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Spaghetti Connections: Synaptonemal Complexes as a Tool to Explore Chromosome Structure, Evolution, and Meiotic Behavior in Fish

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Abstract
Background: The synaptonemal complex (SC) is a protein axis formed along chromosomes during meiotic prophase to ensure proper pairing and crossing over. SC analysis has been widely used to study the chromosomes of mammals and less frequently of birds, reptiles, and fish. It is a promising method to investigate the evolution of fish genomes and chromosomes as a part of complex approach. ***Summary:*** Compared with conventional metaphase chromosomes, pachytene chromosomes are less condensed and exhibit pairing between homologous chromosomes. These features of SCs facilitate the study of the small chromosomes that are typical in fish. Moreover, it allows the study of heteromorphisms in sex chromosomes and supernumerary chromosomes. In addition, it enables the investigation of the pairing between orthologous chromosomes in hybrids, which is crucial for uncovering the causes of hybrid sterility and asexual reproduction, such as gynogenesis or hybridogenesis. However, the application of SC analysis to fish chromosomes is limited by the associated complications. First, in most fish, meiosis does not occur during every season and life stage. Second, different SC preparation methods are optimal for different fish species. Third, commercial antibodies targeting meiotic proteins have been primarily developed against mammalian antigens, and not all of them are suitable for fish chromosomes. ***Key Messages:*** In the present review, we provide an overview of the methods for preparing fish SCs and highlight important studies using SC analysis in fish. This study will be valuable for planning and designing research that applies SC analysis to fish cytogenetics and genomics.

Introduction

Synaptonemal complexes (SCs) are protein structures that are formed along chromosomes during the meiotic prophase to ensure proper pairing and recombination in most eukaryotes, including vertebrates [1, 2]. Under a light microscope, SCs are seen more as "spaghetti" rather than "rods and dots" due to their lower degree of chromatin condensation compared to metaphase. The structure of the SC is evolutionarily conserved in the majority of eukaryotes.

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Although the main components of SCs share structural similarities in various organisms, they may have low sequence homology [3]. SCs consist of lateral elements that hold homologous chromosomes and a central element that binds the lateral elements together. The lateral elements of the SC in vertebrates are composed of several proteins, including Synaptonemal Complex Proteins 2 and 3 (SYCP2 and SYCP3) [4, 5]. The main component of the central element is the SYCP1 protein [6, 7]. SC formation begins at the leptotene stage with an assemblage of lateral elements along homologous chromosomes, represented by two sister chromatids. The sister chromatids are held together by a cohesin complex that includes the protein SMC3 (Structural Maintenance of Chromosomes 3) [8]. The loading of meiosis-specific cohesin complexes provides a scaffold for the recruitment of additional meiosis-specific proteins, including HORMADs (Hop1p/Rev7p/MAD2-domain proteins), whose incorporation into axial elements is essential for subsequent meiotic events, including recombination and formation of SCs [9, 10]. During leptotene, the homologs are attached to the nuclear envelope by their ends and form a configuration known as the "telomere bouquet" [11, 12]. At this stage, the telomeres of all chromosomes congregate, and synapsis is initiated in homologous regions [11]. Double-strand breaks (DSBs), which are prerequisites for crossing over, are introduced in the chromosomes by the topoisomerase-like protein SPO11 [13, 14]. DSBs mediate the co-alignment of homologous chromosomes and are required for their pairing [14]. DNA repair enzymes such as RAD51 are loaded at the DSB sites [15, 16]. RAD51, together with DMC1 (DNA Meiotic Recombinase 1) and other DNA strand exchange proteins, catalyzes the invasion of the 3′DNA strand freed during DSB formation into an intact homologous strand [14, 16]. During zygotene, the central elements of the SCs are assembled, and the conjugation of homologous chromosomes proceeds via a zip-lock mechanism [2, 17]. During pachytene, complete synapsis is achieved, and part of the DSBs is resolved in a crossover way to form mature recombination nodules (RNs) [14, 16]. This is performed by several DNA repair proteins, such as MLH1 (MutL Homolog 1) [18]. Other DSBs are repaired in a non-crossover manner [19]. In cases of limited homology between chromosomes (heteromorphic sex chromosomes, heterozygous chromosomal rearrangements, and orthologous chromosomes in hybrids), synaptic processes may be delayed and continue through early pachytene through a process known as synaptic adjustment [20–23]. During diplotene, the SC is disassembled and the homologs are held together at the points of crossing over, which are called chiasmata at this and later stages [1]. At least one chiasma per chromosome pair is usually required, with a few exceptions, for normal homolog segregation during metaphase [17, 24, 25]. In male tetrapods, gonocytes proliferate and enter meiosis constantly throughout life, whereas in females, SC-bearing oocytes are present only during the exact ontogenetic stages in juveniles and even embryos, and a stockpile of oocytes is fixed at the diplotene stage for a long time [26–28]. However, in some fishes, both male and female gametogenesis is continuous throughout their lifetime, and the meiocyte stock is fulfilled by proliferating gonocytes [29, 30]. During diplotene, in females of all vertebrates except mammals (with the possible exception of monotremes) [31], oocytes tend to accumulate yolk and regulatory transcripts, which requires extreme decondensation of chromosomes, transforming them into giant lampbrush chromosomes [32, 33].

The behavior of SCs, that is, pairing patterns, DSBs, and crossover numbers and localizations, may reveal important details of the chromosome structure. Synapsis and recombination of heterogeneous chromosome pairs, such as sex chromosomes, may reveal the localization of freely recombining and non-recombining regions, regions of Y and W chromosome "degeneration" or expansion, chromosome orientation in fusion-fission events, inversion borders, and other features. As SCs are more decondensed than metaphase chromosomes, they are longer and provide better resolution, which allows studying small chromosomes, such as microchromosomes, and many B chromosomes, which appear as dots in metaphase. Therefore, SC analysis is mainly conducted in studies of sex chromosomes, germline-restricted chromosomes [34], and meiosis in hybrids between species [35] or subspecies and chromosomal races [36]. Therefore, SC analysis is actively used in vertebrate cytogenetics, particularly in mammals [37–39]. It has also been implemented in fish and birds, although less frequently, and recently in pioneering studies on reptiles [34, 40–43]. The classical method of SC visualization involves the silver impregnation of surface-spread spermatocytes and oocytes, followed by light or electron microscopy [44]. The advantage of this method is the possibility of finely analyzing the ultrastructure of SCs; however, the visualization of specific proteins is not possible. Since the early 2000s, the leading technique for SC analysis has been immunofluorescent detection of specific molecular targets with subsequent light microscopy. It allows the discriminate detection of various SC proteins, DSBs, recombination nodules, and chromatin modifications, among others. Fluorescence in situ hybridization (FISH) with specific DNA targets, chromosome painting, and genomic in situ hybridization (GISH) techniques are often coupled with immunofluorescent analysis [39].

In fish, SC analysis has long been used and has revealed notable findings. However, fewer studies have been conducted compared to those in mammals. One possible reason for the relatively few studies is that performing a successful SC study in fish often requires overcoming methodological challenges. First, the different structures and chemistries of gonadal tissues necessitate the adoption of different protocols of SC spreading and fixation for different species, requiring extensive search and optimization. Second, unlike metaphase chromosomes, SC-bearing cells may only be found during certain seasons or life stages in some species. Third, the correspondence between SCs and metaphase chromosomes can be complicated due to differences in chromosome decondensation and difficulty in locating centromeres at the SC stage. This problem is further complicated because some fish are polyploid, and even at the pachytene stage, the complete analysis of SC is extremely difficult (and often impossible) because of the high number of SC elements in the cells. Finally, most commercially available antibodies for meiotic proteins have been developed based on mammalian antigens and are not always suitable for fish proteins.

Methods for working with fish SCs and the results obtained by different groups have not been systematized extensively. In this review, we summarized the current status of state-of-the-art fish SC studies. Our review promotes the adoption of SC analysis in fish as a promising method of cytogenetic analysis and provides researchers with guidance for planning and designing experiments and methodological strategies. This review will also be of interest to researchers studying meiosis and SCs in other organisms.

Methods of SC Preparation and Staining in Fish

Although many methods for obtaining fish SC spreads have been implemented throughout history, few basic techniques have been used frequently in recent studies. The fastest and simplest of these methods has been described by Moens [45]. Initially developed for zebrafish (*Danio rerio*, Cyprinidae, Cypriniformes), it was later successfully used in guppies (*Poecilia reticulata*, Poeciliidae, Cyprinodontiformes) [46], annual killifish (*Nothobranchius*, Nothobranchiidae, Cyprinodontiformes) [47], sturgeons (*Acipenser dauricus*, Acipenseridae, Acipenseriformes) [48], and loaches (*Cobitis*, Cobitidae, Cypriniformes) [49]. The basic technique is as follows.

1. Macerate testis piece or whole testis of a small fish, using needles and forceps, in 1 × phosphate buffered saline (PBS; 4.3 mM Na_2HPO_4, 1.43 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, pH 7.4) to obtain a cloudy suspension. Remove debris using forceps.
2. Prepare a Polysine or Superfrost microscopic slide with drops of hypotonic solution (1 part of PBS and 2 parts of MilliQ water). Usually, six to eight 30 μL drops can be placed on it.
3. Inject 1–2 μL of suspension into each drop, and leave for 20 min.
4. Tilt the slide to remove the solution. Add 500 μL of 2% paraformaldehyde (pH = 7–9) and fix for 3 min. Tilt the slide to remove the solution.
5. Wash the slide in 0.1% Kodak Photo-Flo or Tween-20 and air-dry.

The duration of hypotonic treatment and fixation can vary. This method yields excellent results for guppies, loaches, and *Nothobranchius* males. A similar simple method, with 0.46% KCl as a hypotonic solution, has been used for swamp guppy (*Micropoecilia picta*, Poeciliidae, Cyprinodontiformes) [50].

Campos-Ramos et al. [51] proposed another frequently used method as follows:

1. Macerate a piece of the gonad or the whole gonad of a small fish in Hanks' salt solution using needles and forceps to obtain a cloudy suspension. In the case of small gonads, take 100 μL of Hanks' solution.
2. Place the suspension into a 1.5-mL centrifuge tube and allow it to settle for 20 min at room temperature.
3. In the case of large testes, take 1 mL of the suspension and centrifuge it at 112 *g* for 2 min to remove debris. Take the supernatant and centrifuge it at 112 *g* for 5 min. This step is omitted in the case of small gonads.
4. Take the cell pellet in 20 μL Hank's solution to another tube, add 40 μL of 0.2 M sucrose and 60 μL of 0.2% "Lipsol" detergent (SciLabware LTD, UK) (each at pH = 8.5) to it, and shake gently. In the case of small gonads, take the 100 μL suspension obtained at step (1) and add 50 μL of sucrose and 200 μL of "Lipsol." Incubate the suspension for 10 min at room temperature.
5. Add 80 μL of 4% paraformaldehyde (pH = 8.5) to the suspension of large testis cells and 100 μL to the suspension of small gonad cells, and gently shake. Incubate the suspension at 4°C.
6. For electron microscopy, cover the microscopic slide with a plastic film. Rinse the slide in 0.4% Kodak Photo-Flo. Add 200–250 μL of fixed cell suspension to the slide, and allow it to dry for 4 h. Rinse for 1 min in Photo-Flo and dry again.

A modification of this method was developed by Araya-Jaime et al. [52]. The difference lies in the use of Triton X-100 instead of "Lipsol" and the preparation of the suspension directly on the slide after step (2). Further, the

volumes of reagents used differ from the original method; 20 μL of cell suspension in Hanks' solution is placed onto the slide, and 2 drops of sucrose and Triton X-100 and 10 drops of fixative are used. This method yields excellent results in loach females [53, 54] and some sturgeons [48].

Peters et al. [55] suggested a method that is now frequently used for vertebrates, including fish. We implemented this method for guppies; however, brighter antibody fluorescence was achieved with the method of Moens with an unchanged immunostaining protocol [45]. The method of Peters et al. [55] was optimal for clariid catfish (*Clarias*, Clariidae, Siluriformes) and female mollies (*Poecilia formosa*), as it resulted in better SC spreading. The protocol is as follows:

1. Keep the gonad pieces submerged in a hypotonic solution (30 mM Tris, 50 mM sucrose, 17 mM trisodium citrate dihydrate, and 5 mM ethylene diamine tetraacetic acid (EDTA), pH = 8.2) for 30–60 min. Shear even small gonads to liberate the cells into the hypotonic solution.
2. Place a small piece of gonad into 20 μL of 100 mM sucrose and macerate with forceps to obtain a cell suspension. Remove the debris, add 20 μL of sucrose, and mix with a pipette.
3. Dip a clean polysine slide into a 1% paraformaldehyde solution with 0.15% Triton X-100 (pH = 9.2). Place 20 μL of cell suspension onto a slide and tilt it in different directions to spread the cells on the slide. Dry the slide for at least 2 h in a humid chamber at room temperature, wash the slide in 0.4% Kodak Photo-Flo, and air-dry.

Blokhina et al. [56] propose a similar method. In this method, the first stage is omitted, and the cell suspension is obtained via chemical dissociation of the tissue using collagenase, trypsin, and DNAse I. This method with the omission of step (1) has been successfully used in sticklebacks (*Gasterosteus aculeatus*, Gastrosteidae, Scorpaeniformes) [57] and mollies (*P. formosa* and *P. mexicana*) [58]. Three basic fish SC preparation methods are summarized in Figure 1.

Prior to immunostaining, the slides may be permeabilized in hot 0.01 M sodium citrate buffer (pH = 6). The buffer is heated to 95°C, and the slides are placed there for 20 min. The buffer is then cooled to room temperature for another 20 min and the slides are washed in 1 × PBS. In our practice, we achieved brighter MLH1 signals in the guppies with such pretreatment. The protocol described by Anderson et al. [37] can be used for immunofluorescence staining. First, the slides are incubated in 10% PBT (1 × PBT contains 3% bovine serum albumin and 0.05% Tween-20 in 1 × PBS) for 45 min to reduce nonspecific antibody binding. Then, the antibodies are diluted in 1 × PBT, and 50 μL of antibody solution under a 20 × 60 mm coverslip is used for one slide. Triton X-100 can be used instead of Tween-20. The slides are incubated with the primary antibodies overnight in a humid chamber at 37°C or for 2–3 days at 4°C. Longer incubation can be implemented in the case of weak fluorescence or the absence of visible signals from certain antibodies. After primary staining, the slides are washed three times in PBST (1 × PBS with 0.1% Tween-20) for 5–15 min each. Before applying the secondary antibodies for 1 h at 37°C (alternatively, overnight at 4°C), the slides are incubated in 10% PBT for 45 min, as previously. Finally, the slides are washed with PBST. After drying, the slides are mounted in Vectashield antifade mounting medium with DAPI (Vector Laboratories, cat# H-1000-10, USA) or another medium with DAPI and covered with a 20 × 60 mm coverslip. Then they are observed under a fluorescence microscope using the appropriate fluorescence filters.

Not all commercial mammal-derived antibodies successfully bind to fish proteins. De novo antibodies against fish proteins have been developed in large-scale studies. The commercial antibodies used by us and mentioned in the literature are listed in Table 1.

Applications of SC Analysis in Fish

Mechanisms of Meiosis

First, SC analysis in fish can be used to study the mechanisms of meiosis, including crossover distribution. Such studies have been limited because there are a limited number of fish proteins detectable with mammal-derived commercial antibodies, and the construction of specific antibodies for fish is labor-intensive and time-consuming. Most studies have been performed on zebrafish, a classical model species in developmental biology. Male zebrafish predominantly have one MLH1 focus per bivalent, and these foci are mostly localized distally in the chromosome arms [45]. A similar pattern was observed in the guppy (*P. reticulata*) and swamp guppy (*M. picta*) [46, 50]. In female zebrafish, the total SC length is higher, and MLH1 foci are proportionally more numerous (1:1.55) and more evenly distributed [64]. Similar sexual dimorphism has been observed in the three-spine stickleback (*G. aculeatus*), but not in all populations [65]. Among MLH1-knockout zebrafish mutants, males are mostly sterile because meiosis in most cells does not proceed after metaphase I, and chromosomes do not segregate. By contrast, females are fertile, but their progeny have high levels of deformities and high early mortality rates due to aneuploidies. The few normal fry are triploids that develop from non-segregated eggs [61, 66]. The SC and DSB formation and synaptic progression in zebrafish start at both chromosome ends during the

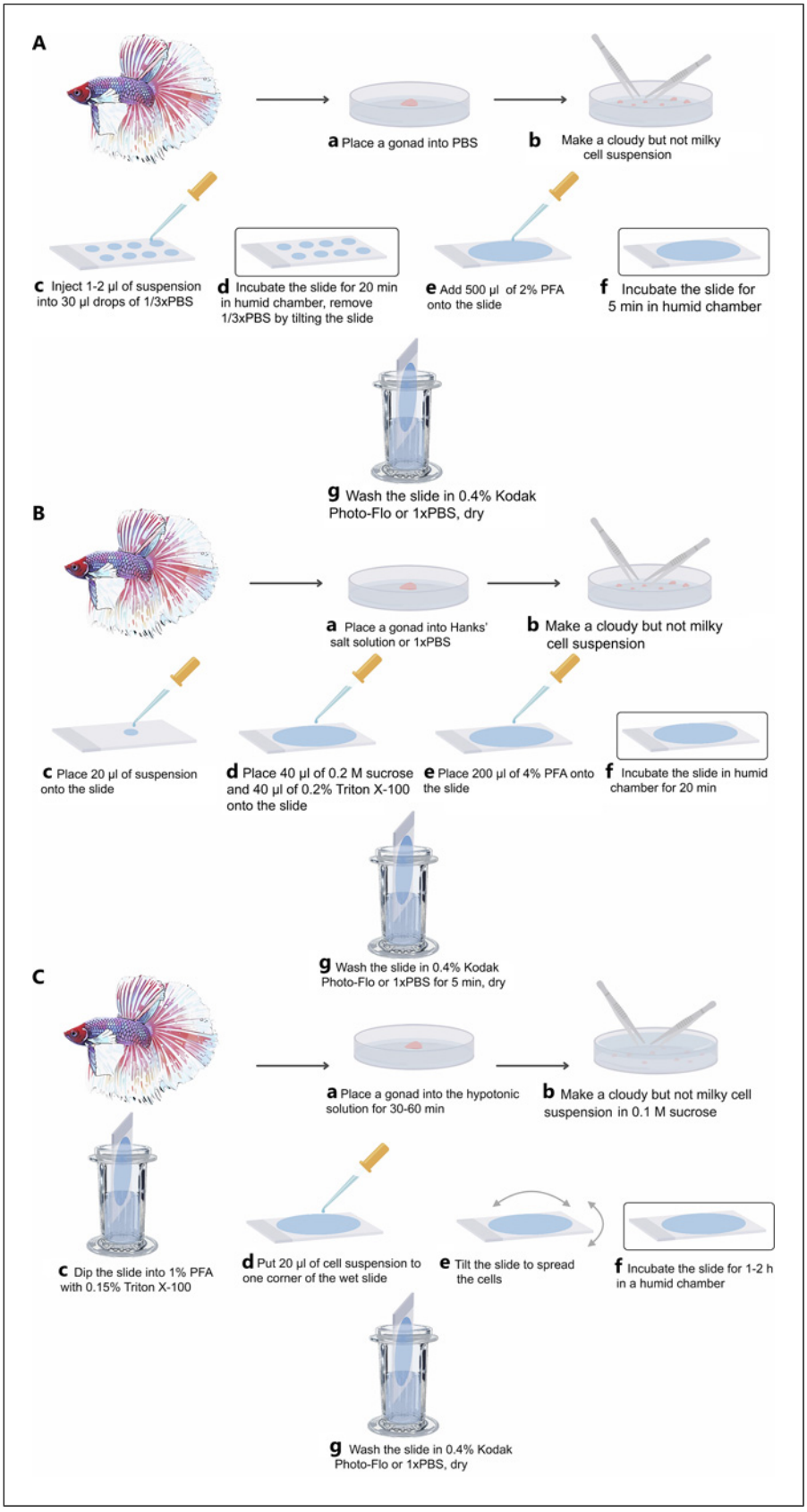


Fig. 1. Schematic presentation of the most used methods of synaptonemal complex (SC) preparation in fish. **A** The method of Moens [45]. **B** Araya-Jaime et al. [52]. **C** Peters et al. [55].

Table 1. List of commercially available antibodies that have been used in synaptonemal complex (SC) studies in fish

Antibodies	Manufacturer	Cat. No.	Use in fish	References
Rabbit polyclonal anti-SYCP3	Abcam	ab15093	Works in all fish	[46–49, 58, 59]
Rabbit anti-hSCP3	Abcam	ab150292	Works in *Danio rerio*	[56]
Rabbit anti-SYCP3	Novus Biologicals	NB300-232	Works in *Danio rerio*	[60]
Mouse anti-hamster SCP3	Abcam	ab97672	Does not work in *Danio rerio*, *Cobitis*, and *Poecilia*	[61]; our data
Anti-SCP1 antibody	Abcam	ab15090	Does not work in *Cobitis, Nothobrancius*, and *Poecilia*	Our data
Anti-SCP1 antibody	GeneTex	GTX15090	Works in *Clarias*, *Cobitis*, and *Poecilia*	Our data
Rabbit anti-SMC3	Invitrogen (Thermo Fisher)	PA529131	Works in *Danio rerio*	[60]
Rabbit polyclonal antibody anti-HORMAD2(C-18)	Santa Cruz	sc-82192	Does not work in *Cobitis* and *Poecilia*	Our data
Rabbit anti-SMC3	Abcam	ab9263	Works in *Gasterosteus aculeatus*	[57]
Rabbit polyclonal anti-H3K9me3	Abcam	ab8898	Works in *Poecilia reticulata*	Our data
Mouse monoclonal anti-MLH1	Abcam	ab14206	Works in guppy, *P. formosa, P. mexicana, M. picta, Nothobranchius spp., Cobitis spp., Betta splendens, Astyanax mexicanus* Does not work in *Alfaro*, *Xiphophorus, Clarias* in our experience	[46, 47, 49, 50, 58, 59]
Monoclonal anti-human MLH1	BD Biosciences Pharmingen	?	Works in *Danio rerio*	[45]
Mouse anti-hMLH1	BD Biosciences	550,838	Does not work in *Danio rerio*	[56]
Chicken polyclonal anti-Rad51	GeneTex	GTX00721	Works in *P. formosa, Clarias*	[58]
Rabbit anti-hRad51	GeneTex	GTX100469	Works in *Danio rerio*	[56]
Rabbit anti-Rad51	Ana-Spec	old: 55,838–2, new: AS-55838	Works in *Danio rerio*	[62]
Mouse anti-Rad51	Invitrogen (Thermo Fisher)	MA5-14419	Works in *Gasterosteus aculeatus*	[57]
Goat anti-hDMC1	Santa Cruz Biotechnology	sc-8973	Does not work in *Danio rerio*	[56]
Mouse anti-hRPA	Sigma-Aldrich	MABE285	Does not work in *Danio rerio*	[56]
Rabbit anti-hRPA	Bethyl	A300–244A	Works in *Danio rerio*	[56]
Mouse anti-hRPA	Santa Cruz	sc-56770	Works in *Danio rerio*	[63]
Human anti-centromere	Antibodies Inc.	15–234	Does not work in fish	Our data
Anti-Centromere CREST antibody	Fitzgerald	90C-CS1058	Works only in *Danio rerio*	[45]; our data

"telomere bouquet" stage. Before synapsis, chromosomes pre-align in telomeric regions via a DSB-dependent mechanism. SPO11-knockout mutants cannot introduce DSBs into their chromosomes and thus have no synapsis and a more distorted phenotype than MLH1-knockout mutants. In both sexes, the effects on fertility are the same as those observed in MLH1 knockouts [62]. By contrast, knockouts of the RAD21L1 cohesin, SMC1β cohesin, and SYCP1 trigger female-to-male sex reversal, as these mutations disrupt female meiosis. In young zebrafish, oogenesis is first initiated in immature gonads, and the male development pathway is activated if it fails for any reason. The first protein is not necessary for male meiosis, and RAD21L1 males are fertile. In SYCP1 and SMC1β knockouts, DSBs are formed, and the homologs start to pair at the telomeric ends, but they do not synapse, and such zebrafish males are sterile. In the SMC1β knockouts, SYCP3 chromosome axes are not formed, and this protein is present only at the telomeres [60, 63, 67]. Notably, fish are very diverse, and the physiology of meiosis in zebrafish does not necessarily reflect the general picture in all fish. Zebrafish are to date the only studied fish species on which human anti-centromere antibodies (CREST) work [45]; this raises an interesting question about the evolution of fish centromere proteins and can be addressed by conducting immunofluorescence studies in more fish, especially those related to zebrafish.

Sex Chromosomes

In other fish species, the main topics addressed by SC analysis are sex chromosome evolution and meiosis in hybrids. The question that arises when studying sex chromosome synapsis and recombination in fish is the identification of sex SCs among autosomal SCs. Fish chromosomes frequently have similar sizes and morphologies, making it challenging to recognize individual bivalents in the SC spread. If sex chromosomes are sufficiently differentiated, they may exhibit delayed abnormal synapsis and synaptic adjustments. The study of sex chromosome pairing in rainbow trout (*Oncorhynchus mykiss*) is a pioneering work in fish SC studies. In rainbow trout, the Y chromosome is shorter than the X chromosome. The sex bivalent completes pairing only when the autosomal bivalents are already paired and is characterized by lateral elements of different lengths. In late pachytene, the X and Y chromosomes adjust their lengths, and the sex bivalent becomes similar to autosomal ones [44]. Synaptic adjustment has recently been studied in detail in the three-spine stickleback (*G. aculeatus*). Herein, it was shown that while the longer X chromosome is shortened to fit the shorter Y chromosome, there is no observed stretching of the Y chromosome. DSBs were also visualized with antibodies against RAD51 along both sex chromosomes and autosomes; this is in contrast with mammals, in which DSB formation along the XY bivalent is suppressed. This indicates an autosome-like meiotic behavior of young sex chromosomes [57].

One of the fish groups in which sex chromosomes have been extensively studied using SC analysis is the tilapia (*Oreochromis* and related genera). In this group, different species have different sex chromosomes, evolved from different ancestral autosomes, and are of different types (XX/XY and ZW/ZZ) [68]. Previous studies have detected synaptic adjustment of the terminal parts of the long arms of chromosome 1 and heterochromatin accumulation in this region. Therefore, this pair has been suggested as the sex chromosome of the Nile tilapia (*O. niloticus*) [69–72]. Subsequent studies have indicated that chromosome 1, corresponding to genetic linkage group (LG) 3, bears a polymorphic heterochromatin block, which gives it a sex chromosome-like meiotic behavior, but the actual sex chromosomes in different strains of Nile tilapia correspond to LG1, LG20, and LG23 [73–76]. Because of their low level of differentiation, they did not have any synaptic abnormalities. However, LG3 frequently acts as a sex chromosome in other tilapia species; for example, the ZW/ZZ system in blue tilapia (*O. aureus*) [51, 77]. A heterochromatic region with reduced recombination has been suggested to act as a pre-adaptation, facilitating the emergence of a sex-determining locus. MLH1 mapping has not yet been performed for any tilapia species [78]. Because chromosome-specific FISH markers exist for tilapias, including *O. niloticus*, crossover mapping could help to identify sex chromosomes and reveal their structures in many species for which chromosome-level genome assemblies are not yet available.

Poeciliids are another group of fishes with well-studied sex chromosomes. In some species, such as platyfish, sex chromosomes are homomorphic and have not been identified during SC examination [79]. In guppies (*P. reticulata*), the first study revealed only fully synapsed SCs, perhaps because only late pachytene cells were examined [80]. Later, synaptic adjustment and delayed pairing due to size differences between the long Y chromosome and short X chromosomes were described based on electron microscopic observations [79]. However, owing to the limited ability to discriminate between specific DNA sequences and proteins in silver-stained SC spreads, these observations were misinterpreted. The authors suggested that pairing cannot start in presumably non-homologous terminal heterochromatic regions and thus concluded that it should initiate in proximal centromeric regions, proceeding terminally. In tilapia, this pattern of synaptic adjustment

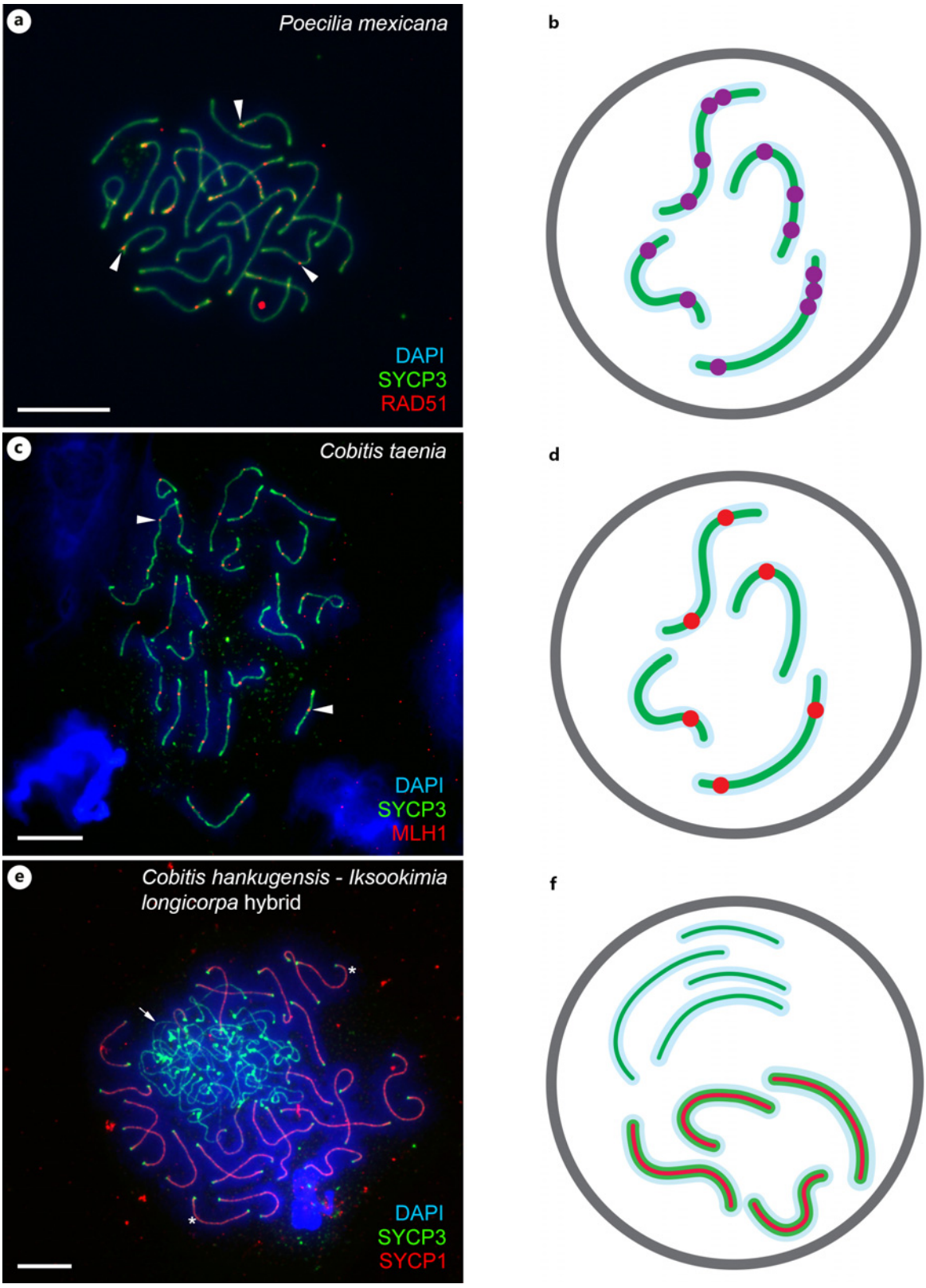
occurs on chromosome 1. However, later, a fluorescence microscopic analysis of male guppy meiosis showed sex and centromeric heterochromatin blocks visualization and MLH1 foci detection [46]. The terminal regions of sex chromosomes were found to be homologous and initiated synapsis, which then proceeded proximally and not vice versa. This case highlights the importance of using diverse approaches in cytogenetic studies. In the swamp guppy *M. picta*, a close relative of the guppy, the Y chromosome is more differentiated and shorter than the X chromosome. Similar to guppies, synapsis is initiated in the terminal parts of chromosomes, and recombination occurs in this region. However, because of the large size differences between sex chromosomes, synaptic adjustment is never fully completed, and the proximal part of the X chromosome remains unpaired throughout prophase [50]. In mammals, unsynapsed chromosome fragments during pachytene lead to meiosis failure and apoptosis, and thus the incompletely synapsed sex bivalent is isolated inside a specific structure called "sex vesicle" [81]. Sex vesicles are not observed in swamp guppy. Therefore, the meiotic mechanisms in this species require further investigation. The mechanisms underlying meiotic sex chromosome inactivation (MSCI) and meiotic silencing of unsynapsed chromatin (MSUC) in fish remain unclear. Many other poeciliid species, such as the common molly (*P. sphenops*) and the western mosquitofish (*Gambusia affinis*), have ZW/ZZ sex chromosomes [82–84]. Considering the small size of these fish, obtaining the gonads of juvenile females for SC analysis can be challenging; thus, the SCs of sex chromosomes have not been studied in these species. However, the SCs of the parthenogenetic species, the Amazon molly (*P. formosa*), were recently obtained and analyzed (see below) [58]. This opens up the possibility of studying the meiotic behavior of sex chromosomes in ZW/ZZ poeciliids, which may lead to more important findings.

Another well-known group of fish with widespread sex chromosomes is the African annual killifish (genus *Nothobranchius*). Some fish in this group have simple XX/XY sex chromosomes, whereas others have multiple $X_1X_2X_1X_2/X_1X_2Y$ sex chromosomes. Sex chromosome synapsis and recombination were analyzed in the turquoise killifish (*N. furzeri*) and *N. kadleci* using immunofluorescence staining for SYCP3 and MLH1. Since the X and Y chromosomes in these species are weakly differentiated, no synaptic abnormalities or skewed MLH1 distributions were detected (although differences were found between sexes in MLH1 distribution across bivalents in general) [47]. In species with $X_1X_2X_1X_2/X_1X_2Y$ sex chromosomes (*N. lourensi, N. guentheri, N. janpapi, N. ditte, N. brieni,* and the closely related *Fundulosoma thierry*), sex trivalents were observed during male meiosis in all sampled animals, and it was concluded that, at least in some of them, multiple sex chromosomes were formed by independent fusions between the ancestral XX/XY sex chromosomes and different autosomes [85].

The wolffish *Hoplias malabaricus* (Erythrinidae, Characiformes) is a species or species complex that is peculiar owing to its sex chromosome evolution. It has seven "karyomorphs" (A–F), with karyomorphs A and E not having known sex chromosomes, B, C, and F having simple XX/XY sex chromosomes, D having $X_1X_2X_1X_2/X_1X_2Y$ multiple sex chromosome system, and G having XX/XY_1Y_2 multiple sex chromosome system. The sex chromosome systems of karyomorphs B, C, and D on one side and F and G on the other side have two independent origins from different autosomes [86, 87]. Despite the appeal of this species for SC studies, only one relevant study has been published to date [88]. Synapsis of the sex trivalent in karyomorph D was investigated by electron microscopy, and full pairing was observed, with nonhomologous pairing of the overhanging ends of the X_1 and X_2 chromosomes. Immunofluorescence investigation of sex chromosome synapsis and recombination is a promising direction for future work.

Meiosis in Hybrids and Polyploids

SC analysis is an important tool for analyzing meiosis in various polyploid and hybrid fish species. The simplest meiotic consequence of interspecific hybridization is distorted ortholog pairing. In closely related species, pairing can be normal except for rearranged chromosomes. The two tilapia species, *O. niloticus* ($2n = 44$) and *O. karongae* ($2n = 38$), differ in chromosome number. Their hybrids are fertile, and 19 fully synapsed SCs – 16 bivalent and three trivalent – are observed in males. This indicates that the karyotypes of *O. karongae* and *O. niloticus* differ in three fusion/fission events [89]. Simultaneously, significant interspecific divergence may cause hybrid sterility even in the case of a conserved chromosome number. In the Neotropical family Serrasalmidae (Characiformes), *Piaractus mesopotamicus* and *Colossoma macropomum*, both with $2n = 54$, produce a sterile hybrid called "tambacu," that is widely cultivated for food. Tambacu spermatocytes show strong genome-wide failure of chromosome pairing with univalents, bivalents, and incomplete bivalents. Although mature sperm is somehow produced, it is genetically dysfunctional but can trigger gynogenetic egg development [90]. The situation in a hybrid between two air-breathing catfish belonging to the Clariidae family, North African catfish (*Clarias gariepinus*) ($n = 28$) and bighead



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catfish (*C. macrocephalus*) ($n = 27$), cultivated for food in Thailand, is similar. Hybrid males show strong genome-wide asynapsis, and while certain cells in certain individuals complete normal-like synapsis and produce small numbers of mature spermatozoa, these spermatozoa have not been proven to be functional [91]. Female hybrids can sometimes produce offspring, although it is unknown whether the reproduction is Mendelian or gynogenetic [92]. In hybrids between the guppy (*P. reticulata*) and the Yucatan molly (*P. velifera*), individual hybrids also differ in the success of meiosis. In one of the two hybrids analyzed, no meiosis or sperm was observed in the testes. In another specimen, most SC-containing cells showed massive asynapsis and incomplete synapsis, a few cells showed complete synapsis, and a few spermatozoa were observed [80]. Hybrids between different medaka species (*Oryzias latipes* and *O. curvinotus*), which show failed ortholog synapsis, exhibit low expression of SYCP1. However, in females, a small number of oocytes undergo endoreplication to produce diploid eggs that develop via gynogenesis [93]. Aberrant synapsis can result from genetic and chromosomal divergences. Crosses of loach species with different phylogenetic distances and karyotype variability showed that hybrids between distantly related species with morphologically similar karyotypes undergo pairing with orthologous chromosomes. By contrast, hybrids between closely related species with different karyotypes exhibit aberrant synapsis during SC formation in both hybrid males and females [49].

Both male and female hybrid fish usually show Mendelian fertility in crosses between closely related species and sterility in crosses between distantly related parental species. However, in hybrid female fish, an intermediate step leads to clonal (gynogenetic) or hemiclonal (hybridogenetic) reproduction [49, 94, 95]. These reproductive mechanisms can be distinguished using SC analysis. For instance, in European loaches of the genus *Cobitis*, the majority of oocytes in hybrid females undergo failed meiosis and apoptosis, similar to those in hybrid males [53, 96]. However, a minor subpopulation of gonocytes (approximately 6%) undergoes premeiotic genome duplication (endoreplication) and becomes tetraploid. During meiosis, pairing in such cells is allowed because each chromosome has a copy to pair with. These oocytes progress beyond pachytene and complete meiosis, resulting in diploid eggs that can develop after activation by the sperm of a sexual species (gynogenetically) [53, 96]. A similar pattern has been observed in gynogenetic triploid hybrids yielding natural populations [96]. Moreover, premeiotic genome endoreplication has been demonstrated in F_1 hybrids [49]. In another well-known gynogenetic hybrid fish, the Amazon molly (*P. formosa*), no premeiotic endoreplication or synapsis between orthologous chromosomes occurs [58, 97]. Its chromosomes form univalent SCs and lack the RAD51 and MLH1 foci, suggesting the absence of DSB formation and crossing over [58]. Moreover, meiosis I is omitted, resulting in unreduced gametes produced by apomixis [54, 97]. Interestingly, triploid hybrid males emerging after occasional fertilization of diploid eggs of *P. formosa* have aberrant pairing, whereas triploid females maintain gynogenetic reproduction, possibly via mechanisms similar to those of diploid *P. formosa* [98].

In addition to providing insights into the clonal reproduction of hybrid fishes, SC analysis revealed the potential mechanism for hemiclonal reproduction in triploid hybrids that produce eggs through hybridogenesis. Triploid hybrid loaches from *Cobitis hankugensis* × *Iksookimia longicorpa* hybrid complex exhibit triploid hybridogenesis, during which a single copied genome is eliminated (for example, *LLH* hybrids between parental species *L* and *H* eliminate the genome *H*), whereas the double-copied genomes (*LL* in *LLH* hybrids) enter normal meiosis, undergo pairing and recombination, and result in haploid gametes [99, 100]. Analysis of SCs showed that a single copy of the genome is eliminated before meiosis in a portion of the gonocytes [54]. After genome elimination, the remaining two genomes form bivalents. However, most gonocytes maintain their original ploidy level and form a mixture of univalents and bivalents during pachytene [54]. Such oocytes are probably eliminated during the pachytene checkpoint due to synaptic failure. Immunostained SC spreads of pure fish species *P. mexicana*

Fig. 2. Pachytene chromosome spreads (**a**, **b**, **c**) with immunostaining for SYCP3 (**a–f**), RAD51 (**a**), MLH1 (**c**), and SYCP1 (**e**) proteins and their schematic representations (**b**, **d**, **f**) in pure fish species and a triploid hybrid. **a** Immunostaining with antibodies against RAD51 (pointed by arrowheads) and SYCP3 indicates the presence of DSBs along the bivalents of *Poecilia mexicana*. **b** Schematic representation of RAD51 staining (violet circles), lateral elements of synaptonemal complexes (SCs) (green), and chromatin (blue) staining. **c** Staining for MLH1 (pointed by arrowheads) and SYCP3 indicates the presence of crossing-over loci on the bivalents of *Cobitis taenia*. **d** Schematic representation of MLH1 staining (red circles), lateral elements of SCs (green), and chromatin (blue) staining. **e** Staining for lateral (SYCP3) and central (SYCP1) components of SCs shows bivalents (indicated by asterik) and univalents (indicated by arrows) on SC spreads of the triploid *C. hankugensis* × *Iksookimia longicorpa* hybrid. **f** Schematic representation of lateral (green) and central (red) elements of SCs and chromatin (blue). The methods of SC preparation and staining are described in [58, 96]. Scale bar represents 10 μm.

(Fig. 2a, b), *C. taenia* (Fig. 2c, d), and the triploid female hybrid *C. hankugensis* × *I. longicorpa* (Fig. 2e, f) are represented in Figure 2.

Some hybrid fish do not experience premeiotic chromosome duplication but are polyploid. This also leads to the normalization of meiosis and sexual reproduction in both sexes. During the subsequent evolution of such hybrids, their parental genomes diverge, resulting in "re-diploidization," making them functionally diploid. This process can be tracked using SC analysis. Both quadrivalents and bivalents are observed in SC spreads at early stages in some tetraploid loach species of the genera *Misgurnus* and *Cobitis*, as well as in Kaluga (*A. dauricus*) [48, 101]. In some fish, such as the white sturgeon (*A. transmontanus*), at later stages, the chromosomes of the parental genomes do not attempt to pair with each other, and multivalents are not observed [102].

Exploration of B Chromosomes

B chromosomes, which are usually even smaller than fish A-chromosomes, are also more easily studied by SC analysis than by metaphase chromosome analysis because of the decondensed chromatin at the meiotic prophase. B chromosomes are widespread in some species of Characidae. In the red-eye tetra (*Moenkhausia sanctaefilomenae*) from the Paraná River, B chromosomes act as sex chromosomes, and 0–2 B microchromosomes are present in male metaphase. SC analysis in *M. sanctaefilomenae* revealed both bivalent and univalent configurations, indicating that the B chromosomes are homologous [103]. In the Mexican tetra (*Astyanax mexicanus*), a blind cavefish, 1–3 B microchromosomes are present in male metaphases and can be visualized by SC analysis [58]. In *Psalidodon scabripinnis* and a related species, *P. paranae,* the B chromosome is not related to sex determination but is a large metacentric macrochromosome. Based on C-banding, it was suggested to be an isochromosome. SC and meiotic metaphase analysis showed that it folds twice, self-pairs at pachytene, and presents a ring configuration with one chiasma at metaphase, which confirms this suggestion [104, 105]. In summary, SC analysis provides a powerful lens to investigate the intricacies of meiotic physiology and reproductive mechanisms in diverse fish species, offering valuable insights into chromosome behavior, sex chromosome evolution, and the challenges posed by hybridization and polyploidy.

Conclusion

SC analysis has multiple applications in chromosomal studies of fish. Coupled with modern methods, such as immunostaining, it is a powerful tool for studying chromosome behavior in fish meiosis in detail. In this review, we highlighted the importance of SC analysis in research on the physiology of fish meiosis, sex chromosome structure, polyploidy, and interspecies hybridization, corroborating its indispensable role in these fields, which should not be overlooked. A successful SC study depends on preparation quality and dictates the overall research success. The many factors governing SC analysis success in fish, namely, choosing the optimal life stage and season for sampling, the optimal method of SC preparation, and the optimal staining technique, including antibodies and FISH probes, must all be carefully considered. This review may serve as a resourceful guide for researchers, offering a reference for optimizing future investigations in fish and other species.

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Conflict of Interest Statement

The authors have no conflict of interest to declare.

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Author Contributions

Conceptualization, data curation, and writing – original draft: A.L. and D.D.; funding acquisition: K.S.; visualization: A.L., D.D., and K.S.; writing – review and editing: A.L,. D.D., S.S., T.P., W.S., and K.S. All authors have read and agreed to publish the final version of the manuscript.

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