associated with the major rDNA loci [Mayr et al., 1985; Amemiya and Gold, 1986; Schmid and Guttenbach, 1988]. To date, this association has been observed not only in a number of teleostean groups but also in representatives of non-teleostean lineages [Ráb et al., 1999; Gornung, 2013]. Inside Osteoglossiformes, an association of GC-rich heterochromatin with the NOR was also previously reported in *A. gigas* [da Rosa et al., 2009]. Our results in *Pantodon* showed multiple cytogenetically detectable CMA_3^+ sites, one of them associated with the major rDNA, making its genome a remarkable exception from the general pattern found among fishes.

A considerable fraction of the eukaryotic genome consists of repetitive DNA sequences, which include multigene families, satellites, microsatellites, and transposable elements (TEs) [Jurka et al., 2005]. Although the rDNA multigene families have been reported in a small number of teleosts, their distribution in only 1 chromosome pair represents the most frequent condition [Gornung, 2013]. In *A. gigas* and in some Mormyridae, the major 18S rRNA genes were also confined to 1 chromosome pair, not homologous across species [Marques et al., 2006; da Rosa et al., 2009; Ozouf-Costaz et al., 2015]. Accordingly, in *P. buchholzi* only 1 chromosome pair each bears 18S and 5S rDNA. In this way, the presence of only two 5S and 18S rDNA sites and the association of GC-rich heterochromatin with the major rDNA loci in representatives of the ancient Osteoglossiformes, reinforces the idea that such phenotypes may represent the ancestral state for teleost fishes. In turn, the cytogenetic mapping of the multigene family U2 snRNA in *P. buchholzi* provided insights into the genome composition of this species, resulting in useful markers for comparative genomics at the chromosomal level. This snRNA showed a dispersed distribution over the chromosomes, with some specific accumulation/amplification near the telomeric regions. Until now, there is no information about the distribution pattern of U2 snRNA for other osteoglossiform fishes. However, our findings are quite similar to those reported in other teleosts, such as *Amphichthys cryptocentrus, Batrachoides manglae, Porichthys plectrodon*, and *Thalassophryne maculosa* (Batrachoidiformes, Batrachoididae), where these sequences are widely scattered throughout the genome [Úbeda-Manzanaro et al., 2010].

Microsatellites or simple sequence repeats are substantial components of the repetitive DNA, and there are speculations that they may have derived from TEs [Kidwell, 2002]. These repeats are usually located in the heterochromatic regions of fish genomes. In fact, the dinucleotide sequences $(CA)_{15}$ and $(GC)_{15}$ were accumulated in the subtelomeric regions of all chromosomes, while the other microsatellites showed a dispersed distribution over chromosomes including euchromatic and heterochromatic regions, despite some accumulation of $(GAA)_{10}$ in the telomeric regions. Contrasting with the present data, only the $(GAA)_{10}$ microsatellite produced a clear and abundant hybridization pattern in the pericentromeric/telomeric heterochromatin of some Mormyridae species [Ozouf-Costaz et al., 2015]. TEs constitute another important class of repetitive DNA widely studied in many organisms. The ubiquitous presence of TEs in heterochromatic regions can be correlated with a possible structural role or with the reduced selective pressure acting on these regions, due to their poor gene content [Hua-Van et al., 2005]. However, despite their preferential localization in noncoding regions, their distribution pattern in heterochromatin and euchromatin is extremely variable in fish genomes [Volff et al., 2003; Ferreira et al., 2011]. Among TEs, *Rex6* represents a reverse transcriptase encoding retrotransposon that shows a broad distribution and genome organization in several fish species [Volff et al., 1999; Cioffi and

Bertollo, 2012; Yano et al., 2014], which can be found both compartmentalized in the heterochromatic regions or scattered across the genome [Ferreira et al., 2011]. *P. buchholzi* also shares this last condition, as *Rex6* signals are broadly distributed over the whole chromosome complement of this species.

Conclusion

In summary, we analyzed the chromosomes of *P. buchholzi,* the sole species of the family Pantodontidae, using standard and molecular cytogenetic approaches. Our study is the first one to offer reliable chromosomal data for *P. buchholzi* from individuals of known origin (i.e., from the Niger River basin) by both conventional and molecular cytogenetic protocols, and certainly, additional data from the Congo River region are now needed for further comparative population analyses.

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Statement of Ethics

All experiments followed ethical and anesthesia conducts in accordance with the Ethics Committee (process number CEUA 1926260315).

Disclosure Statement

The authors declare no conflicts of interest.

References

Amemiya CT, Gold JR: Chromomycin A_3 stains nucleolus organizer regions of fish chromosomes. Copeia 1:226–231 (1986).

Arai R: Fish Karyotypes. A Check List (Springer, Tokyo 2011).

Bănărescu P: Zoogeography of Fresh Waters. General Distribution and Dispersal of Freshwater Animals (Aula-Verlag, Wiesbaden 1990).

Berra TM: Freshwater Fish Distribution (University of Chicago Press, Chicago 2007).

Bertollo LAC, Cioffi MB, Moreira-Filho O: Direct chromosome preparation from freshwater teleost fishes, in Ozouf-Costaz C, Pisano E, Foresti F, Almeida Toledo LF (eds): Fish Cytogenetic Techniques (Ray-Fin Fishes and Chondrichthyans), pp 21–26 (CRC Press, Enfield 2015).

Cioffi MB, Bertollo LAC: Chromosomal distribution and evolution of repetitive DNAs in fish, in Garrido-Ramos MA (ed): Repetitive DNA. Genome Dynamics, vol 7, pp 197–221 (Karger, Basel 2012).

Cioffi MB, Martins C, Bertollo LAC: Comparative chromosome mapping of repetitive sequences. Implications for genomic evolution in the fish, *Hoplias malabaricus.* BMC Genet 10:34 (2009).

Cross I, Merlo A, Manchado M, Ifante C, Canavate JP, Rebordinos L: Cytogenetic characterization of the sole *Solea senegalensis* (Teleostei: Pleuronectiformes: Soleidae): Ag-NOR, $(GATA)_n$, $(TTAGGG)_n$ and ribosomal genes by one-color and two-color FISH. Genetica 128:253–259 (2006).

da Rosa R, Rubert M, Caetano-Filho M, Giuliano-Caetano L: Conserved cytogenetic features in the Amazonian Arapaima, *Arapaima gigas* (Schinz 1822) from Jamari River, Rondônia–Brazil. Open Biol J 2:91–94 (2009).

Donsakul T, Magtoon W: A Chromosome Study on Three Species of Notopterid Fishes, *Notopterus notopterus, N. chitala* and *N. blanci* from Thailand (Tech Pap Agric Biol, Srinakharinwirot University 1990).

Ferreira DC, Porto-Foresti F, Oliveira C, Foresti F: Transposable elements as a potential source for understanding the fish genome. Mob Genet Elements 1:112–117 (2011).

Gornung E: Twenty years of physical mapping of major ribosomal RNA genes across the teleosts: a review of research. Cytogenet Genome Res 141:90–102 (2013).

Greenwood PH, Thomson KS: The pectoral anatomy of *Pantodon buchholzi* Peters (a freshwater flying fish) and the related Osteoglossidae. J Zool 135:283–301 (1960).

Greenwood PH, Wilson MVH: Bonytongues and their allies, in Paxton JR, Eschmeyer WN (eds): Encyclopedia of Fishes, ed 2, pp 80–84 (Academic Press, San Diego 1998).

Greenwood PH, Rosen DE, Weitzman SH, Myers GS: Phyletic studies teleostean fishes, with a provisional classification of living forms. Bull Am Mus Nat Hist 131:339–456 (1966).

Hilton EJ: Comparative osteology and phylogenetic systematics of fossil and living bonytongue fishes (Actinopterygii, Teleostei, Osteoglossomorpha). Zool J Linn Soc 137:1–100 (2003).

Hirata J, Urushido T: Karyotypes and DNA content in the Osteoglossiformes (in Japanese with English abstract). Sci Rep Res Inst Evol Biol 9:83–90 (2000).

Howell WM, Black DA: Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia 36:1014–1915 (1980).

Hua-Van A, Le Rouzic A, Maisonhaute C, Capy P: Abundance, distribution and dynamics of retrotransposable elements and transposons: similarities and differences. Cytogenet Genome Res 110:426–440 (2005).

Inoue JG, Kumazawa Y, Miya M, Nishida M: The historical biogeography of the freshwater knifefishes using mitogenomic approaches: a Mesozoic origin of the Asian notopterids (Actinopterygii: Osteoglossomorpha). Mol Phylogenet Evol 51:486–499 (2009).

Jurka J, Kapitonov VV, Pavlicek A, Klonowski P, Kohany O, Walichiewicz J: Repbase update, a database of eukaryotic repetitive elements. Cytogenet Genome Res 110:462–467 (2005).

Kershaw DR: The cranial osteology of the 'Butterfly Fish', *Pantodon buchholzi* Peters. Zool J Linn Soc 49:5–19 (1970).

Kidwell MG: Transposable elements and the evolution of genome size in eukaryotes. Genetica 115:49–63 (2002).

Krysanov EY, Golubtsov AS: Karyotypes of four fish species from the Nile and Omo-Turkana basins in Ethiopia, East Africa. J Ichthyol 54: 889–992 (2014).

Kubát Z, Hobza R, Vyskot B, Kejnovský E: Microsatellite accumulation on the Y chromosome in *Silene latifolia*. Genome 51:350–356 (2008).

Lavoué S: Was Gondwanan breakup the cause of the intercontinental distribution of Osteoglossiformes? A time-calibrated phylogenetic test combining molecular, morphological, and paleontological evidence. Mol Phylogenet Evol 99:34–43 (2016).

Lavoué S, Sullivan JP: Simultaneous analysis of five molecular markers provides a well-supported phylogenetic hypothesis for the living bony-tongue fishes (Osteoglossomorpha: Teleostei). Mol Phylogenet Evol 33:171–185 (2004).

Lavoué S, Miya M, Arnegard ME, McIntyre PB, Mamonekene V, Nishida M: Remarkable morphological stasis in an extant vertebrate despite tens of millions of years of divergence. Proc Biol Sci 278:1003–1008 (2011).

Levan A, Fredga K, Sandberg AA: Nomenclature for centromeric position on chromosomes. Hereditas 52:201–220 (1964).

Li GQ, Wilson MVH: Phylogeny of Osteoglossomorpha, in Stiassny ML, Parenti LR, Johnson JD (eds): Interrelationships of Fishes, pp 163–174 (Academic Press, New York 1996).

Marques DK, Venere PC, Galetti PM Jr: Chromosomal characterization of the bonytongue *Arapaima gigas* (Osteoglossiformes: Arapaimidae). Neotrop Ichthyol 4:215–218 (2006).

Mayr B, Ráb P, Kalat M: Localization of NORs and counterstain-enhanced fluorescence studies in *Perca fluviatilis* (Pisces, Percidae). Genetica 67:51–56 (1985).

Mu XD, Wang XJ, Song HM, Yang YX, Luo D, et al: Mitochondrial DNA as effective molecular markers for the genetic variation and phylogeny of the family Osteoglossidae. Gene 511: 320–325 (2012).

Nayyar RP: Karyotype studies in the genus *Notopterus* (Lacepede). The occurrence and fate of univalent chromosomes in spermatocytes of *N. chitala*. Genetica 36:398–406 (1965).

Near TJ, Eytan RI, Dornburg A, Kuhn KL, Moore JA, et al: Resolution of ray-finned fish phylogeny and timing of diversification. Proc Natl Acad Sci USA 109:13698–13703 (2012).

Nelson JS: Fishes of the World, ed 4 (John Wiley & Sons, Inc., Hoboken 2006).

Ozouf-Costaz C, Coutanceau JP, Bonillo C, Belkadi L, Fermon Y, et al: First insights into karyotype evolution within the family Mormyridae. Cybium 39:227–236 (2015).

Pendás AM, Morán P, Freije JP, Garcia Vazquez E: Chromosomal mapping and nucleotide sequence of two tandem repeats of Atlantic salmon 5S rDNA. Cytogenet Cell Genet 67: 31–36 (1994).

Pinkel D, Straume T, Gray J: Cytogenetic analysis using quantitative, high sensitivity, fluorescence hybridization. Proc Natl Acad Sci USA 83:2934–2938 (1986).

Ráb P, Rábová M, Reed KM, Phillips RB: Chromosomal characteristics of ribosomal DNA in the primitive semionotiform fish, longnose gar *Lepisosteus osseus*. Chromosome Res 7: 475–480 (1999).

Rishi KK, Singh J: Chromosomes of *Notopterus notopterus* (Pallas) (Notopteridae: Clupeiformes). Chromosome Inform Service 34: 10–11 (1983).

Saidel WM, Strain GF, Fornari SK: Characterization of the aerial escape response of the African butterfly fish, *Pantodon buchholzi* Peters. Env Biol Fish 71:63–72 (2004).

Sallan LC: Major issues in the origins of ray-finned fish (Actinopterygii) biodiversity. Biol Rev 89:950–971 (2014).

Sambrook JH, Russell DW: Molecular Cloning (Cold Spring Harbor Laboratory Press, New York 2001).

Schmid M: Chromosome banding in Amphibia. IV. Differentiation of GC- and AT-rich chromosome regions in Anura. Chromosoma 77: 83–103 (1980).

Schmid M, Guttenbach M: Evolutionary diversity of reverse (R) fluorescent chromosome bands in vertebrates. Chromosoma 97:101–114 (1988).

Shen XY, Kwan HY, Thevasagayam NM, Prakki SRS, Kuznetsova IS, et al: The first transcriptome and genetic linkage map for Asian arowana. Mol Ecol Resour 14:622–635 (2014).

Silawong K, Aoki S, Supiwong W, Tanomtong A, Khakhong S, et al: The first chromosomal characteristics of nucleolar organizer regions (NORs) in grey featherback fish, *Notopterus notopterus* (Osteoglossiformes, Notopteridae) by conventional and Ag-NOR staining techniques. Cytologia 77:279–285 (2012).

Srivastava MD: The structure and behavior of chromosomes in six freshwater teleosts. Cellule 65:93–98 (1964).

Stewart DJ: Re-description of *Arapaima agassizii* (Valenciennes), a rare fish from Brazil (Osteoglossomorpha: Osteoglossidae). Copeia 2013:38–51 (2013a).

Stewart DJ: A new species of *Arapaima* (Osteoglossomorpha: Osteoglossidae) from the Solimões River, Amazonas State, Brazil. Copeia 2013:470–476 (2013b).

Sumner AT: Simple technique for demonstrating centromeric heterochromatin. Exp Cell Res 75:304–306 (1972).

Supiwong W, Tanomtong A, Khakhong S, Silawong K, Aoki S, Sanoamuang L: The first chromosomal characteristics of nucleolar organizer regions and karyological analysis of clown knife fish, *Chitala ornata* (Osteoglossiformes, Notopteridae) by T-lymphocyte cell culture. Cytologia 77:393–399 (2012).

Suzuki A, Taki Y, Urushido T: Karyotypes of two species of arowana, *Osteoglossum bicirrhosum* and *O. ferreirai*. Jpn J Ichthyol 29:220–222 (1982).

Takai A, Ojima Y: C-banded karyotype and nucleolus organizer regions of a notopterid fish, *Notopterus chitala* (Notopteridae, Osteoglossiformes). Chromosome Sci 2:35–38 (1998).

Taverne L: Les Ostéoglossomorphes marins de l'Eocène du Monte Bolca (Italie): *Monopteros* Volta 1796, *Thrissopterus* Heckel, 1856 et *Foreyichthys* Taverne, 1979. Considérations sur la phylogénie des Téléostéens ostéoglossomorphes. Studi e Ricerche sui Giacimenti Terziari di Bolca (Museo Civico di Storia Naturale, Verona 1998).

Teugels GG: Pantodontidae, in Paugy D, Lévêque C, Teugels GG (eds): The Fresh and Brackish Water Fishes of West Africa, vol 1, pp 148–150 (IRD Editions, Paris 2003).

Úbeda-Manzanaro M, Merlo MA, Palazón JL, Cross I, Serasquete C, Rebordinos L: Chromosomal mapping of the major and minor ribosomal genes, $(GATA)_n$ and U2 snRNA gene by double-colour FISH in species of the Batrachoididae family. Genetica 138:787–794 (2010).

Urushido T, Takahashi E, Taki Y: Karyotypes of three species of fishes in the order Osteoglossiformes. Chromosome Inform Service 18: 20–22 (1975).

Uyeno T: A comparative study of chromosomes in the teleostean fish order Osteoglossiformes. Jpn J Ichthyol 20:211–217 (1973).

Volff JN, Korting C, Sweeney K, Schartl M: The non-LTR retrotransposon *Rex3* from the fish *Xiphophorus* is widespread among teleosts. Mol Biol Evol 16:1427–1438 (1999).

Volff JN, Bouneau L, Ozouf-Costaz C, Fischer C: Diversity of retrotransposable elements in compact pufferfish genomes. Trends Genet 19:674–678 (2003).

Völker M, Ráb P: Direct chromosome preparation from regenerating fish fin tissue, in Ozouf-Costaz C, Pisano E, Foresti F, Almeida-Toledo LF (eds): Fish Cytogenetic Techniques (Ray-Fin Fishes and Chondrichthyans), pp 37–41 (CRC Press, Enfield 2015).

Wilson MVH, Murray AM: Osteoglossomorpha: phylogeny, biogeography, and fossil record and the significance of key African and Chinese fossil taxa, in Cavin L, Longbottom A, Richter M (eds): Fishes and the Break-up of Pangaea, pp 185–219 (Geological Society of London, London 2008).

Yano CF, Poltronieri J, Bertollo LAC, Artoni RF, Liehr T, Cioffi MB: Chromosomal mapping of repetitive DNAs in *Triportheus trifurcatus* (Characidae, Characiformes): insights into the differentiation of the Z and W chromosomes. PLoS One 9:e90946 (2014).

7.6. Publication VI

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Author contribution: optimization of GISH and CGH experiments on different fish species, image processing, co-drafting the manuscript

I declare that the contribution of Zuzana Majtánová to the publication Symonová et al. (2015), as stated here, is true.

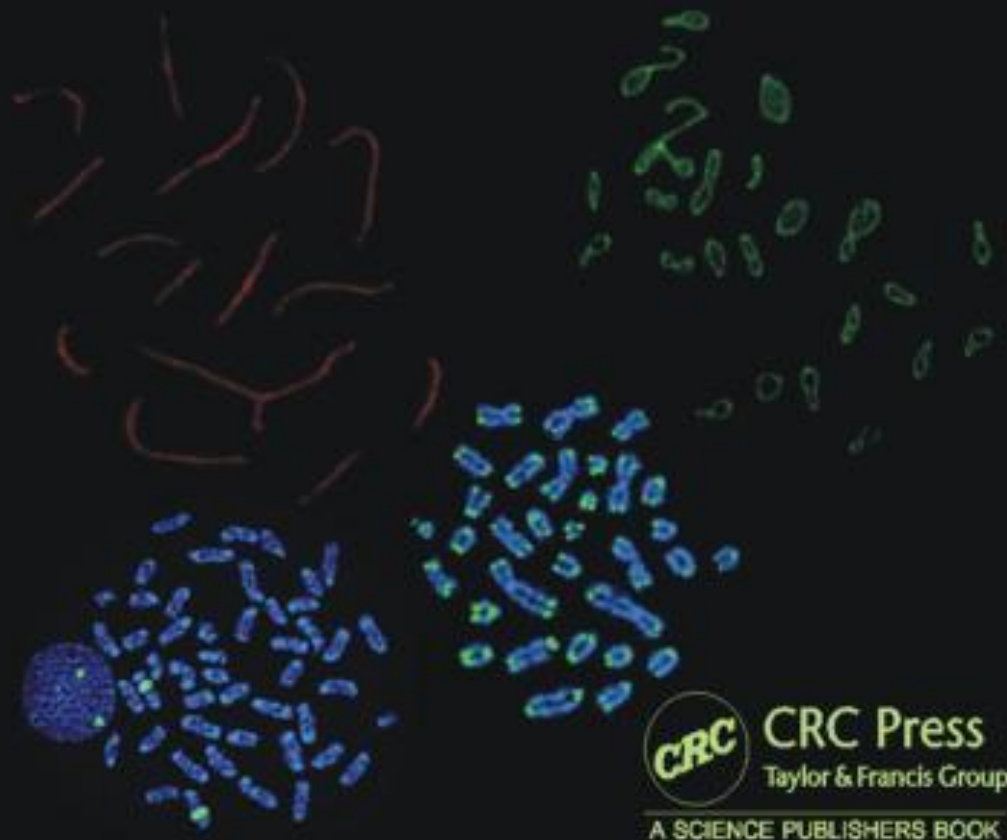
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16

Characterization of Fish Genomes by GISH and CGH

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Principles

Using total genomic DNA (gDNA) as a probe for *in situ* hybridization (ISH) experiments is a modified FISH (fluorescence *in situ* hybridization) technique that becomes an increasingly relevant approach in non-clinical research, including fish molecular cytogenetics. GISH (genomic *in situ* hybridization) and CGH (comparative genomic hybridization) are two basic types of ISH experiments using gDNA as probes. These methods utilize DNA-DNA hybridization and sequence similarities to visualize genome specific DNA on metaphase spreads. They allow for comprehensive analysis of entire genomes:

1. In GISH the labelled gDNA of one organism is used as a probe and hybridized to target chromosomal DNA of another, usually a hybrid organism, to discriminate chromosomes originating in one of the parental species.
2. CGH enables the detection of unbalanced chromosomal rearrangements. In clinical application, one reference gDNA probe (healthy control) and one studied gDNA probe (patient, DNA from tumor cells) are cohybridized to reference metaphase spreads only. In molecular cytogenetics this method enables comparison of two differentially labelled gDNA probes on

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chromosomes of one or both tested genomes (i.e., reciprocal scheme when chromosomes preparations of both genomes are available). In principle, in a typical CGH experiment, one of the compared gDNA always belongs to the genome that is identical with the genome of chromosomes tested. The experiment may be designed as intraspecies or interspecies CGH. The intraspecies design may involve a comparison of male and female gDNA to identify sex-specific chromosomes (regions) or a comparison of a tumour genome (patient) with healthy DNA (reference) on normal chromosomes. The interspecies design may involve gDNA of two or several species compared against each other. For an example and specific details in fish see Symonová et al. (2013a).

A variety of modifications of CGH and GISH exist based on the tested system and DNA/chromosome probes available, in the end blurring the differences between these two approaches. Actually, the most striking difference is in the target genome—a hybrid of compared organisms (mostly GISH) or a pure species more or less related to the compared organisms (mostly CGH). Both approaches employ an unlabelled competitor DNA, *sensu* Sealey et al. (1985) or the Cot1-DNA, *sensu* Landegent et al. (1987). Total gDNA of another species, phylogenetically not closely related, is used unlabelled and sheared (physically or enzymatically) to about 100 bp long fragments as competitor DNA to suppress hybridization of unspecific repetitive sequences, blurring the species' specific signal.

Applications and Limitations

GISH has been routinely used especially in plant cytogenetics to discriminate parental genomes in allopolyploids, e.g., Takahashi et al. (1999), Matoba et al. (2007) and Lim et al. (2007). In conventional GISH experiments, labelled gDNA from one species and unlabelled competitor DNA from another species are simultaneously applied to chromosome spreads to map chromosomal distribution of DNA of the labelled genome. Later, comparative GISH (cGISH) technology has been introduced in plants She et al. (2012). In cGISH experiments, the labelled gDNA of one species hybridizes to chromosomes of more or less related species without the application of competitor DNA; thus, the hybridization signals represent DNA sequences in common between the two species. Therefore, cGISH technology is a useful tool for comparative genome analysis to visualize chromosomal distribution of conserved DNA sequences.

CGH has been developed as a tool for solid tumour cytogenetics, to analyze chromosomal imbalances in entire genomes (Kallioniemi et al. 1992). Since cells of solid tumours usually do not produce usable chromosome spreads, it is possible to compare their genome with a reference (healthy) genome in the form of gDNA to normal (healthy) chromosomes. An analogous situation can be found, when an entire genome comparison of more fish species is desirable.

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However, in some species no usable chromosome spreads are available. In this case, only their gDNA can be used for comparison with chromosomes of other species.

In fishes, male/female CGH was applied to mitotic and meiotic chromosomes of the zebrafish *Danio rerio*, the platyfish *Xiphophorus maculatus* and the guppy *Poecilia reticulata* to localize sex-specific chromosome regions and track early stages of sex chromosome differentiation (Traut and Winking 2001). Application of CGH has also proven to be a good tool in genomic comparison within sympatric species pairs of *Coregonus* as shown by Symonová et al. (2013b). Cross-species GISH using labelled cDNA of tilapia *Oreochromis niloticus* were used to compare syntenic chromosomal regions across the genomes of several South American and African cichlid species (Valente et al. 2009). Identification of species-specific chromosomes in an allotetraploid gynogenetically reproducing female genome of *Carassius auratus gibelio* was documented using GISH by Zhu and Gui (2007). A similar study was performed by Knytl et al. (2013) in European *Carassius* and revealed a natural allotetraploid female.

Another example of the useful application of GISH in fishes is the identification of nuclear genomic constituents in hybrid spined loaches. Across Europe, the spined loach of the genus *Cobitis* produced 2n, 3n and 4n clonal lineages. The all-female clones originated by hybridization and subsequent genome addition in pre-Holocene times and reproduce by gynogenesis. The high morphological, and in some cases biochemical, similarity makes proper identification of their biotypes and studies on chromosomal rearrangements difficult. For instance, genomes of *C. tanaitica* and *C. taenia* in their hybrids cannot be distinguished by allozyme studies, but they can be clearly identified by GISH. Moreover, homologous and homoeologous chromosomes of hybrids cannot be distinguished in the normal DAPI stained metaphase. Here, as an example, we show results of a GISH experiment with labelled gDNA from both parental species as probes to discriminate genomic composition of the *C. 2elongatoides-1tanaitica* hybrid. GISH clearly distinguishes the two haploid sets of the *C. elongatoides* genome and one haploid set of the *C. tanaitica* chromosomes in this hybrid (see Fig. 1).

Protocol

The following protocols are modified combinations of procedures described by Cremer et al. (2008), Bi and Bogart (2006), Traut et al. (1999) and references therein.

Chromosome spreads should be prepared in advance, so that chromosome aging can be carried out.

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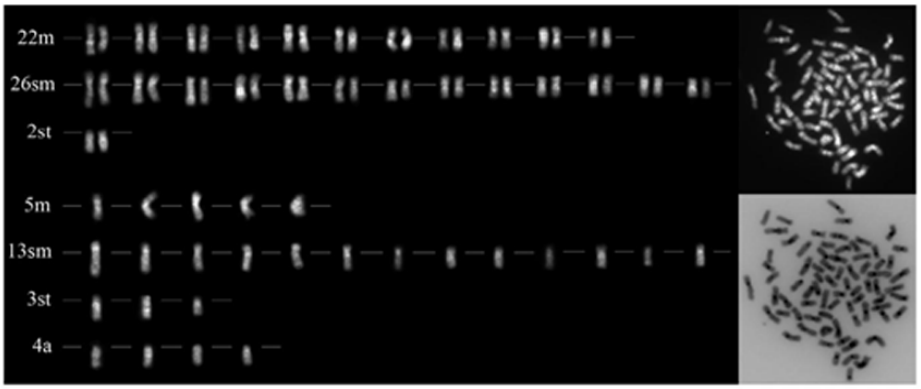


Figure 1. Representative metaphases of the *Cobitis 2elongatoides-1tanaitica* hybrid visualized by GISH with *C. elongatoides* and *C. tanaitica* genomic probes. Homologous and homoeologous chromosomes of triploid hybrids cannot be distinguished in normal DAPI stained metaphase, however, the GISH clearly distinguishes the *C. elongatoides* (shown as those with green fluorescence) and *C. tanaitica* chromosomes (shown with red fluorescence) in *C. 2elongatoides-1tanaitica* hybrid.

Color image of this figure appears in the color plate section at the end of the book.

1. gDNA isolation and specific competitor/Cot1-DNA preparation

The phenol-chloroform method of gDNA isolation, *sensu* Graham (1978) can be employed as well as using commercial kits, e.g., DNeasy Blood & Tissue Kit (Qiagen). The total DNA amount required for one GISH/CGH experiment is related to the amount of the competitor or Cot1-DNA. According to standardized protocols, e.g., Cremer et al. (2008), 1 µg of gDNA per genome probe is prepared. Therefore, when using a competitor or Cot1-DNA in 3 to 50 times higher amount than the probe, as much as 4 to 51 µg of gDNA is required for one experiment and one genome. Application of competitor DNA involves using additional unlabelled gDNA sonicated into 100–200 bp long fragments in the final concentration 3 to 50 times higher than the probe. The intensity and number of cycles of sonication has to be tested for each DNA probe individually. A crucial step is cooling the vessel containing DNA after each cycle of sonication to avoid an excess of degradation. The concentration of the competitor DNA has to be measured after the sonication, because during this step, some DNA can be lost. The preparation of the Cot1-DNA is described by Trifonov et al. (2009).

In this step, a way of gDNA amplification may become useful, e.g., GenomiPhi DNA amplification kits by GE Healthcare or Amersham Biosciences as well as a way of DNA concentration using a vacuum concentrator machine.

Particularly for CGH, it is crucial to isolate a higher amount of gDNA of very good quality in terms of its purity and similar fragment size. Therefore, the conventional phenol-chloroform method is rather suggested for this step. In the case only when a little amount of the starting material (tissue) is available, the Chelex® 100 gDNA extraction can be employed as described by Walsh et al. (1991).

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2. Chromosome spreads preparation and aging

Chromosome spreads can be produced by either of the methods described in this book. Other possibilities to produce chromosome spreads in fishes are lymphocyte culture described by Fujiwara et al. (2001) or the direct preparation from cephalic kidneys described by Ráb and Roth (1988).

To improve the adhesion of chromosomes to the glass surface and to make the structure of the chromosome compact and thus enable optimal access of the labelled DNA to the target DNA, aging of the chromosomal preparations is highly recommended. The routinely applied and the easiest way is dry heat aging through one of these incubations: 7 days at RT, 3 to 8 hrs at 37°C, or 1 to 3 hrs at 60°C. The exposition of chromosome preparations to higher temperature also reduces the effects of cytoplasm remnants over chromosome spreads, when stained with DAPI.

A possible option is chemical aging, by exposing a slide with 150–200 µl of EtOH covered with a coverslip to 94°C for 2 to 20 sec (in a thermocycler). The chemical aging can be carried out when it is desirable to shorten the whole protocol. For more details, see Henegariu et al. 2001.

3. Chromosome spread pre-treatment

Pre-treatment of chromosome spreads prior to ISH allows

- decreasing the background, which results in an unspecific signal, by removing cytoplasm remnants, RNA and proteins that can bind to probe and to detection reagents.
- improving the access of DNA probes to the target DNA.

Pre-treatment is performed according to the following steps:

a) Brief equilibration in 2x SSC, 5 min, RT.
b) RNase treatment: Drop 200 µl/slide of RNase solution (20 µg/µl), cover with cover slips and incubate for 1 to 3 hrs at 37°C.
c) Wash in PBS, 5 min, RT.
d) Pepsinization: incubate slides in a coplin jar with pepsinization solution for 1 to 3 min at 37°C in a water bath.
e) Wash in 4x SSC, 5 min at RT.

4. Probe preparation, DNA labelling by Nick Translation (NT)

The best method of gDNA probe labelling is the Nick Translation (NT). This procedure can be carried out either using a commercial kit (e.g., the Roche Nick Translation Mix, Invitrogen BioNick™ DNA Labelling System or Abbott Molecular Nick Translation Kit) according to the manufacturer's instructions or according to the following protocol (the price per reaction in the commercial kits is much higher than when chemicals are purchased separately). The DNA probe can be labelled either directly with dUTP coupled with fluorochromes

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(e.g., Cy™3-dUTP, FITC-dUTP, Rhodamin-dUTP, Alexa Fluor® products, etc.) or indirectly with dUTP coupled with haptens (e.g., biotin and digoxigenin). When working with a directly labelled DNA probe, more steps of the protocol must be performed protected from a direct light source because the risk of fluorochrome bleaching during the manipulations is higher; on the other side, the use of an indirectly labelled gDNA probe can increase the risk of an unspecific background during hybridization signal analysis.

NT reaction:

1 µg gDNA
5 µl 10x NT Buffer
5 µl NT dNTPs-mix
5 µl 0.1 M mercaptoethanol
1 to 2.5 µl modified dUTP 1 mM (i.e., Biotin-dUPT/Digoxigenin-dUTP)
1 µl DNA polymerase I (0.1 U/µL)
1 µl DNase I (2,000 U/mL, diluted 1:10–200 with ddH_2O)
x µl ddH_2O q.s.p. 50 µl/reaction

According to the standard protocol (e.g., Cremer et al. 2008 and/or manufacturers of the commercial kits), the NT reaction involves incubation for 90 min at 15°C. However, this time strongly depends on the starting gDNA quality and the length of its fragments. Even when gDNA of the recommended fragments size (i.e., 3 to 5 kbp) is used, the time required for reaching the proper resulting fragment size (200 to 500 bp) may vary strongly and needs to be tested in each batch/species individually prior to preparing a higher amount of probes.

For this purpose, either commercial Biotin-dUTP/Digoxigenin-dUTP (e.g., Roche) are available, or there is a possibility to perform coupling of hapten succinimidyl esters (in DMSO) to aminoallyl-dUTP as described by Cremer et al. (2008). In the latter case, the cost is 100 to 300 times lower than using commercial biotin/digoxigenin-dUTPs.

Check with the 0.8% agarose gel electrophoresis, whether the length of DNA fragments is in the range of 200 to 500 bp. Probes should be kept on ice during electrophoresis. When longer fragments occur, additional time of NT incubation would be necessary (see "Troubleshooting"). As soon as the right fragment size is obtained, stop the NT reaction by following these steps (or as indicated by the NT kit manufacturer):

1. Add 2 ul 0.5 M EDTA, pH 8, and mix well.
2. Incubate 10 min at 65°C.
3. Cool on ice.

The NT product can either be stored at –20°C for up to several years or can be immediately used as follows:

a) Mix the labelled probes that will be hybridized together. When the competitor/Cot1-DNA is used, add this DNA in the concentration 3- to

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50-fold the concentration of one probe DNA. The competitor DNA can be derived from one genome only, usually from the same one as the chromosome preparation originated. However, different modifications according to experimental scheme are possible.

b) Add following reagents:
5 µg unlabelled salmon sperm* DNA for each probe
0.1 vol. 3 M Natrium (Sodium) Acetate pH 5.2
2.5 vol. 96% EtOH

*when working with salmonid fishes, use BSA as blocking reagent instead of salmon sperm.

c) Mix all components gently.
d) Incubate at least for 30 min at –20°C (this step can be prolonged for up to several days).
e) Centrifuge at 13,000 RPM for 15 to 20 min.
f) Discard the supernatant.
g) Add 200 ul of 70% undenatured ethanol to wash out the saline components such as sodium acetate.
h) Centrifuge at 13,000 RPM for 15 to 20 min.
i) Discard the supernatant.
j) Dry the pellet carefully with an absorbent and antistatic laboratory wipe. Let it dry for 1 to 2 minutes at 60°C or 10 to15 min at 37°C until all the liquid has evaporated. When possible, a vacuum centrifuge can be used in this step instead of 37°C/60°C incubation.
k) Re-suspend the pellet in 20 to 30 µl of the hybridization buffer and shake overnight at 37°C. In the case of direct labelling, cover the tubes on the shaker with aluminium foil to prevent the fluorochrome from bleaching.
l) On the next day, check whether the pellet has re-suspended completely.
m) After re-suspension, the hybridization probes can be used in further steps. When they are not used, they can be stored at –20°C for up to several months.

5. Denaturation of chromosome spreads and labelled DNA probe

a) Preheat 70% formamide/2x SSC at least for 30 min at 76°C in a coplin jar.
b) Denature the chromosome spreads in the coplin jar with 70% formamide/2x SSC for 3 min at 76°C.

Note: The time and temperature may vary considerably (10 s to 4 min/72 to 76°C) and must be adjusted specifically for each fish group.

c) Dehydrate slides in 70% and 80% ice-cold ethanol (in coplin jars on ice), and in 96% ethanol, at RT, 3 min each, and let it air-dry until usage (not longer than several hours).
d) Denature the DNA probe in a 1.5 ml Eppendorf tube in a water bath or a heating block at 86°C for 6 min and place immediately on ice for at least 10 min or until use.

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Note: In the case of higher or too high proportion of repetitive sequences and no possibility to add competitor/Cot1-DNA, an optional step of pre-annealing can be included to the protocol by incubating the denaturated DNA probe for 90 min at 37°C in order to suppress the repetitive sequences.

This optional step is based on the higher re-association kinetics of repetitive sequences that will preferentially re-associate within the probe DNA. The negative effect of repetitive sequences is thus suppressed by their mutual re-association. However, this step is not as effective as using the unlabelled competitor/Cot1-DNA, which should be preferred in most cases.

6. Setup of hybridization

Pipette the denatured DNA mixture (20 to 30 µl) onto the denatured chromosome preparation and carefully cover with a cover slip (24 x 50 mm). Place slides in a humid chamber (e.g., metal box with grid filled distilled water) and incubate for 48 hrs to 72 hrs at 37°C. To improve the hybridization reaction, this step can be prolonged for up to 5 days.

7. Washing and detection of indirectly labelled probes*

a) Place slides in 4x SSC/0.2% Tween 20, 5 min, RT, to help removing the cover slip.
b) Wash slides in 50% formamide 2x SSC at 45°C, twice for 10 minutes, occasionally shaking them, in coplin jars.
c) Wash slides, in 1x SCC at 43 to 45°C, 3 times, for 7 min, occasionally shaking them, in coplin jars.
d) Apply a blocking treatment (500 to 800 µl 3% BSA/4x SSC/0.2% Tween 20) onto each slide and cover with a cover slip, incubate for at least 20 min at 37°C.
e) Briefly wash in 4x SSC/0.2% Tween 20 at RT.
f) In the meanwhile centrifuge the stock solution of antibodies for 3 min at 5,000 to 8,000 RPM before preparing the following mixture.
g) First antibody layer: e.g., Anti- Digoxigenin-Fluorescein + 0.5% BSA/PBS (1:20, v/v)**.
h) Pipette 150 µl of the mixture per slide, cover with 24 x 60 mm cover slip and incubate for 45 to 60 min at 37°C.
i) Wash in 4x SSC/0.2% Tween 20 at 42 to 45°C 3 times, for 7 min, occasionally shaking them.
j) Apply blocking treatment (500 to 800 µl 3%BSA/4x SSC/0.2% Tween 20) on the slide, cover by cover slip, incubate at least for 20 min at 37°C.
k) Briefly wash in 4x SSC/0.2% Tween 20.
l) Second antibody layer: e.g., Cy™-3-Streptavidin + 10% NGS/PBS (1:100, v/v).**/***

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m) Pipette 150 µl of the mixture per slide, cover with 24 x 60 mm cover slip and incubate for 45–60 min at 37°C.
n) Wash in 4x SSC/0.2% Tween 20 at 42 to 45°C 3 times, for 7 min, occasionally shaking.
o) Add a drop of DAPI containing an antifade (e.g., Vectashield) on each slide, close by a cover slip (24 x 50 mm) and seal with a colourful nail polish.

*Note 1: In the case of directly labelled DNA probes (i.e., when fluorochrome is directly incorporated into the probe using, e.g., Cy3-dUTP or Fluorescein-dUTP) you can omit steps d to m.

**Note 2: The exact concentration of the antibody should be determined by the user, manufacturer's recommendations usually indicate a higher concentration than necessary.

***Note 3: In order to shorten the detection time it is possible to apply a combined solution of antibodies of both layers at once. We recommend such a simplification only if the conventional protocol works well.

Antibodies are sensitive to light therefore the steps involving fluorochromes should be performed in darkness.

8. Microscope analysis and data acquisition

For the last steps an epifluorescent microscope equipped with filters compatible with the fluorochromes used is required. An acquisition software enabling acquisition of each of the RGB channels separately in black and white regime and enabling their superimposition in pseudo-coloured composite images in RGB is also necessary. The final digital processing can be performed by using for, e.g., Adobe Photoshop software. Details on microscopy and imaging systems for ISH methods are provided by Iourov (2009), for details on fluorescent optical filters, see Sommerauer and Feuerbacher (2009).

9. Re-using chromosome preparations

After a CGH/GISH experiment it is possible to re-use slides, e.g., for a FISH experiment or for other kinds of chromosomes staining/banding:

a) Pre-heat two coplin jars with 4x SSC/0.1% Tween at 37°C.
b) Remove the nail polish from the cover slip and slide using a lancet.
c) Incubate the slide with cover slip in the pre-heated coplin jar with 4x SSC/0.1% Tween for 30 min at 37°C; shake each 10 min or perform this step in a shaking water bath or a rocking platform in an incubator.
d) Let the cover slip slide down the slide.

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e) Incubate the slide without the cover slip in the second pre-heated coplin jar with 4x SSC/Tween for 30 min at 37°C shaking or occasionally shaking it.
f) Dehydrate slides in coplin jars in 70%, 80% and 96% EtOH, 3 min each at RT and let them air-dry.
g) Incubate slides in coplin jar with fixative (methanol/acetic acid 3:1 v/v) for 30 min at RT and then let them air-dry.
h) Perform aging of the slide as described. After dehydration in 70, 80 and 96% ethanol, slides are ready to be re-used or stored at –20°C.
i) Prior to repeated denaturation, the RNase and pepsin treatment are not necessary anymore. However, to be sure that no previously hybridized DNA will remain on chromosomes, it is advisable to prolong denaturation time of the slide by 30 s - 1 min. Optionally, it is also possible to increase the temperature of denaturation slightly (this has to be tested).

Troubleshooting

Quality and spreading of chromosomes

This is a crucial factor influencing considerably the overall hybridization as well as the evaluation of the resulting pattern. Generally, the better the chromosome spreading, the better hybridization and its evaluation.

Aging

Slides that were not aged properly can result in: 1. weak or uneven DAPI staining and/or 2. a puffy appearance of chromosomes after hybridization and/or 3. a weak hybridization signal.

Nick translation

The most important factor influencing the time required for the reaction and the length of the DNA fragment is the proportion of dUTP conjugated to haptens.

Precipitation of pellet

In the case that the pellet has not precipitated, it is necessary to check whether the appropriate amount of EDTA has been used to stop the NT reaction (as indicated by the manufacturer of the commercial NT kits). Optionally, it is possible to prolong the freezing (–20°C) of the mixture of gDNA, EtOH, NaAc and salmon sperm for up to several days (when necessary and/or to improve the precipitation of the pellet).

Unspecific background signal

The bond between antigen and unspecifically attached hapten can result in an unspecific background signal. This can be reduced or avoided by increasing the concentration of BSA in the BSA/4x SSC solution or prolonged incubation with this solution at 37°C. As an alternative, 0.4 M NaCl can be used. Washing

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steps during detection are also crucial to get rid of unspecific background signals—the coplin jars have to be preheated to the desired temperature. If possible, use a shaking water bath or a rocking platform in an incubator set to the required temperature. Should this be impossible, at least occasionally gently shake the coplin jars with slides.

Laboratory set up

Equipment

Major

Agarose electrophoresis with documentation system; centrifuge; vortex; water bath; incubator/oven; mini-centrifuge; freezer (–20°C); epifluorescence microscope equipped with CCD camera; shaking water bath or rocking platform in an incubator (optional).

Minor

1.5 ml microtubes; coplin jars; microscopic slides with cover slips; nail polish; micropipette (1–1000 µl) with tips; fine forceps; timers.

Biochemicals and reagents

Ethanol 96%; formamide (Sigma-Aldrich); agarose in an electrophoresis buffer; dNTP-Mix (0.5 mM dATP, dCTP, dGTP+ 0.1 mM dTTP); 0.1 M mercaptoethanol (Merck); DNA polymerase I (Roche); DNase I (Roche; 100 mg diluted 1:250 in ddH_2O); biotin-16-dUTP (Roche); digoxigenin-11-dUTP (Roche); hapten succinimidyl esters (biotin, digoxigenin, dinitrophenol); aminoallyl-dUTP (Invitrogen); 1 M Tris-HCl pH 7.75; 2 M glycine pH 80.2 M sodium bicarbonate pH 8.3; BSA; PBS; SSC; Tween; 3 M natrium (sodium) Acetate; hybridization buffer; salmon sperm DNA; antifade (Vectashield); commercial kits: DNeasy Blood & Tissue Kit (Qiagen); nick translation mix for *in situ* probes (Roche).

FITC-streptavidin (Invitrogen) 1:20 to 1:100 dilution with PBS containing 10% FBS or dilution for specific application should be determined by the investigator to obtain the best conditions.

Cy™3-streptavidin (Invitrogen) 1:20 to 1:100 dilution with PBS containing 10% NGS.

Anti-digoxigenin-fluorescein, Fab fragments (Roche) 1:15 to 1:20 dilution with PBS containing 0.5% BSA.

Anti-digoxigenin-rhodamin, Fab fragments (Roche) 1:15 to 1:20 dilution with PBS containing 0.5% BSA.

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Stock and working solutions

NT dNTP-Mix
0.5 mM dATP
0.5 mM dCTP
0.5 mM dGTP
0.1 mM dTTP
10x NT buffer
1 ml 1 M Tris-HCl, pH 7.5
100 ul 1 M $MgCl_2$
10 ul 10% BSA
890 ul ddH_2O
Store in 100 ul aliquots at –20°C.

Hybridization buffer (*sensu* Cremer et al. (2008))

5 ml formamide
2 ml 50% dextran sulfate
1 ml 20x SSC
400 µl 1M natrium-phosphate buffer
100 µl 10% SDS
200 µl 50x Denhardt's solution
1.3 ml ddH2O (total volume 10 ml), store at –20°C.

50x Denhardt's solution (or Sigma-Aldrich)

2 g BSA
2 g Ficoll (type 400)
2 g polyvinylpyrrolidone
200 ml ddH_2O
Filter to sterilize, divide into aliquots, and store at –20°C.

Pepsinization solution (0.005% pepsin in 0.0 1M HCl)

50 µl 10% pepsin
10 ml 0.1 M HCl
90 ml ddH_2O
Warm up to 37°C, store at –20°C.

RNase solution

10 µl RNase aliquot (25 mg/1.25 ml ddH_2O)
1 ml 2x SSC.

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References

Bi, K. and J.P. Bogart. 2006. Identification of intergenomic recombinations in unisexual salamanders of the genus *Ambystoma* by genomic *in situ* hybridization (GISH). Cytogenet. Genome Res. 112(3-4): 307–312.

Cremer, M., F. Grasser, Ch. Lanctôt, S. Müller, M. Neusser, R. Zinner, I. Solovei and T. Cremer. 2008. Multicolor 3D fluorescence *in situ* hybridization for imaging interphase chromosomes. pp. 205–239. *In*: Hancock, R. (ed.). The Nucleus: Volume I: Nuclei and Subnuclear Components, Methods in Molecular Biology™. Volume 463. Humana Press.

Fujiwara, A., C. Nishida-Umehara, T. Sakamoto, N. Okamoto, I. Nakayama and S. Abe. 2001. Improved fish lymphocyte culture for chromosome preparation. Genetica. 111: 77–89.

Graham, D.E. 1978. The isolation of high molecular weight DNA from whole organisms of large tissue masses. Anal. Biochem. 85: 609–613.

Henegariu, O., N.A. Heerema, L.L. Wright, P. Bray-Ward, D.C. Ward and G.H. Vance. 2001. Improvements in cytogenetic slide preparations: controlled chromosome spreading, chemical aging and gradual denaturing. Cytometry 43: 101–109.

Iourov, I. 2009. Microscopy and imaging systems. pp. 75–84. *In*: Liehr, T. (ed.). Fluorescence *In Situ* Hybridization (FISH)—Application Guide. Springer-Verlag, Berlin, Heidelberg.

Kallioniemi, A., O.P. Kallioniemi, D. Sudar, D. Rutovitz, J.W. Gray, F. Waldman and D. Pinkel. 1992. Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. Science 258(5083): 818–821.

Knytl, M., L. Kalous, R. Symonová, K. Rylková and P. Ráb. 2013. Chromosome studies of European cyprinid fishes: cross-species painting reveals natural allotetraploid origin of *Carassius* female with 206 chromosomes. Cytogenet. Genome Res. 139(4): 276–283.

Landegent, J.E., N. Jansen in de Wal, R.W. Dirks, F. Baao and M. van der Ploeg. 1987. Use of whole cosmid cloned genomic sequences for chromosomal localization by non-radioactive *in situ* hybridization. Hum. Genet. 77(4): 366–370.

Lim, K.I., R. Matyášek, A. Kovařík and A. Leitch. 2007. Parental origin and genome evolution in the allopolyploid *Iris versicolor*. Ann. Bot-London. 100: 219–224.

Matoba, H., A. Soejima and Y. Hoshi. 2007. Identification of parental genomes and genomic organization in *Aster microcephalus* var. Ovatus. J. Plant Res. 120: 585–593.

Ráb, P. and P. Roth. 1988. Cold-blooded vertebrates. pp. 115–124. *In*: Balicek, P., J. Forejt and J. Rubes (eds.). Methods of Chromosome Analysis. Cytogenet. Sect. Cs. Biol. Soc. Publishers, Brno.

Sealey, P.G., P.A. Whittaker and E.M. Southern. 1985. Removal of repeated sequences from hybridisation probes. Nucleic Acids Res. 13(6): 1905–22.

She, Ch.-W., Y.-Ch. Song and X.-H. Jiang. 2012. Comparative genomic *in situ* hybridization analysis on the chromosomes of five grass species with rice genomic DNA probe. Afr. J. Biotechnol. 11(39): 9508–9515.

Sommerauer, M. and I. Feuerbacher. 2009. Optical filters for FISH. pp. 85–98. *In*: Liehr, T. (ed.). Fluorescence *In Situ* Hybridization (FISH)—Application Guide. Springer-Verlag, Berlin, Heidelberg.

Symonová, R., M. Flajšhans, A. Sember, M. Havelka, D. Gela, T. Kořínková, M. Rodina, M. Rábová and P. Ráb. 2013a. Molecular cytogenetics in artificial hybrid and highly polyploid

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sturgeons: an evolutionary story narrated by repetitive sequences. Cytogenet. Genome Res. 141(2-3): 153–162.
Symonová, R., Z. Majtánová, A. Sember, G.B.O. Staaks, J. Bohlen, J. Freyhof, M. Rábová and P. Ráb. 2013b. Genome differentiation in a species pair of coregonine fishes: an extremely rapid speciation driven by stress activated retrotransposons mediating extensive ribosomal DNA multiplications. BMC Evol. Biol. 13: 42.
Takahashi, C., J.A. Marshall, M.D. Bennett and I.J. Leitch. 1999. Genomic relationships between maize and its wild relatives. Genome 42: 1201–1207.
Traut, W. and H. Winking. 2001. Meiotic chromosomes and stages of sex chromosome evolution in fish: zebrafish, platyfish and guppy. Chrom. Res. 9(8): 659–672.
Traut, W., K. Sahara, T.D. Otto and F. Marec. 1999. Molecular differentiation of sex chromosomes probed by comparative genomic hybridization. Chromosoma. 108: 173–180.
Trifonov, V.A., N.N. Vorobieva and W. Rens. 2009. FISH with or without COT1 DNA. pp. 99–109. *In*: Liehr, T. (ed.). Fluorescence *In Situ* Hybridization (FISH)—Application Guide. Springer-Verlag, Berlin, Heidelberg.
Valente, G.T., C.H. Schneider, M.C. Gross, E. Feldberg and C. Martins. 2009. Comparative cytogenetics of cichlid fishes through genomic *in situ* hybridization (GISH) with emphasis on *Oreochromis niloticus*. Chrom. Res. 17: 791–799.
Walsh, P.S., D.A. Metzger and R. Higuchi. 1991. Chelex® 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques 10: 506–513.
Zhu, H.P. and J.F. Gui. 2007. Identification of genome organization in the unusual allotetraploid form of *Carassius auratus gibelio*. Aquaculture 265(1-4): 109–117.

Chapter 16

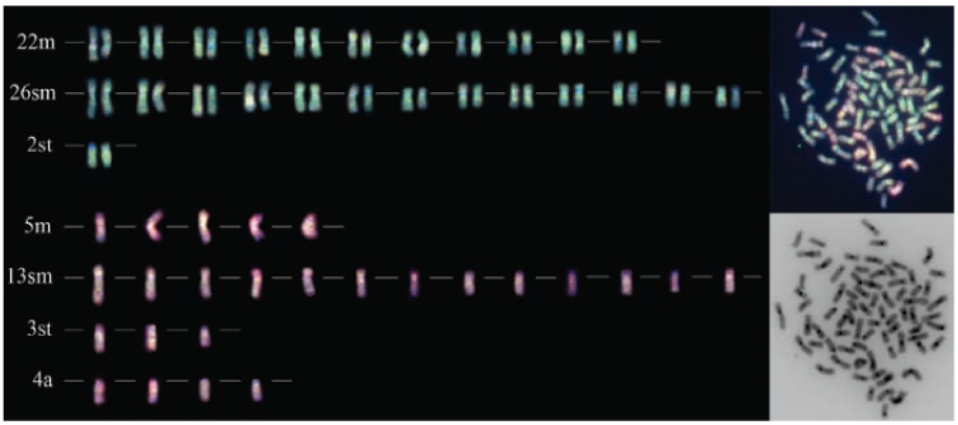


Figure 1. Representative metaphases of the *Cobitis 2elongatoides-1tanaitica* hybrid visualized by GISH with *C. elongatoides* and *C. tanaitica* genomic probes. Homologous and homoeologous chromosomes of triploid hybrids cannot be distinguished in normal DAPI stained metaphase, however, the GISH clearly distinguishes the *C. elongatoides* (shown as those with green fluorescence) and *C. tanaitica* chromosomes (shown with red fluorescence) in *C. 2elongatoides-1tanaitica* hybrid.

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8. REFERENCES

Alfaro, M. E., F. Santini, C. Brock, H. Alamillo, A. Dornburg, D. L. Rabosky, G. Carnevale, and L. J. Harmon. 2009. Nine exceptional radiations plus high turnover explain species diversity in jawed vertebrates. Proc. Natl. Acad. Sci. 106:13410–13414.

Allendorf, F. W., and G. H. Thorgaard. 1984. Tetraploidy and the evolution of salmonid fishes - Springer. Pp. 1–53 *in* Evolutionary genetics of fishes. Springer US.

Amemiya, C. T., and J. R. Gold. 1986. Chromomycin A_3 stains nucleolus organizer regions of fish chromosomes. Copeia 1986:226–231.

Amores, A., A. Force, Y.-L. Yan, L. Joly, C. Amemiya, A. Fritz, R. K. Ho, J. Langeland, V. Prince, Y.-L. Wang, M. Westerfield, M. Ekker, and J. H. Postlethwait. 1998. Zebrafish Hox clusters and vertebrate genome evolution. Science 282:1711–1714.

Aparicio, S., J. Chapman, E. Stupka, N. Putnam, J. Chia, P. Dehal, A. Christoffels, S. Rash, S. Hoon, A. Smit, M. D. S. Gelpke, J. Roach, T. Oh, I. Y. Ho, M. Wong, C. Detter, F. Verhoef, P. Predki, A. Tay, S. Lucas, P. Richardson, S. F. Smith, M. S. Clark, Y. J. K. Edwards, N. Doggett, A. Zharkikh, S. V. Tavtigian, D. Pruss, M. Barnstead, C. Evans, H. Baden, J. Powell, G. Glusman, L. Rowen, L. Hood, Y. H. Tan, G. Elgar, T. Hawkins, B. Venkatesh, D. Rokhsar, and S. Brenner. 2002. Whole-genome shotgun assembly and analysis of the genome of *Fugu rubripes*. Science 297:1301–1310.

Arai, R. 2011. Fish karyotypes: a check list. 2011 edition. Springer, Tokyo ; New York.

Arias-Rodríguez, L., S. Páramo-Delgadillo, W. M. Contreras-Sánchez, and C. A. Álvarez-González. 2009. Cariotipo del pejelagarto tropical *Atractosteus tropicus* (Lepisosteiformes: Lepisosteidae) y variación cromosómica en sus larvas y adultos. Rev. Biol. Trop. 57:529–539.

Arkhipova, I. R., M. A. Batzer, J. Brosius, C. Feschotte, J. V. Moran, J. Schmitz, and J. Jurka. 2012. Genomic impact of eukaryotic transposable elements. Mob. Dna 3:19.

Arratia, G. 2000. Remarkable teleostean fishes from the Late Jurassic of southern Germany and their phylogenetic relationships. Foss. Rec. 3:137–179.

Belyayev, A. 2014. Bursts of transposable elements as an evolutionary driving force. J. Evol. Biol. 27:2573–2584.

Bemis, W. E., E. K. Findeis, and L. Grande. 1997. An overview of Acipenseriformes. Environ. Biol. Fishes 48:25–71.

Bernardi, G. 2005. Structural and evolutionary genomics: natural selection in genome evolution. Elsevier.

Bernardi, G., E. O. Wiley, H. Mansour, M. R. Miller, G. Orti, D. Haussler, S. J. O'Brien, O. A. Ryder, and B. Venkatesh. 2012. The fishes of Genome 10K. Mar. Genomics 7:3–6.

Boroń, A. 1999. Banded karyotype of spined loach *Cobitis taenia* and triploid *Cobitis* from Poland. Genetica 105:293–300.

Boroń, A. 1992. Karyotype study of diploid and triploid *Cobitis taenia* (Pisces, Cobitidae) from Vistula river basin. Cytobios 72:201–206.

Boroń, A. 2003a. Karyotypes and cytogenetic diversity of the genus *Cobitis* (Pisces, Cobitidae) in Poland: a review. Cytogenetic evidence for a hybrid origin of some *Cobitis* triploids. Folia Biol. (Praha) 51 Suppl:49–54.

Boroń, A. 2003b. Replication banding patterns in the spined loach, *Cobitis taenia* L. (Pisces, Cobitidae). Genetica 119:51–55.

Boroń, A., M. Culling, and A. Pułym. 2003. Cytogenetic characteristics of the fish genus *Cobitis* from England. Folia Biol. (Praha) 51 Suppl:13–16.

Braasch, I., A. R. Gehrke, J. J. Smith, K. Kawasaki, T. Manousaki, J. Pasquier, A. Amores, T. Desvignes, P. Batzel, J. Catchen, A. M. Berlin, M. S. Campbell, D. Barrell, K. J. Martin, J. F. Mulley, V. Ravi, A. P. Lee, T. Nakamura, D. Chalopin, S. Fan, D. Wcisel, C. Cañestro, J. Sydes, F. E. G. Beaudry, Y. Sun, J. Hertel, M. J. Beam, M. Fasold, M. Ishiyama, J.

Johnson, S. Kehr, M. Lara, J. H. Letaw, G. W. Litman, R. T. Litman, M. Mikami, T. Ota, N. R. Saha, L. Williams, P. F. Stadler, H. Wang, J. S. Taylor, Q. Fontenot, A. Ferrara, S. M. J. Searle, B. Aken, M. Yandell, I. Schneider, J. A. Yoder, J.-N. Volff, A. Meyer, C. T. Amemiya, B. Venkatesh, P. W. H. Holland, Y. Guiguen, J. Bobe, N. H. Shubin, F. Di Palma, J. Alföldi, K. Lindblad-Toh, and J. H. Postlethwait. 2016. The spotted gar genome illuminates vertebrate evolution and facilitates human-teleost comparisons. Nat. Genet. 48:427–437.

Broughton, R. E., R. Betancur-R., C. Li, G. Arratia, and G. Ortí. 2013. Multi-locus phylogenetic analysis reveals the pattern and tempo of bony fish evolution. PLoS Curr. 5.

Bucciarelli, G., G. Bernardi, and G. Bernardi. 2002. An ultracentrifugation analysis of two hundred fish genomes. Gene 295:153–162.

Calton, M., and T. Denton. 1974. Chromosomes of chocolate gourami - cytogenetic anomaly. Science 185:618–619.

Chalopin, D., M. Naville, F. Plard, D. Galiana, and J.-N. Volff. 2015. Comparative analysis of transposable elements highlights mobilome diversity and evolution in vertebrates. Genome Biol. Evol. 7:567–580.

Charlesworth, B., P. Sniegowski, and W. Stephan. 1994. The evolutionary dynamics of repetitive DNA in Eukaryotes. Nature 371:215–220.

Choleva, L., A. Apostolou, P. Ráb, and K. Janko. 2008. Making it on their own: sperm-dependent hybrid fishes (*Cobitis*) switch the sexual hosts and expand beyond the ranges of their original sperm donors. Philos. Trans. R. Soc. B Biol. Sci. 363:2911–2919.

Choleva, L., K. Janko, K. De Gelas, J. Bohlen, V. Šlechtová, M. Rábová, and P. Ráb. 2012. Synthesis of clonality and polyploidy in vertebrate animals by hybridization between two sexual species. Evol. Int. J. Org. Evol. 66:2191–2203.

Choleva, L., Z. Musilova, A. Kohoutova-Sediva, J. Paces, P. Rab, and K. Janko. 2014. Distinguishing between incomplete lineage sorting and genomic introgressions: complete fixation of allospecific mitochondrial DNA in a sexually reproducing fish (*Cobitis*; Teleostei), despite clonal reproduction of hybrids. PLoS ONE 9:e80641.

Cioffi, M. B., and L. a. C. Bertollo. 2012. Chromosomal distribution and evolution of repetitive DNAs in fish. Genome Dyn. 7:197–221.

Cioffi, M. de B., L. A. Carlos Bertollo, M. A. Villa, E. A. de Oliveira, A. Tanomtong, C. F. Yano, W. Supiwong, and A. Chaveerach. 2015. Genomic organization of repetitive DNA elements and its implications for the chromosomal evolution of channid fishes (Actinopterygii, Perciformes). PLoS One 10:e0130199.

Clark, M. S. 2003. Genomics and mapping of Teleostei (bony fish). Comp. Funct. Genomics 4:182–193.

Costantini, M., R. Cammarano, and G. Bernardi. 2009. The evolution of isochore patterns in vertebrate genomes. BMC Genomics 10:146.

Crête-Lafrenière, A., L. K. Weir, and L. Bernatchez. 2012. Framing the Salmonidae family phylogenetic portrait: a more complete picture from increased taxon sampling. PLoS ONE 7:e46662.

Crow, K. D., C. D. Smith, J.-F. Cheng, G. P. Wagner, and C. T. Amemiya. 2012. An independent genome duplication inferred from Hox paralogs in the American paddlefish--a representative basal ray-finned fish and important comparative reference. Genome Biol. Evol. 4:937–953.

de Boer, J. G., R. Yazawa, W. S. Davidson, and B. F. Koop. 2007. Bursts and horizontal evolution of DNA transposons in the speciation of pseudotetraploid salmonids. BMC Genomics 8:422.

de Oliveira, M. L. M., R. Utsunomia, J. C. Pansonato-Alves, P. C. Scacchetti, C. C. Primo, M. R. Vicari, R. F. Artoni, L. Centofante, O. Moreira-Filho, C. Oliveira, and F. Foresti. 2016. Microstructural chromosome reorganization in the genus *Trichomycterus* (Siluriformes: Trichomycteridae). Neotropical Ichthyol. 14:e150084.

Deiana, A. M., A. Cau, S. Salvadori, E. Coluccia, R. Cannas, A. Milia, and J. Tagliavini. 2000. Major and 5S ribosomal sequences of the largemouth bass *Micropterus salmoides* (Perciformes, Centrarchidae) are localized in GC-rich regions of the genome. Chromosome Res. 8:213–218.

Dion-Cote, A.-M., S. Renaut, E. Normandeau, and L. Bernatchez. 2014. RNA-seq reveals transcriptomic shock involving transposable elements reactivation in hybrids of young lake whitefish species. Mol. Biol. Evol. 31:1188–1199.

Dion-Côté, A.-M., R. Symonová, F. C. Lamaze, Š. Pelikánová, P. Ráb, and L. Bernatchez. 2016. Standing chromosomal variation in Lake Whitefish species pairs: the role of historical contingency and relevance for speciation. Mol. Ecol., doi: 10.1111/mec.13816.

Dion-Côté, A.-M., R. Symonová, P. Ráb, and L. Bernatchez. 2015. Reproductive isolation in a nascent species pair is associated with aneuploidy in hybrid offspring. Proc. R. Soc. Lond. B Biol. Sci. 282:20142862.

Eagle, S. H., and T. J. Crease. 2012. Copy number variation of ribosomal DNA and Pokey transposons in natural populations of *Daphnia*. Mob. DNA 3:4.

Eickbush, T. H. 2002. R2 and related site-specific non-long terminal repeat retrotransposons. Pp. 813–835 *in* Mobile DNA II. ASM Press Washington, DC.

Faircloth, B. C., L. Sorenson, F. Santini, and M. E. Alfaro. 2013. A phylogenomic perspective on the radiation of ray-finned fishes based upon targeted sequencing of ultraconserved elements (UCEs). PLoS ONE 8:e65923.

Ferreira, D. C., F. Porto-Foresti, C. Oliveira, and F. Foresti. 2011. Transposable elements as a potential source for understanding the fish genome. Mob. Genet. Elem. 1:112–117.

Filipski, J., J. Thiery, and G. Bernardi. 1973. Analysis of Bovine Genome by Cs_2SO_4-Ag^+ Density Gradient Centrifugation. J. Mol. Biol. 80:177–197.

Flicek, P., M. R. Amode, D. Barrell, K. Beal, K. Billis, S. Brent, D. Carvalho-Silva, P. Clapham, G. Coates, S. Fitzgerald, L. Gil, C. G. Girón, L. Gordon, T. Hourlier, S. Hunt, N. Johnson, T. Juettemann, A. K. Kähäri, S. Keenan, E. Kulesha, F. J. Martin, T. Maurel, W. M. McLaren, D. N. Murphy, R. Nag, B. Overduin, M. Pignatelli, B. Pritchard, E. Pritchard, H. S. Riat, M. Ruffier, D. Sheppard, K. Taylor, A. Thormann, S. J. Trevanion, A. Vullo, S. P. Wilder, M. Wilson, A. Zadissa, B. L. Aken, E. Birney, F. Cunningham, J. Harrow, J. Herrero, T. J. P. Hubbard, R. Kinsella, M. Muffato, A. Parker, G. Spudich, A. Yates, D. R. Zerbino, and S. M. J. Searle. 2014. Ensembl 2014. Nucleic Acids Res. 42:D749-755.

Fontana, F., M. Lanfredi, L. Congiu, M. Leis, M. Chicca, and R. Rossi. 2003. Chromosomal mapping of 18S-28S and 5S rRNA genes by two-colour fluorescent in situ hybridization in six sturgeon species. Genome 46:473–477.

Fujiwara, A., S. Abe, E. Yamaha, F. Yamazaki, and M. C. Yoshida. 1998. Chromosomal localization and heterochromatin association of ribosomal RNA gene loci and silver-stained nucleolar organizer regions in salmonid fishes. Chromosome Res. Int. J. Mol. Supramol. Evol. Asp. Chromosome Biol. 6:463–471.

Galetti, P. M., C. T. Aguilar, and W. F. Molina. 2005. An overview of marine fish cytogenetics. Hydrobiologia 420:55–62.

Grande, L. 2010. An empirical synthetic pattern study of gars (Lepisosteiformes) and closely related species, based mostly on skeletal anatomy the resurrection of Holostei. American Society of Ichthyologists and Herpetologists, Lawrence, Kan.

Grande, L., and W. E. Bemis. 1998. A comprehensive phylogenetic study of amiid fishes (Amiidae) based on comparative skeletal anatomy. an Empirical Search for Interconnected Patterns of Natural History. J. Vertebr. Paleontol. 18:1–696.

Gregory, T. R. 2015. Animal Genome Size Database.

Gregory, T. R. 2005. Genome Size Evolution in Animals. Gregory, T. Ryan; Nat Hist Museum, Dept Entomol, Cromwell Rd, London SW7 5BD, UK.

Havelka, M., D. Bytyutskyy, R. Symonová, P. Ráb, and M. Flajšhans. 2016. The second highest chromosome count among vertebrates is observed in cultured sturgeon and is associated with genome plasticity. Genet. Sel. Evol. 48:12.

Helfman, G., B. B. Collette, D. E. Facey, and B. W. Bowen. 2009. The diversity of fishes: biology, evolution, and ecology. 2 edition. Wiley-Blackwell, Oxford.

Helland, I. P., L. A. Vøllestad, J. Freyhof, and T. Mehner. 2009. Morphological differences between two ecologically similar sympatric fishes. J. Fish Biol. 75:2756–2767.

Hilton, E. J. 2003. Comparative osteology and phylogenetic systematics of fossil and living bony-tongue fishes (Actinopterygii, Teleostei, Osteoglossomorpha). Zool. J. Linn. Soc. 137:1–100.

Hoegg, S., and A. Meyer. 2005. Hox clusters as models for vertebrate genome evolution. Trends Genet. TIG 21:421–424.

Howell, W. 1982. Selective Staining of nucleolus organizer regions (NORs). Cell Nucl. 11:89–142.

Howell, W. M., and D. A. Black. 1980. Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia 36:1014–1015.

Hurley, I. A., R. L. Mueller, K. A. Dunn, E. J. Schmidt, M. Friedman, R. K. Ho, V. E. Prince, Z. Yang, M. G. Thomas, and M. I. Coates. 2007. A new time-scale for ray-finned fish evolution. Proc. R. Soc. Lond. B Biol. Sci. 274:489–498.

Inman, R. 1966. A denaturation map of lanbda phage dna molecule determined by electron microscopy. J. Mol. Biol. 18:464-.

Inoue, J. G., Y. Kumazawa, M. Miya, and M. Nishida. 2009. The historical biogeography of the freshwater knifefishes using mitogenomic approaches: a mesozoic origin of the Asian notopterids (Actinopterygii: Osteoglossomorpha). Mol. Phylogenet. Evol. 51:486–499.

Inoue, J. G., M. Miya, B. Venkatesh, and M. Nishida. 2005. The mitochondrial genome of Indonesian coelacanth *Latimeria menadoensis* (Sarcopterygii : Coelacanthiformes) and divergence time estimation between the two coelacanths. Gene 349:227–235.

Jaillon, O., J.-M. Aury, F. Brunet, J.-L. Petit, N. Stange-Thomann, E. Mauceli, L. Bouneau, C. Fischer, C. Ozouf-Costaz, A. Bernot, S. Nicaud, D. Jaffe, S. Fisher, G. Lutfalla, C. Dossat, B. Segurens, C. Dasilva, M. Salanoubat, M. Levy, N. Boudet, S. Castellano, V. Anthouard, C. Jubin, V. Castelli, M. Katinka, B. Vacherie, C. Biémont, Z. Skalli, L. Cattolico, J. Poulain, V. de Berardinis, C. Cruaud, S. Duprat, P. Brottier, J.-P. Coutanceau, J. Gouzy, G. Parra, G. Lardier, C. Chapple, K. J. McKernan, P. McEwan, S. Bosak, M. Kellis, J.-N. Volff, R. Guigó, M. C. Zody, J. Mesirov, K. Lindblad-Toh, B. Birren, C. Nusbaum, D. Kahn, M. Robinson-Rechavi, V. Laudet, V. Schachter, F. Quétier, W. Saurin, C. Scarpelli, P. Wincker, E. S. Lander, J. Weissenbach, and H. Roest Crollius. 2004. Genome duplication in the teleost fish *Tetraodon nigroviridis* reveals the early vertebrate proto-karyotype. Nature 431:946–957.

Jakubczak, J. L., W. D. Burke, and T. H. Eickbush. 1991. Retrotransposable elements R1 and R2 interrupt the rRNA genes of most insects. Proc. Natl. Acad. Sci. U. S. A. 88:3295–3299.

Janko, K., J. Bohlen, D. Lamatsch, M. Flajshans, J. T. Epplen, P. Ráb, P. Kotlík, and V. Šlechtová. 2007a. The gynogenetic reproduction of diploid and triploid hybrid spined

loaches (*Cobitis*: Teleostei), and their ability to establish successful clonal lineages on the evolution of polyploidy in asexual vertebrates. Genetica 131:185–194.

Janko, K., M. Flajšhans, L. Choleva, J. Bohlen, V. Šlechtová, M. Rábová, Z. Lajbner, V. Šlechta, P. Ivanova, I. Dobrovolov, M. Culling, H. Persat, J. Kotusz, and P. Ráb. 2007b. Diversity of European spined loaches (genus *Cobitis* L.): an update of the geographic distribution of the *Cobitis taenia* hybrid complex with a description of new molecular tools for species and hybrid determination. J. Fish Biol. 71:387–408.

Janko, K., J. Kotusz, K. De Gelas, V. Šlechtová, Z. Opoldusová, P. Drozd, L. Choleva, M. Popiołek, and M. Baláž. 2012. Dynamic formation of asexual diploid and polyploid lineages: multilocus analysis of *Cobitis* reveals the mechanisms maintaining the diversity of clones. PLoS ONE 7:e45384.

Janko, K., V. P. Vasil'ev, P. Ráb, M. Rábová, V. Šlechtová, and E. D. Vasil'eva. 2005. Genetic and morphological analyses of 50-chromosome spined loaches (*Cobitis*, Cobitidae, Pisces) from the Black Sea basin that are morphologically similar to C. taenia, with the description of a new species. Folia Zool. 54:405–420.

Kasahara, M. 2007. The 2R hypothesis: an update. Curr. Opin. Immunol. 19:547–552.

Kasahara, M., K. Naruse, S. Sasaki, Y. Nakatani, W. Qu, B. Ahsan, T. Yamada, Y. Nagayasu, K. Doi, Y. Kasai, T. Jindo, D. Kobayashi, A. Shimada, A. Toyoda, Y. Kuroki, A. Fujiyama, T. Sasaki, A. Shimizu, S. Asakawa, N. Shimizu, S.-I. Hashimoto, J. Yang, Y. Lee, K. Matsushima, S. Sugano, M. Sakaizumi, T. Narita, K. Ohishi, S. Haga, F. Ohta, H. Nomoto, K. Nogata, T. Morishita, T. Endo, T. Shin-I, H. Takeda, S. Morishita, and Y. Kohara. 2007. The medaka draft genome and insights into vertebrate genome evolution. Nature 447:714–719.

Kato, A., J. M. Vega, F. Han, J. C. Lamb, and J. A. Birchler. 2005. Advances in plant chromosome identification and cytogenetic techniques. Curr. Opin. Plant Biol. 8:148–154.

Kirtiklis, L., and M. Jankun. 2006. Chromosome analysis in coregonid individuals in the interspecific hybridization zone. J. Appl. Ichthyol. 22:401–403.

Knytl, M., L. Kalous, R. Symonová, K. Rylková, and P. Ráb. 2013. Chromosome studies of European cyprinid fishes: cross-species painting reveals natural allotetraploid origin of a *Carassius* female with 206 chromosomes. Cytogenet. Genome Res. 139:276–283.

Kohn, M., J. Högel, W. Vogel, P. Minich, H. Kehrer-Sawatzki, J. A. M. Graves, and H. Hameister. 2006. Reconstruction of a 450-My-old ancestral vertebrate protokaryotype. Trends Genet. TIG 22:203–210.

Kuraku, S., and A. Meyer. 2009. The evolution and maintenance of Hox gene clusters in vertebrates and the teleost-specific genome duplication. Int. J. Dev. Biol. 53:765–773.

Lartillot, N. 2012. Phylogenetic patterns of GC-biased gene conversion in placental mammals, and the evolutionary dynamics of recombination landscapes. Mol. Biol. Evol. mss239.

Lavoué, S. 2016. Was Gondwanan breakup the cause of the intercontinental distribution of Osteoglossiformes? A time-calibrated phylogenetic test combining molecular, morphological, and paleontological evidence. Mol. Phylogenet. Evol. 99:34–43.

Lavoué, S., M. Miya, M. E. Arnegard, P. B. McIntyre, V. Mamonekene, and M. Nishida. 2010. Remarkable morphological stasis in an extant vertebrate despite tens of millions of years of divergence. Proc. R. Soc. B-Biol. Sci., doi: 10.1098/rspb.2010.1639.

Lavoué, S., and J. P. Sullivan. 2004. Simultaneous analysis of five molecular markers provides a well-supported phylogenetic hypothesis for the living bony-tongue fishes (Osteoglossomorpha: Teleostei). Mol. Phylogenet. Evol. 33:171–185.

Li, G., and M. V. H. Wilson. 1996. Phylogeny of Osteoglossomorpha. Pp. 163–174 *in* M. L. J. Stiassny, L. R. Parenti, and J. Johnson, eds. Interrelationships of Fishes. Academic Press, New York.

Lu, J., E. Peatman, H. Tang, J. Lewis, and Z. Liu. 2012. Profiling of gene duplication patterns of sequenced teleost genomes: evidence for rapid lineage-specific genome expansion mediated by recent tandem duplications. BMC Genomics 13:246.

Ludwig, A., N. M. Belfiore, C. Pitra, V. Svirsky, and I. Jenneckens. 2001. Genome duplication events and functional reduction of ploidy levels in sturgeon (*Acipenser*, *Huso* and *Scaphirhynchus*). Genetics 158:1203–1215.

Macqueen, D. J., and I. A. Johnston. 2014. A well-constrained estimate for the timing of the salmonid whole genome duplication reveals major decoupling from species diversification. Proc. R. Soc. B-Biol. Sci. 281:20132881.

Maisey, J. G., C. Chesek, and D. Finnin. 1996. Discovering Fossil Fishes. 1st edition. Henry Holt & Co, New York, NY.

Maneechot, N., C. F. Yano, L. A. Carlos Bertollo, N. Getlekha, W. F. Molina, S. Ditcharoen, B. Tengjaroenkul, W. Supiwong, A. Tanomtong, and M. de B. Cioffi. 2016. Genomic organization of repetitive DNAs highlights chromosomal evolution in the genus *Clarias* (Clariidae, Siluriformes). Mol. Cytogenet. 9:4.

Mank, J. E., and J. C. Avise. 2006. Phylogenetic conservation of chromosome numbers in Actinopterygiian fishes. Genetica 127:321–327.

Marais, G. 2003. Biased gene conversion: Implications for genome and sex evolution. Trends Genet. 19:330–338.

Martins, C., and A. P. Wasko. 2004. Organization and evolution of 5S ribosomal DNA in the fish genome. Pp. 335–363 *in* Focus on genome research.

Martins, C., A. P. Wasko, C. Oliveira, and J. M. Wright. 2000. Nucleotide sequence of 5S rDNA and localization of the ribosomal RNA genes to metaphase chromosomes of the Tilapiine cichlid fish, *Oreochromic niloticus*. Hereditas 133:39–46.

Matthey, R. 1945. L' evolution de la formule chromosomiale chez les vertebrees. Experientia 1:50–56.

Mayr, B., P. Rab, and M. Kalat. 1985. Localization of NORs and counterstain-enhanced fluorescence studies in *Perca fluviatilis* (Pisces, Percidae). Genetica 67:51–56.

Mehner, T., K. Pohlmann, C. Elkin, M. T. Monaghan, B. Nitz, and J. Freyhof. 2010. Genetic population structure of sympatric and allopatric populations of Baltic ciscoes (*Coregonus albula* complex, Teleostei, Coregonidae). BMC Evol. Biol. 10:85.

Meyer, A., and Y. Van de Peer. 2005. From 2R to 3R: evidence for a fish-specific genome duplication (FSGD). Bioessays 27:937–945.

Morescalchi, M. A., I. Liguori, L. Rocco, and V. Stingo. 2007. Karyotypic characterization and genomic organization of the 5S rDNA in *Erpetoichthys calabaricus* (Osteichthyes, Polypteridae). Genetica 131:209–216.

Mu, X., X. Wang, H. Song, Y. Yang, D. Luo, D. Gu, M. Xu, C. Liu, J. Luo, and Y. Hu. 2012. Mitochondrial DNA as effective molecular markers for the genetic variation and phylogeny of the family Osteoglossidae. Gene 511:320–325.

Mugal, C. F., P. F. Arndt, and H. Ellegren. 2013. Twisted signatures of GC-biased gene conversion embedded in an evolutionary stable karyotype. Mol. Biol. Evol. 30:1700–1712.

Nakatani, Y., H. Takeda, Y. Kohara, and S. Morishita. 2007. Reconstruction of the vertebrate ancestral genome reveals dynamic genome reorganization in early vertebrates. Genome Res. 17:1254–1265.

Near, T. J., R. I. Eytan, A. Dornburg, K. L. Kuhn, J. A. Moore, M. P. Davis, P. C. Wainwright, M. Friedman, and W. L. Smith. 2012. Resolution of ray-finned fish phylogeny and timing of diversification. Proc. Natl. Acad. Sci. U. S. A. 109:13698–13703.

Nelson, J. S., T. C. Grande, and M. V. H. Wilson. 2016. Fishes of the World. 5 edition. Wiley, Hoboken, New Jersey.

Normark, B. B. 1999. Evolution in a putatively ancient asexual aphid lineage: recombination and rapid karyotype change. Evolution 53:1458.

Ocalewicz, K., P. Woznicki, and M. Jankun. 2008. Mapping of rRNA genes and telomeric sequences in Danube salmon (*Hucho hucho*) chromosomes using primed in situ labeling technique (PRINS). Genetica 134:199–203.

Ohlberger, J., T. Mehner, G. Staaks, and F. Hölker. 2008a. Temperature-related physiological adaptations promote ecological divergence in a sympatric species pair of temperate freshwater fish, *Coregonus* spp. Funct. Ecol. 22:501–508.

Ohlberger, J., G. Staaks, T. Petzoldt, T. Mehner, and F. Hölker. 2008b. Physiological specialization by thermal adaptation drives ecological divergence in a sympatric fish species pair. Evol. Ecol. Res. 10:1173–1185.

Ohno, S. 1970. Evolution by gene duplication. Springer-Verlag.

Ohno, S., J. Muramoto, C. Stenius, L. Christian, W. A. Kittrell, and N. B. Atkin. 1969. Microchromosomes in holocephalian, chondrostean and holostean fishes. Chromosoma 26:35–40.

Ojima, Y., and T. Yamano. 1980. A chromosome study of the longnose gar, *Lepisosteus osseus*. Chromosome Inf Serv 7–8.

Oliveira, C., J. M. Wright, and F. Foresti. 2002. Chromosome Structure in Fishes. Pp. 103–108 *in* R. C. Sobit, G. Obe, and R. S. Athwal, eds. Some aspects of chromosome structure and functions. Springer Netherlands.

Pereira, C. S. A. 2013. Introgressive hybridization in Iberian cyprinid fishes:a cytogenomic approach to homoploid Leuciscinae. Ph.D. Thesis.

Pereira, C. S. A., P. Rab, and M. J. Collares-Pereira. 2013. Chromosomes of Iberian Leuciscinae (Cyprinidae) revisited: evidence of genome restructuring in homoploid hybrids using dual-color FISH and CGH. Cytogenet. Genome Res. 141:143–152.

Pessia, E., A. Popa, S. Mousset, C. Rezvoy, L. Duret, and G. A. B. Marais. 2012. Evidence for widespread GC-biased gene conversion in Eukaryotes. Genome Biol. Evol. 4:787–794.

Phillips, R., and P. Ráb. 2001. Chromosome evolution in the Salmonidae (Pisces): an update. Biol. Rev. Camb. Philos. Soc. 76:1–25.

Pisano, E., C. Ozouf-Costaz, and F. Foresti. 2007. Fish Cytogenetics. CRC Press.

Post, A. 1974. Chromosomes of tree species of family Gonostomatidae (Osteichthyes, Stomiatoidei). Pp. 51–55 *in* Results of research cruises of FRV "Walther Hertwig" to South-America. XXXIV.

Pucci, M. B., P. Barbosa, V. Nogaroto, M. C. Almeida, R. F. Artoni, J. C. Pansonato-Alves, F. Foresti, O. Moreira-Filho, and M. R. Vicari. 2014. Population differentiation and speciation in the genus *Characidium* (Characiformes: Crenuchidae): effects of reproductive and chromosomal barriers. Biol. J. Linn. Soc. 111:541–553.

Putnam, N. H., T. Butts, D. E. K. Ferrier, R. F. Furlong, U. Hellsten, T. Kawashima, M. Robinson-Rechavi, E. Shoguchi, A. Terry, J.-K. Yu, E. L. Benito-Gutiérrez, I. Dubchak, J. Garcia-Fernàndez, J. J. Gibson-Brown, I. V. Grigoriev, A. C. Horton, P. J. de Jong, J. Jurka, V. V. Kapitonov, Y. Kohara, Y. Kuroki, E. Lindquist, S. Lucas, K. Osoegawa, L. A. Pennacchio, A. A. Salamov, Y. Satou, T. Sauka-Spengler, J. Schmutz, T. Shin-I, A. Toyoda, M. Bronner-Fraser, A. Fujiyama, L. Z. Holland, P. W. H. Holland, N. Satoh, and D. S. Rokhsar. 2008. The amphioxus genome and the evolution of the chordate karyotype. Nature 453:1064–1071.

Ráb, P., M. Rábová, K. M. Reed, and R. B. Phillips. 1999. Chromosomal characteristics of ribosomal DNA in the primitive semionotiform fish, longnose gar *Lepisosteus osseus*. Chromosome Res. Int. J. Mol. Supramol. Evol. Asp. Chromosome Biol. 7:475–480.

Ráb, P., K. M. Reed, F. a. P. deLeon, and R. B. Phillips. 1996. A new method for detecting nucleolus organizer regions in fish chromosomes using denaturation and propidium iodide staining. Biotech. Histochem. 71:157–163.

Rábová, M., Š. Pelikánová, L. Choleva, and P. Ráb. 2007. Cytogenetics of bisexual species and their asexual hybrid clones in European spined loaches, genus *Cobitis*. II. Mapping of telomeric (TTAGGG)n sequences and DAPI-positive heterochromatins in four parental species. P. 59 *in* ECI XII European Congress of Ichthyology, Book of Abstracts. Cavtat, Croatia.

Rábová, M., P. Ráb, and C. Ozouf-Costaz. 2001. Extensive polymorphism and chromosomal characteristics of ribosomal DNA in a loach fish, *Cobitis vardarensis* (Ostariophysi, Cobitidae) detected by different banding techniques and fluorescence in situ hybridization (FISH). Genetica 111:413–422.

Rábová, M., M. Völker, Š. Pelikánová, and P. Ráb. 2015. Sequential chromosome banding in fishes. Pp. 92–102 *in* C. Ozouf-Costaz, E. Pisano, F. Foresti, and L. F. de A. Toledo, eds. Fish Cytogenetic Techniques. CRC Press.

Rampin, M., K. Bi, J. P. Bogart, and M. J. Collares-Pereira. 2012. Identifying parental chromosomes and genomic rearrangements in animal hybrid complexes of species with small genome size using genomic *in situ* hybridization (GISH). Comp. Cytogenet. 6:287–300.

Robinson, T. J., and F. Yang. 2012. Molecular cytogenetics: karyotype evolution, phylogenomics and future prospects. Heredity 108:1–3.

Rossi, A. R., and E. Gornung. 2005. Cytogenetic analysis of three Italian populations of *Coregonus lavaretus* (Pisces, Salmoniformes) with chromosomal localization of major and minor ribosomal genes, and telomeric repeats. Hereditas 142:15–21.

Saitoh, K., W.-J. Chen, and R. L. Mayden. 2010. Extensive hybridization and tetrapolyploidy in spined loach fish. Mol. Phylogenet. Evol. 56:1001–1010.

Sakai, C., F. Konno, O. Nakano, T. Iwai, T. Yokota, J. Lee, C. Nishida-Umehara, A. Kuroiwa, Y. Matsuda, and M. Yamashita. 2007. Chromosome elimination in the interspecific hybrid medaka between *Oryzias latipes* and *O. hubbsi*. Chromosome Res. 15:697–709.

Sallan, L. C. 2014. Major issues in the origins of ray-finned fish (Actinopterygii) biodiversity. Biol. Rev. 89:950–971.

Salvadori, S., E. CoLuccia, and A. M. Deiana. 2015. C-banding. Pp. 88–91 *in* C. Ozouf-Costaz, E. Pisano, F. Foresti, and L. de Almeida, eds. Fish Cytogenetic Techniques. CRC Press.

Sayers, E. W., T. Barrett, D. A. Benson, E. Bolton, S. H. Bryant, K. Canese, V. Chetvernin, D. M. Church, M. DiCuccio, S. Federhen, M. Feolo, I. M. Fingerman, L. Y. Geer, W. Helmberg, Y. Kapustin, D. Landsman, D. J. Lipman, Z. Lu, T. L. Madden, T. Madej, D. R. Maglott, A. Marchler-Bauer, V. Miller, I. Mizrachi, J. Ostell, A. Panchenko, L. Phan, K. D. Pruitt, G. D. Schuler, E. Sequeira, S. T. Sherry, M. Shumway, K. Sirotkin, D. Slotta, A. Souvorov, G. Starchenko, T. A. Tatusova, L. Wagner, Y. Wang, W. J. Wilbur, E. Yaschenko, and J. Ye. 2011. Database resources of the National Center for Biotechnology Information. Nucleic Acids Res. 39:D38–D51.

Schluter, D., and A. Rambaut. 1996. Ecological speciation in postglacial fishes. Philos. Trans. R. Soc. Lond. B Biol. Sci. 351:807–814.

Schmid, M., and M. Guttenbach. 1988. Evolutionary diversity of reverse (R) fluorescent chromosome bands in vertebrates. Chromosoma 97:101–114.

Schneider, M. C., and D. M. Cella. 2010. Karyotype conservation in 2 populations of the parthenogenetic scorpion *Tityus serrulatus* (Buthidae): rDNA and its associated heterochromatin are concentrated on only one chromosome. J. Hered. 101:491–496.

Schulz, M., and J. Freyhof. 2003. *Coregonus fontanae*, a new spring-spawning cisco from Lake Stechlin, northern Germany (Salmoniformes: Coregonidae). Ichthyol. Explor. Freshw. 14:209–216.

Šlechtová, V., J. Bohlen, and H. H. Tan. 2007. Families of Cobitoidea (Teleostei; Cypriniformes) as revealed from nuclear genetic data and the position of the mysterious genera *Barbucca*, *Psilorhynchus*, *Serpenticobitis* and *Vaillantella*. Mol. Phylogenet. Evol. 44:1358–1365.

Soltis, P., and D. E. Soltis. 2012. Polyploidy and genome evolution. Springer Science & Business Media.

Sumner, A. T. 2003. Constitutive heterochromatin. Pp. 84–96 *in* Chromosomes. Blackwell Science Ltd.

Sun, Y., Y. Huang, X. Li, C. C. Baldwin, Z. Zhou, Z. Yan, K. A. Crandall, Y. Zhang, X. Zhao, M. Wang, A. Wong, C. Fang, X. Zhang, H. Huang, J. V. Lopez, K. Kilfoyle, Y. Zhang, G. Ortí, B. Venkatesh, and Q. Shi. 2016. Fish-T1K (Transcriptomes of 1,000 Fishes) Project: large-scale transcriptome data for fish evolution studies. GigaScience 5.

Sunnucks, P., P. R. England, A. C. Taylor, and D. F. Hales. 1996. Microsatellite and chromosome evolution of parthenogenetic *Sitobion* aphids in Australia. Genetics 144:747–756.

Suzuki, A., and J. Hirata. 1991. Chromosomes and DNA contents of *Amia calva*. Chromosome Inf. Serv. 50:34–37.

Suzuki, D., M. C. Brandley, and M. Tokita. 2010. The mitochondrial phylogeny of an ancient lineage of ray-finned fishes (Polypteridae) with implications for the evolution of body

elongation, pelvic fin loss, and craniofacial morphology in Osteichthyes. BMC Evol. Biol. 10:21.

Symonová, R., M. Flajšhans, A. Sember, M. Havelka, D. Gela, T. Kořínková, M. Rodina, M. Rábová, and P. Ráb. 2013a. Molecular cytogenetics in artificial hybrid and highly polyploid sturgeons: An evolutionary story narrated by repetitive sequences. Cytogenet. Genome Res. 141:153–162.

Symonová, R., Z. Majtánová, A. Sember, G. B. O. Staaks, J. Bohlen, J. Freyhof, M. Rábová, and P. Ráb. 2013b. Genome differentiation in a species pair of coregonine fishes: an extremely rapid speciation driven by stress-activated retrotransposons mediating extensive ribosomal DNA multiplications. BMC Evol. Biol. 13:42.

Symonová, R., A. Sember, Z. Majtánová, and P. Ráb. 2015. Characterization of fish genomes by GISH and CGH. Pp. 118–131 *in* C. Ozouf-Costaz, E. Pisano, F. Foresti, and L. de Almeida, eds. Fish Cytogenetic Techniques. CRC Press.

Symonová R., Majtánová Z., Arias-Rodriguez L., Mořkovský L., Kořínková T., Cavin L., Johnson Pokorná M., Doležálková M., Flajšhans M., Normandeau E., Ráb P., Meyer A. & Bernatchez L. *in press*. Unique genome organisation in gars (Lepisosteidae: Actinopterygii) shows more similarities to mammals than to any other ray-finned fishes. Journal of Experimental Zoology Part B: Molecular and Develomental Evolution. Invited manuscript to the special issue „Genome of the spotted gar“.

Taylor, E. B. 1999. Species pairs of north temperate freshwater fishes: Evolution, taxonomy, and conservation. Rev. Fish Biol. Fish. 9:299–324.

Taylor, J. S., I. Braasch, T. Frickey, A. Meyer, and Y. Van de Peer. 2003. Genome duplication, a trait shared by 22,000 species of ray-finned fish. Genome Res. 13:382–390.

Taylor, M. I., and M. A. Alexandrou. 2012. Whole genome duplication accelerates speciation in Corydoradinae catfishes. Pp. S1-2, 7–10 *in* ICPHB 2012 – International Conference on Polyploidy, Hybridization and Biodiversity. Průhonice, Czech Republic.

Thomson, R. C., and H. B. Shaffer. 2010. Rapid progress on the vertebrate tree of life. Bmc Biol. 8:19.

Timusk, E. R., M. M. Ferguson, H. K. Moghadam, J. D. Norman, C. C. Wilson, and R. G. Danzmann. 2011. Genome evolution in the fish family salmonidae: generation of a brook charr genetic map and comparisons among charrs (Arctic charr and brook charr) with rainbow trout. BMC Genet. 12:68.

Traut, W., and H. Winking. 2001. Meiotic chromosomes and stages of sex chromosome evolution in fish: zebrafish, platyfish and guppy. Chromosome Res. Int. J. Mol. Supramol. Evol. Asp. Chromosome Biol. 9:659–672.

Uyeno, T. 1973. A comparative study of chromosomes in the teleostean fish order fi Osteoglossiformes. Jpn. J Ichthyol 211–217.

Uyeno, T., and G. Smith. 1972. Tetraploid origin of karyotype of *Catostomid* fishes. Science 175:644-.

Valente, T. G., H. C. Schneider, C. M. Gross, E. Feldberg, and C. Martins. 2009. Comparative cytogenetics of cichlid fishes through genomic in-situ hybridization (GISH) with emphasis on *Oreochromis niloticus*. Chromosome Res. Int. J. Mol. Supramol. Evol. Asp. Chromosome Biol. 17:791–799.

Vandepoele, K., W. De Vos, J. S. Taylor, A. Meyer, and Y. Van de Peer. 2004. Major events in the genome evolution of vertebrates: paranome age and size differ considerably between ray-finned fishes and land vertebrates. Proc. Natl. Acad. Sci. U. S. A. 101:1638–1643.

Vasil'ev, V. P., and E. D. Vasil'eva. 2008. Comparative karyological analysis of mud loach and spined loach species (genera *Misgurnus* and *Cobitis*) from the Far East region of Russia. Folia Zool. 57:51–59.

Vasil'ev, V. P., and E. D. Vasil'eva. 2011. Comparative karyology of species of the genera *Misgurnus* and *Cobitis* (Cobitidae) from the Amur River Basin in connection with their taxonomic relations and the evolution of karyotypes. J. Ichthyol. 48:1–13.

Venkatesh, B. 2003. Evolution and diversity of fish genomes. Curr. Opin. Genet. Dev. 13:588–592.

Welch, J. L. M., D. B. M. Welch, and M. Meselson. 2004. Cytogenetic evidence for asexual evolution of bdelloid rotifers. Proc. Natl. Acad. Sci. U. S. A. 101:1618–1621.

Werren, J. H. 2011. Selfish genetic elements, genetic conflict, and evolutionary innovation. Proc. Natl. Acad. Sci. U. S. A. 108 Suppl 2:10863–10870.

Wicker, T., F. Sabot, A. Hua-Van, J. L. Bennetzen, P. Capy, B. Chalhoub, A. Flavell, P. Leroy, M. Morgante, O. Panaud, E. Paux, P. SanMiguel, and A. H. Schulman. 2007. A unified classification system for eukaryotic transposable elements. Nat. Rev. Genet. 8:973–982.

Wilson, M. V. H., and A. M. Murray. 2008. Osteoglossomorpha: Phylogeny, biogeography, and fossil record and the significance of key African and Chinese fossil taxa. Geol. Soc. Lond. Spec. Publ. 1:185–219.

Wolfe, K. H. 2001. Yesterday's polyploids and the mystery of diploidization. Nat. Rev. Genet. 2:333–341.

Xu, P., X. Zhang, X. Wang, J. Li, G. Liu, Y. Kuang, J. Xu, X. Zheng, L. Ren, G. Wang, Y. Zhang, L. Huo, Z. Zhao, D. Cao, C. Lu, C. Li, Y. Zhou, Z. Liu, Z. Fan, G. Shan, X. Li, S. Wu, L. Song, G. Hou, Y. Jiang, Z. Jeney, D. Yu, L. Wang, C. Shao, L. Song, J. Sun, P. Ji, J. Wang, Q. Li, L. Xu, F. Sun, J. Feng, C. Wang, S. Wang, B. Wang, Y. Li, Y. Zhu, W.

Xue, L. Zhao, J. Wang, Y. Gu, W. Lv, K. Wu, J. Xiao, J. Wu, Z. Zhang, J. Yu, and X. Sun. 2014. Genome sequence and genetic diversity of the common carp, *Cyprinus carpio*. Nat. Genet. 46:1212–1219.

Yang, L., T. Sado, M. V. Hirt, E. Pasco-Viel, M. Arunachalam, J. Li, X. Wang, J. Freyhof, K. Saitoh, A. M. Simons, M. Miya, S. He, and R. L. Mayden. 2015. Phylogeny and polyploidy: Resolving the classification of cyprinine fishes (Teleostei: Cypriniformes). Mol. Phylogenet. Evol. 85:97–116.

Yu, X., and X. Yu. 1990. A schizothoracine fish species *Diptychus dipogon* with a very high number of chromosomes. Chromosome Inf Serv. 48:17–18.

Zhang, X., M. T. Eickbush, and T. H. Eickbush. 2008. Role of recombination in the long-term retention of transposable elements in rRNA gene loci. Genetics 180:1617–1626.

Zhu, H.-P., and J.-F. Gui. 2007. Identification of genome organization in the unusual allotetraploid form of *Carassius auratus gibelio*. Aquaculture 265:109–117.