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CYTOGENETICS (T LIEHR, SECTION EDITOR)

Conventional Cytogenetic Approaches—Useful and Indispensable Tools in Discovering Fish Biodiversity

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Abstract

Purpose of Review Fishes exhibit the greatest biodiversity among extant vertebrates. In fact, about 34,000 fish species are currently estimated, of which ~25% are living in Neotropical freshwaters. Currently, several leading-edge studies using molecular biology procedures have largely contributed to the investigation of the fish genomic architecture at the chromosomal level. In this review, we intend to demonstrate that conventional cytogenetics is also a powerful procedure to identify and clarify both individual and inter- or intrapopulational fish characteristics and to unveil their biodiversity.

Recent Findings Intra- or interpopulational chromosomal characteristics, revealing dramatic processes of evolution and cryptic divergence and even speciation, as well as unusual cases of interspecific hybridization, clonal reproduction, and sex chromosome differentiation, were, and still are, unmistakably discovered among fishes by using conventional, i.e., non-molecular cytogenetic procedures.

Summary In this review, we aim to demonstrate that conventional cytogenetics constitutes a powerful and indispensable tool in characterizing the hidden biodiversity of the ichthyofauna. We focus on some key examples that clearly illustrate the importance and the efficiency of this approach.

Keywords Fish · Conventional cytogenetics · Polymorphism · Cryptic speciation · Clonal reproduction

Introduction

Fishes are the richest vertebrate group, with more than 34,000 valid species taxonomically recognized, about 19,000 of them living in freshwater, of which 50% can be found in Neotropical region [1••]. Therefore, it is not surprising that such enormous biodiversity implies a number of phylogenetic and taxonomical challenges. Although species can be identified by means of different phenotypic characters, particularly cytogenetic studies have proved to be an important tool in numerous cases. Changes in chromosome number and structure have been correlated with several environmentally adapted traits [2, 3••]; especially in fishes, such evolutionary processes might be tightly linked with considerable genome plasticity, as they are characterized by impressively elevated tolerance to chromosomal changes [4]. Such dynamics might

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represent a powerful evolutionary driver, opening diverse routes to increase biodiversity and, as a by-product, to enhance the value of the cytogenetic data in the exploration of fish biodiversity and conservation biology [5]. Actually, fish cytogenetics has proved to be an important source of valuable information for both basic and applied science (Fig. 1).

Besides the Giemsa staining, conventional cytogenetic studies include the chromosome banding techniques (i.e., C-, G-, R-, Q-, and H banding, silver nitrate staining of nucleolar organizer regions known as Ag-NORs and combined DAPI/Chromomycin A_3 staining). During the last decades, fish cytogenetics has experienced a remarkable development with advent of new analytical methodologies. More specifically, fluorescence in situ hybridization (FISH)-based technologies have allowed the mapping of diverse repetitive sequences and genes of interest on fish chromosomes. In addition, whole chromosome painting (WCP) and comparative genomic hybridization (CGH) enabled uncovering the extent of homology between chromosomes of closely or distantly related species, as well as the presence of sex-linked sequences [6•]. Similarly to other biological disciplines, the onset of cytogenetics is inseparably linked with seminal discoveries made during investigation of human cells. Improvements include in cell culture application of colchicine for cell cycle arrest, hypotonic treatment for better chromosome spreading, chromosomal staining and banding approaches, and other molecular procedures allowing considerable methodological advances, including the diagnosis of chromosomal syndromes since the 1960s (see Ferguson-Smith [7] for a comprehensive review). Application of Ag-NORs represents most likely one of the most exciting examples of conventional cytogenetic performance in clinical studies in the way that the activity (and particularly the size) of NORs in the cells might be directly correlated with the activity of specific brain regions [8]. In fact, despite the exponential growth of studies harnessing state-of-the-art cytogenetics, one should not forget that many initial discoveries of unusual karyotype characteristics, including polymorphisms, polytypy, polyploidy, and sex and B chromosomes (Bs), were a priori achieved by pioneer studies based on conventional methodologies (e.g., [9–12]).

In this paper, we intend to draw attention to a set of specific examples of freshwater and marine fish taxa, in order to illustrate how conventional cytogenetics allows to get more insights into biological features of species and populations, unmasking their unforeseen diversity and, in some cases, exposing probable ongoing speciation processes. We first take a closer look at highly diverse Neotropical freshwater ichthyofauna, where evergrowing number of reports points to hidden and underscored biodiversity, with high amount of cryptic species being left unsolved in previous taxonomic analyses (Sections "The Enigmas of Outstanding Neotropical Fish Biodiversity," "The Tale of Wolf Fish *Hoplias malabaricus* (Characiformes, Erythrinidae)," and "*Astyanax scabripinnis*: Unveiling its Biodiversity Through Cytogenetic Investigations"). Nonetheless, even research centered on Neotropical marine ichthyofauna is not devoid of analogous challenges, as discussed in Section "Cytogenetics of Marine Fishes: Contributions to Evolutionary Investigation." Next, we would like to direct attention towards European diploid-polyploid unisexually reproducing complexes in spined loach fishes (Section "European Spined Loaches of the Genus *Cobitis* (Cypriniformes, Cobitoidea): a Hidden Biodiversity

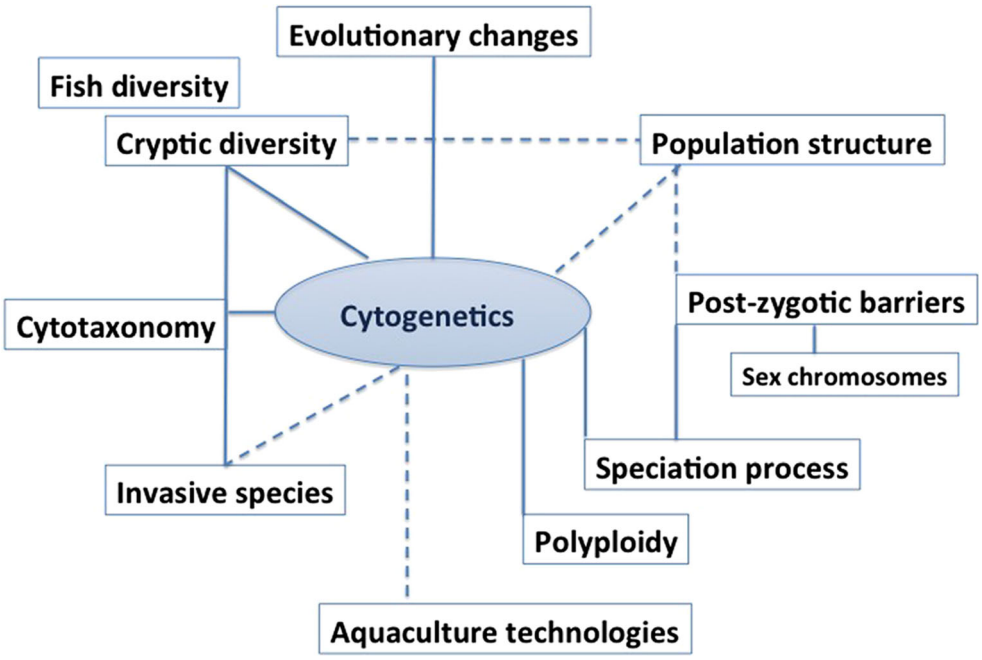

Fig. 1 An illustrative interaction network among different basic and applied science fields, having the cytogenetics as direct or indirect supporting data nucleus for each one of the different approaches there represented

Just in Front of Our Doors"). We will also touch on cytogenetics of sturgeons, with emphasis on their outstanding tolerance to interspecific hybridization (Section "Sturgeons, Shovelnoses, Paddlefishes (Chondrostei: Acipenseriformes): When Elevated Ploidy Level Coupled with Interspecific Hybridization Means No Constraints/Problem"), and we will close the review by pointing on the impact of conventional cytogenetics-based protocols on fish sex chromosome identification (Section "Conventional Cytogenetics Shedding Light on Sex Chromosome Evolution in Fishes").

The Enigmas of Outstanding Neotropical Fish Biodiversity

Neotropical ichthyofauna covers roughly 9100 species, of which about 5200 belong to freshwater lineages, representing the richest freshwater worldwide [1••, 13••, 14]. Such species richness is not surprising given the geological history of South-American continent that shaped, branched, and fragmented the water bodies, as well as the wealth of ecological niches that might be utilized [13••]. However, it is even wider than previously thought, as new and/or cryptic species were and are still identified by distinct investigative methods [15, 16]. Accordingly, species with wide geographical distribution cover hidden taxonomic entities, representing more previously cryptic species inside a species complex. Examples with cytogenetically different karyomorphs, i.e., different karyotype forms, were already found in diverse fish groups [15, 17–25]. In many of these cases, the absence of hybrid forms suggesting gene flow restriction was documented by several complementary approaches. Such findings reinforce the view that different karyomorphs may already have diverged enough to develop certain post-zygotic reproductive barriers. As morphological characters are often not resolute, such hidden biodiversity provides challenges to taxonomical efforts. This problematics is, of course, not restricted to Neotropical fishes (see [26]), neither to fishes at all (e.g., [27]).

The Tale of Wolf Fish *Hoplias malabaricus* (Characiformes, Erythrinidae)

The Erythrinidae fish family comprises a small but widespread group throughout the Neotropical region, composed of three recognized genera—*Hoplias* (Gill, 1903), *Hoplerythrinus* (Gill, 1985), and *Erythrinus* (Scopoli, 1777)—and at least 18 species [14]. Most of erythrinids display a remarkable preference for various types of lentic environments [28], and due to their sedentary habits, they are usually not able to overcome certain natural physical barriers, such as waterfalls and large rapids. Such situation had undoubtedly considerable impact on reduced gene flow among populations and, consequently, to the stepwise increase in their genetic difference [10, 29]. Indeed, erythrinid fishes usually possess large karyotypic variation (with a single exception in *Hoplias lacerdae* group), where pioneer conventional cytogenetic reports showed extensive variability among conspecific populations concerning diploid chromosome numbers (2n), karyotype compositions, and sex chromosome systems [10, 30–31, 32••].

Particularly in the wolf fish *Hoplias malabaricus*, conventional cytogenetic investigations were sufficient enough to characterize the main features of the hidden biodiversity of this taxon. Thus, *H. malabaricus* became one of the most exemplary models for studying karyotype evolution among Neotropical fishes. This species complex is composed of seven major karyomorphs (A–G) that differ by 2n (ranging from 39 to 42 elements in the complement), karyotype composition, and three distinct male-heterogametic sex chromosome systems [10, 33–35]. It is important to note that such an evolutionary scenario among karyomorphs was already suggested based on investigation of Giemsa-stained karyotypes [33]. The implications of these seminal studies were further complemented and strengthened by the physical mapping of repetitive DNAs by FISH, CGH, and WCP [10, 35–37]. It is also suggested that post-zygotic reproductive barriers have already been established among karyomorphs, as might be inferred from the absence of inter-karyomorph hybrid forms in regions of sympatry [30].

When taking a closer look at sex chromosomes of *H. malabaricus*, conventional cytogenetics again provided the first important insights into this matter. In karyomorph B (2n = 42 in both sexes), a well-differentiated XY sex chromosome system makes the major difference that distinguishes this karyomorph from the closely related karyomorph A (2n = 42 in both sexes), where such sex system is not recognizable. A remarkable feature of X-chromosome in karyomorph B is an otherwise rarely seen preferential accumulation of heterochromatin and repetitive sequences on X-chromosome instead of allosome Y [38]. In karyomorph F (♂40,XY/♀40,XX), the X- and Y-chromosomes differ only by a distinct heterochromatic block in the short arm of the Y-chromosome [39]. Later, the revealance of uneven distribution of repetitive DNAs in this region, together with a male specific site on the Y-chromosome, confirmed the presence of a nascent sex chromosome system [34, 40].

In turn, karyomorph D (♂39/♀40) is characterized by an X_1X_2Y sex chromosome system. Giemsa-stained mitotic and meiotic spreads together with several chromosome banding methods demonstrated that the large-sized neo-Y-chromosome emerged through a tandem fusion [41]. Moreover, a stabilized pachytene sex trivalent occurs in male meiosis, as well as asynapsis in the putative sex-specific region [42, 43]. It was also evidenced that this multiple sex system was derived from a nascent XY sex chromosomes

occurring in the karyomorph C (♂40,XY/♀40,XX), in which the X-chromosome differs from the Y-chromosome by a discrete accumulation of a GC-rich heterochromatin [43].

Finally, conventional cytogenetics revealed that karyomorph G (♂41,XY_1Y_2/♀40,XX) possess XY_1Y_2 neo-sex chromosomes, with indirect but solid evidences about their tentative homology with particular chromosome pairs found in related karyomorphs E and F [31, 33]. This notion was recently confirmed by showing homology between karyomorph's F and G sex chromosomes through complementary molecular cytogenetic protocols [35].

Taken together, *H. malabaricus* is a textbook example illustrating the usefulness of conventional cytogenetic approaches as effective tools in evidencing hidden biodiversity and in deciphering issues associated with species complexes. In this case, initial basic cytogenetic data indeed sparked the interest and laid foundation for subsequent efforts that uncovered differential patterns of sex chromosome evolution among karyomorphs, particularly in terms of their either common and/or independent origin [10, 40].

Astyanax scabripinnis: Unveiling its Biodiversity Through Cytogenetic Investigations

The genus *Astyanax* represents another Neotropical freshwater assemblage in which the accuracy of species identification was repeatedly put in question. It is represented by more than 100 species and considered as "insertae sedis" in the Characidae family ([44]). Indeed, this group was previously recognized as problematic encompassing variant forms among hydrographic systems. As to *Astyanax scabripinnis*, six subspecies were described by Fowler (1948) [45].

Notably, conventional cytogenetics clearly contributed to discover the hidden biodiversity within *A. scabripinnis*. Six populations from different Brazilian watersheds, initially recognized within this taxon, were investigated using chromosomal characteristics, with the addition of some morphometric data [46]. Five karyomorphs in terms of 2n, karyotype organization and distribution of the constitutive heterochromatin were documented. On the other hand, morphometric analyses discriminated only four populations, clearly differentiated by their chromosomal data. At the same time, morphological traits were sufficient enough to distinguish two populations having similar chromosomal patterns. In conclusion, it was evident that the six populations could be perfectly diagnosed by their chromosomal and/or morphological features, with the indication of a species complex inside taxon *A. scabripinnis* [46]. Although acknowledging that not all of such six species were later confirmed as members of the *A. scabripinnis* species group, this study is pioneering in highlighting the biodiversity within this taxon. Later reports following the same scheme of integrated multi-approach investigation, such as those of Mizoguchi and Martins Santos (1998) [47], showed similar results reaffirming the natural biodiversity within *A. scabripinnis*.

Such approaches played a significant role in taxonomic progresses, by the clear demonstration that *A. scabripinnis* require a re-definition concerning its actual systematic diversity. In fact, advances of taxonomic studies identified new species inside the *A. scabripinnis* complex with more than 30 species until now recognized [48–51].

In addition to taxonomic treatments, conventional cytogenetics also provided significant advances in characterizing several other biological traits within *A. scabripinnis*, such as natural triploidy [52–54], hermaphroditism, unequal sex ratio, and presence of Bs, among others. The first studies characterizing macro and micro B elements in this species [55–57] were followed by several others, improving the knowledge about the properties of such chromosomes. In this sense, Vicente et al. (1996) [58] showed a marked distortion of the sex ratio among populations, with a female predominance and the possible association of such a feature with the higher incidence of Bs in that sex. In addition, a gradual change in the population frequency of these Bs was also recognized, with a sharp decrease from higher to lower altitudes [59]. Significantly, *A. scabripinnis* also shows a considerable proportion of hermaphroditism, and a comparative study among populations from different habitats showed distinct frequencies of hermaphrodite individuals, varying from 1.9 to 9.8% [60]. Nonetheless, further investigations pointed to the occurrence of a functional hermaphroditism, with possible sex reversal for both males and females, and its apparent association with some environmental factors, such as temperature and population density, but without correlation with Bs [61].

To sum up, *A. scabripinnis* stands out as another very attractive taxon for evolutionary investigations. The source of its inherent karyotype variability might lie, again, in the populational structure as the sub-populations are restricted to the headwaters of small streams, where the great rivers act as natural barriers for gene-flow. Therefore, they can give rise to isolated demes that are fated to follow different evolutionary paths, allowing the consequent expansion of *A. scabripinnis* biodiversity.

Cytogenetics of Marine Fishes: Contributions to Evolutionary Investigation

Marine ecosystems encompass vast and complex areas, some of which, such as coral reefs, provide shelter for one third of all known fish species [62]. The physical and environmental characteristics of marine environments have spurred and shaped enormous fish biodiversity and promoted their particular phyletic diversification, but our understanding of real species richness is still far from complete, with hundreds of new species being described every year [63, 64].

However, marine fishes usually display less karyotypic diversification than the freshwater ones [65], and several hypotheses have been put forward to explain this pattern, including the difference in the number of biogeographic barriers [66, 67]. In fact, many marine fish groups have retained an ancestral karyotype composed of 2n = 48 acrocentric chromosomes (e.g., [68–70]), even after longer evolutionary time frames (e.g., [71]). This condition stands out in Percomorpha [65], which includes the majority of marine fish species [1••]. Some conditions, such as large populations, high dispersive potential, either as larvae or migrating adults [72, 73], and vast distribution areas, associated with the historical connectivity of the oceans, seem to act as extrinsic buffering factors for karyotype changes in marine environments [65]. On the other hand, some marine groups are also characterized by a dynamic chromosomal evolution [74], with a wide spectrum of karyotypic changes. In such groups, the contribution of the conventional cytogenetics in characterizing phyletic divergences can be expressed into three main levels: (a) intraspecific or interpopulational chromosomal variations of specific chromosomal regions, representing initial steps of phyletic differentiation; (b) intraspecific numerical and structural karyotype variations, often expressed as evolutionarily transitional karyomorphs; and (c) interspecific karyotype diversification, identifying cryptic taxa.

The most pronounced cases of intraspecific variability among marine species are those related to Ag-NORs in reef fishes, which present high evolutionary dynamism [75, 76], exhibiting significant polymorphisms in size and locus numbers (e.g., [77]). The distribution and frequency of Ag-NORs may differ among populations of different marine regions, indicating populational structuring within the distribution area of particular taxon [78, 79].

Numerical and structural intraspecific karyotype variations are frequent in some marine groups and may be correlated with the initial steps of karyotype divergence. Although pericentric inversions are considered as the main source of changes on marine fish karyotypes [80], Robertsonian (Rb) fusions are also frequent and might persist temporarily in heterozygous constitution as polymorphisms. Populations of *Chromis* and *Dascyllus* species (Pomacentridae) exhibit marked numerical/structural polymorphisms due to Rb fusions [81–83]. In fact, the variable karyotypic patterns of *Dascyllus aruanus* (2n = 28–32), *Dascyllus trimaculatus* (2n = 47–48), and *Dascyllus reticulatus* (2n = 34–36) contrast with the conserved patterns found in other species, namely in *Dascyllus melanurus* (2n = 48 st-a) [84]. Likewise, extensive chromosomal polymorphisms occur within the speciose Gobiidae family (e.g., [85, 86]). Particularly in populations of *Gobius paganellus*, karyomorphs derived from structural polymorphisms may exhibit 2n = 46, 47, 48 (NF = 48), 2n = 46 (NF = 46–47), or 2n = 46–47 (NF = 47), documenting chromosomal fusions, inversions, and deletions [87].

Marine fishes displaying a low degree of phenetic differentiation are a challenge for taxonomy. In this respect, a large number of nominal species have been subdivided as a function of divergent genetic patterns [63]. As a contribution, conventional basic cytogenetic data have also significantly helped to identify cryptic species. Karyotypic analyses of *Bathygobius soporator* (Gobiidae) populations from Brazilian Western Atlantic showed 2m+6st+40a chromosomes (2n = 48; NF = 56), with a largely dissimilar pattern from those of the Atlantic Rocas Atoll, which has 28st+20a chromosomes (2n = 48; NF = 76) [88]. The recognition of a new species was subsequently emphasized by molecular markers [89] and recently described as *Bathygobius brasiliensis* [90]. In the Black Sea, cytogenetic patterns helped to identify two cryptic species of the *Ponticola* genus from western and eastern regions, i.e., *P. odessicus* with 2n = 30–35; NF = 46 and *P. eurycephalus* with 2n = 46; NF = 46, respectively [86].

Bioinvasions have been characterized as a growing phenomenon in marine environments [91], including hybridization with native taxons [92]. Such events may be favored by interspecific karyotype similarities since it imposes less postzygotic restrictions [93]. Cytogenetic data have also assisted in characterizing invasive or potentially invasive species [94, 95] and, in last case, in estimating possible risks to native fauna.

European Spined Loaches of the Genus *Cobitis* (Cypriniformes, Cobitoidea): a Hidden Biodiversity Just in Front of Our Doors

Spined loach, *Cobitis taenia*, is considered to be one of the most widely distributed freshwater fish species in Eurasia. Until recently, it was believed that it ranges from Japan across Siberia, central Russia, Europe, and Northern Africa, and a number of subspecies have been described in this vast area. However, detailed studies revealed that many of such "subspecies" represent in fact distinct taxonomic units (e.g., [96, 97]) and *C. taenia* sensu Berg [98] must be considered as *taenia* complex or group. In fact, recent phylogenetic studies demonstrated that the European representatives of the genus *Cobitis* include five major mitochondrial lineages [99]. Their karyotypes are highly evolutionarily diversified but retain almost the same diploid chromosome number 2n = 50 (reviewed in [11, 100]). Exceptions include 2n decreasing by chromosomal rearrangements (e.g., 2n = 48 for European *C. taenia*, Asian *C. takatsuensis*, and one form of *C. biwae* and 2n = 40 for *C. sinensis*) or increasing via polyploidization events (large race of *C. biwae* and *C. striata* in Japan, polyploid forms in *C. taenia* species complex, [101]). The remarkable diversity of their karyotypes undoubtedly reflects different rates of chromosomal changes in particular lineages.

In several European species of this lineage, an extensive structural polymorphism of major rDNA sites of the presence/

absence type was detected by means of conventional and/or molecular cytogenetic techniques [102–106], but the nature of such unusual polymorphism is not properly understood at present. Four of such species (*C. taenia*, *C. elongatoides*, *C. tanaitica*, and *C. taurica*) have been identified as parental for hybrid diploid-polyploid complexes ([106, 107] and references therein), and conventional cytogenetics at the level of the basic Giemsa-stained chromosomes is still extensively used as a tool in studying the enormous hybrid diversity of these loaches across Europe [5, 108–110]. The evolutionary scenario suggested that during several of the last interglaciation cycles, all four species came into reproductive contact several times, resulting in reciprocal and polyphyletic origins of nearly all female clonal hybrids, that reproduce via gynogenesis, i.e., they act as sperm "parasites" requiring sexual male sperm to trigger embryonic development [106, 111]. A review of hybrid diversity across Europe has documented the presence of hybrids with nearly all possible genome combinations [101]. In addition, Choleva et al. [111] found the genome of *C. strumicae*, a representative of Bicanestrinia (another lineage within the genus *Cobitis*) in combinations with genomes of some abovementioned species. The karyotypes of all these species differ remarkably between each other by the ratio of m and st-a chromosomes, in combination with the chromosome numbers, and can be practically used for the unambiguous determination of the genome compositions in diploid, triploid, and tetraploid clonal hybrids. Additionally, mtDNA and nuclear markers have led to the discovery of an even higher genetic diversity in the hybrid specimens, as those with the same genomic combinations can possess mtDNA markers from different parental species due to their reciprocal origin [101]. Cytogenetics therefore continues to play an important and critical role in the discovery, screening, evaluation, and understanding of diversity within this European fish model.

Sturgeons, Shovelnoses, Paddlefishes (Chondrostei: Acipenseriformes): When Elevated Ploidy Level Coupled with Interspecific Hybridization Means No Constraints/Problem

Acipenseriformes (sturgeons, shovelnoses, and paddlefishes) represent one of the earliest radiations of ray-finned fishes, an ancient group known to be at least as old as early Jurassic (some 200–175 Myr). Due to their basal position in actinopterygian clade, all living members of the order Acipenseriformes are referred to as "living fossils", literally "fishes which forgot to extinct". This fish group together with gars and bowfin (genera *Lepisosteus*, *Atractosteus*, and *Amia*) diverged from other ray-finned fishes before whole genome duplication [112–114] in teleostean lineage. Twenty-seven extant species are recognized in two families—Acipenseridae (sturgeons) and Polyodontidae (paddlefishes). The latter includes American paddlefish *Polyodon spathula* and practically extinct Chinese paddlefish *Psephurus gladius*. Unlike other actinopterygians, acipenserids possess karyotypes composed of several macro- and many small, dot-like microchromosomes of gradually decreasing size. Additionally, they include species with three distinct ploidy levels: (i) paleotetraploid (2n = 120), (ii) paleo-octaploid with ~ 240 to 270 chromosomes, and (iii) paleo-dodecaploid with ~ 360 chromosomes. Such ploidy diversity has been robustly evidenced by direct karyotyping, flow cytometry, and cell size imaging [115]. Paleo-octaploid and paleo-dodecaploid sturgeons thus represent the few vertebrates with the highest chromosome counts. Beside ploidy diversity, the genomes of sturgeons show another peculiarity: the species with different ploidy levels easily hybridize both in nature and captivity, producing the progeny of intermediate ploidy level [116, 117•, 118]; e.g., crossing of sterlet *A. ruthenus* (2n = 120) and Russian sturgeon *A. gueldenstaedtii* (2n = ~ 240) produces intermediate hybrid (2n = ~ 180). Moreover, such hybrids are fully fertile and produce hybrids again with intermediate ploidy level depending on ploidy of respective parents, e.g., crossing of hybrid with 2n = ~ 180 and sterlet *A. ruthenus* (2n = 120) produces hybrid with 2n = ~ 150, while crossing of the same hybrid with Russian sturgeon *A. gueldenstaedtii* (2n = ~ 240) produced hybrid with 2n = ~ 210 [119]. The spontaneously arisen triploid Siberian sturgeon *A. baerii* male (3n = ~ 360) was fully fertile, and its crossing with sterlet, *A. ruthenus* female (2n = 120) or Russian sturgeon, *A. gueldenstaedtii* female (2n = 240) produced hybrids with intermediate constitutions, 240 and 300 chromosomes, respectively [116, 117•]. Using such crossing approach and combined with naturally occurring polyploid levels, the continuous ploidy series, (paleo) 4n ~ 120, (paleo) 5n ~ 150, (paleo) 6n ~ 180, (paleo) 7n ~ 210, (paleo) 8n ~ 240, (paleo) 9n ~ 270, (paleo) 10n ~ 300, (paleo) 11n ~ 240 × 420, (paleo) 12n ~ 360, (paleo) 13n ~ 390, (paleo) 14n ~ 420 could be achieved [115]. However, experimental induction of triploidy in the shortnose sturgeon *A. brevirostrum* (2n = ~ 360) yielded individuals with ~ 540 chromosomes [100], a vertebrate with the highest known chromosome number. The possible reason of the apparent and obvious differences among each particular ploidy levels—~ 30 chromosomes—might be that such "semi-haploid" units of ~ 30 chromosomes may still reflect an original haploid genome of extinct evolutionary diploid ancestor of sturgeons. Such ploidy and hybrid diversity has no parallel among vertebrates, and it strongly supports the hypothesis of Vasil'ev and Vasil'eva (1982) [108] that evolutionary polyploid sturgeons might be the result of reticulate, i.e., hybrid speciation. Both extant paddlefish species are also of paleotetraploid origin, but their tetraploidy appears to be of another origin than in sturgeons ([120].

Conventional Cytogenetics Shedding Light on Sex Chromosome Evolution in Fishes

In this last topic, we would like to discuss shortly is the impact of conventional cytogenetics on sex chromosome research. It is long-time well-known that teleost fishes possess strikingly diverse sex determination systems and, to date, they include also at least nine different sex chromosome constitutions (simple and multiple ones and their variations) described in only about 5% of cytogenetically explored taxa [100, 121]. Fish sex chromosomes display many specific features which repeatedly occur in many fish lineages such as (i) independent emergence from different autosome pairs in closely related species or even within species, (ii) patchy distribution throughout phylogeny, (iii) frequent turnovers of sex chromosome systems, which goes (at least in part) hand in hand with (iv) overall relatively young evolutionary age of fish sex chromosomes and this last aspect is indisputably mirrored in (v) often very low level of morphological divergence between both counterparts in either male-heterogametic (XY) or female-heterogametic (ZW) systems (e.g., [122–124]). Hence, conventional cytogenetics alone is often unable to uncover the entire sex chromosome diversity within a given fish group. Nonetheless, there are specific cases, when such reports immediately point to unusual patterns of sex chromosome evolution, worthy further investigation. Besides a presence of different sex chromosome systems within particular species, we would like to stress also the occasional presence of sex chromosome systems marked by the difference in 2n between sexes (such as ZZ/Z0, XX/X0-derived systems and majority of neo-sex systems including the most prevalent X_1X_2/X_1X_2Y and XX/XY_1Y_2 ones) [125]. Focusing particularly on multiple/neo-sex chromosome systems, the first fish taxon found to exhibit such type of constitution was Mexican cyprinodontid *Megupsilon aporus* [126], and since then, many other cases have been described across the teleost phylogeny (reviewed in [127]). As the number of studies providing evidence for a role of emerging (neo-) sex chromosomes and their turnover in processes such as ecological adaptation, speciation, or genomic conflict are constantly growing [128–130], the pioneer Giemsa-based reports might gain more importance by laying foundation for broader exploration of such tentative evolutionary associations in wider taxonomical scale.

Lastly, we cannot leave without a note that also C-banding procedure proved useful in sex chromosome detection in cases when their morphology is yet identical, and hence, they are hardly recognizable from each other under conventional Giemsa staining. To give one clear example, a distinct C-band on just one homolog from the pair of homomorphic sex chromosomes distinguished female-restricted W-chromosome from its Z counterpart in *Poecilia sphenops* [131]. Analogous cases involving C-banding usage might be found also in other vertebrate lineages such as *Paroedura* geckos [132].

Conclusion

In view of all the research advances available today, one may think that conventional procedures would no longer be necessary or important [133]. The purpose of this article was to demonstrate that this view is in fact not reflecting the reality, using some key examples among fishes. Actually, fish cytogenetics has proved to be an important source of information for both basic and applied science (Fig. 1). Particularly in the biodiversity field, where so many species are still awaiting for detailed investigations, conventional cytogenetics is not only informative, but also indispensable, for uncovering a set of evolutionary or biological attributes that are still waiting for their discovery: hidden inside species and populations.

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Compliance with Ethical Standards

Conflict of Interest The authors reported no potential conflicts of interest relevant to this article.

Human and Animal Rights and Informed Consent All reported studies/experiments with human or animal subjects performed by the authors have been previously published and complied with all applicable ethical standards.

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