FISH as a chromosome identification strategy to delineate karyotypic evolution in vertebrates

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ABSTRACT

Fluorescence *in situ* hybridization (FISH) is a powerful technique to directly localize cloned DNA probes onto chromosomes. This approach is essential to delimit cytogenetic analysis, especially in karyotypic evolution study. In this review, the various applications of this method were summarized: chromosomal localization of repetitive DNA sequence, construction of chromosome mapping with single copy DNA sequences, cross-species chromosome painting, comparative genomic hybridization and genomic *in situ* hybridization, allowing a rapid and comprehensive analysis of the process of chromosomal rearrangement in vertebrates. The compartmental data putting together from several applications of FISH strategies provide insight into the likely constitution of a vertebrate ancestral karyotype and lead us to understand some of the intricacies of karyotypic evolution.

Keywords: chromosome, FISH, vertebrate, karyotypic evolution

INTRODUCTION

For several decades, chromosomes are classically karyotyped, i.e., the analysis of characteristic banding patterns along the chromosomal length. This attribute has disadvantage of the limited resolution which is problematic with the analysis of rearranged chromosomes in karyotypic evolution. Gall and Pardue (1969) developed the hybridization of radioactively labeled rRNA to tissue squashes allowing the *in situ* visualization of the complementary sequences, the rDNA, within cells. Then, *in situ* hybridization, especially the non-radioactive of fluorescence *in situ* hybridization (FISH) has been further flourished to authorize the direct visualization and localization of selected DNA regions onto metaphase chromosomes and/or interphase nuclei. This approach is principally based on the specific pairing of two complementary nucleic acid sequences, the probe and the target sequences on chromosomes, and the hybridized probes are subsequently detected *via* fluorochromes using epifluorescence microscopy. Because of high topographic resolution of FISH, it is thus a prerequisite tool for genome analysis. The process of chromosomal rearrangement in vertebrates has been significantly enhanced by the development of several new FISH techniques such as chromosome painting and high-resolution gene mapping with direct R-banding FISH. Therefore, FISH strategies can be applied to genome research not only for map-rich animal species to extend abundant resource but also for map-poor species to construct chromosome maps and to investigate their course of chromosome evolution. Here, the application of FISH strategies is introduced to identify chromosome of vertebrates through comparative genomics for delineating karyotypic evolution in vertebrate species.

Identification of chromosome map position

Identification of chromosome map position is one of the most important steps to be considered with FISH analysis. There are several structural banding

techniques such as G-, Q-, C-, R- and (DA)-DAPI banding to specific determine chromosomes and their locations by chromosome mapping. However, structural banding methods are affected by many relatively uncontrollable components that result in partially banded metaphase spreads in which chromosome recognition is limited. Furthermore, they might be interfered with *in situ* hybridization procedure by either reducing the efficiency, masking the probe signal, or having low banding resolution (Lichter *et al.*, 1990). Recently, replication banding has successfully improved the methodology by removing all of the variables that affect structural banding procedures and has been assembled simultaneously with FISH method (Iannuzzi *et al.*, 1989). For instance, the direct R-banding FISH method established by Takahashi *et al.* (1990) has accomplished precise sub-chromosomal mapping of DNA clones. Fluorescence hybridization signals could be detected on the chromosome where replication patterns were obtained by Hoechst 33258 after incorporation of 5′-bromodeoxyuridine (BrdU) into cells at the late DNA synthesis stage (S) (Takahashi *et al.*, 1990). This aspect permits advantageously simultaneous visualization of FISH signals on banded chromosomes, which are imperative for definite sub-chromosomal localization of DNA clones. Moreover, fluorescent Hoechst bands, which are indistinguishable from G-bands, are observed on the same metaphase spread. They are amenable consequently to easily identify each chromosome (Fig. 1 and 2).
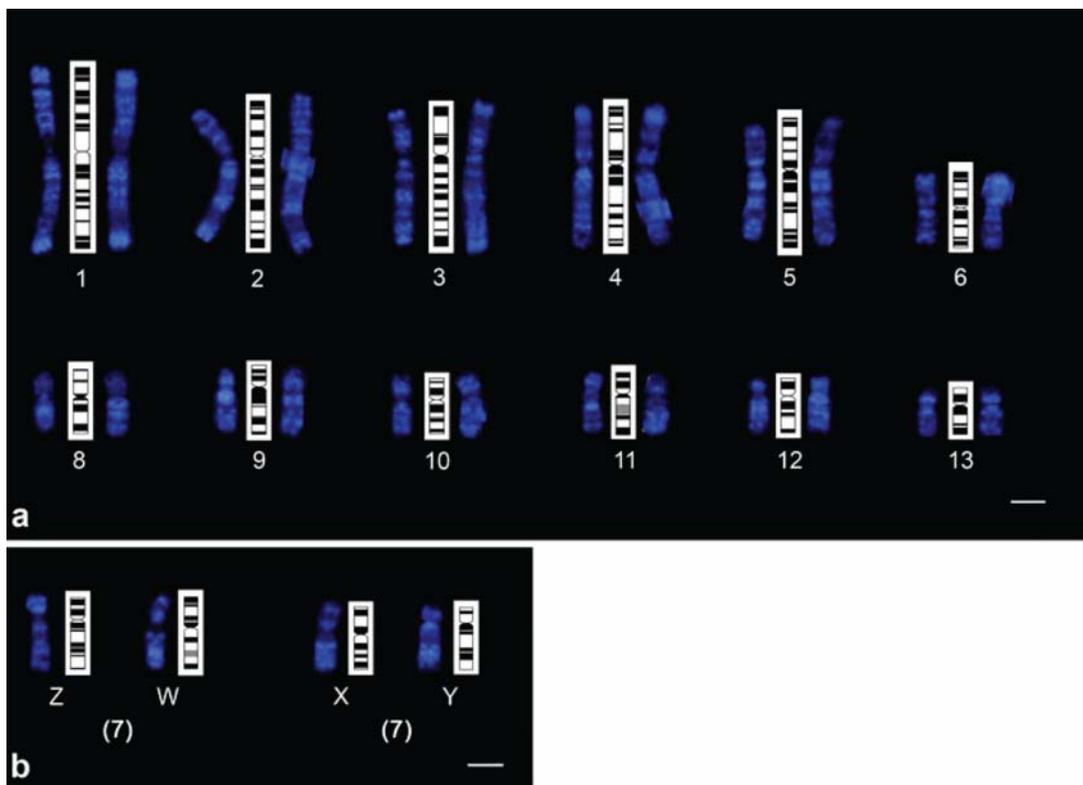
Fig. 1 Hoechst-banded karyotype of *R. rugosa* and its ideogram. (a) Hoechst-stained chromosomes of the ZW-individual and the XY-individual are demonstrated on the left and the right for each autosomal pair, respectively, and their ideograms are shown in the middle. (b) Hoechst-banded patterns of the ZW and XY sex chromosomes and their ideograms. Scale bars represent 5 mm (reprinted after modification with permission from Uno *et al.*, 2008). See color figure on the journal website.
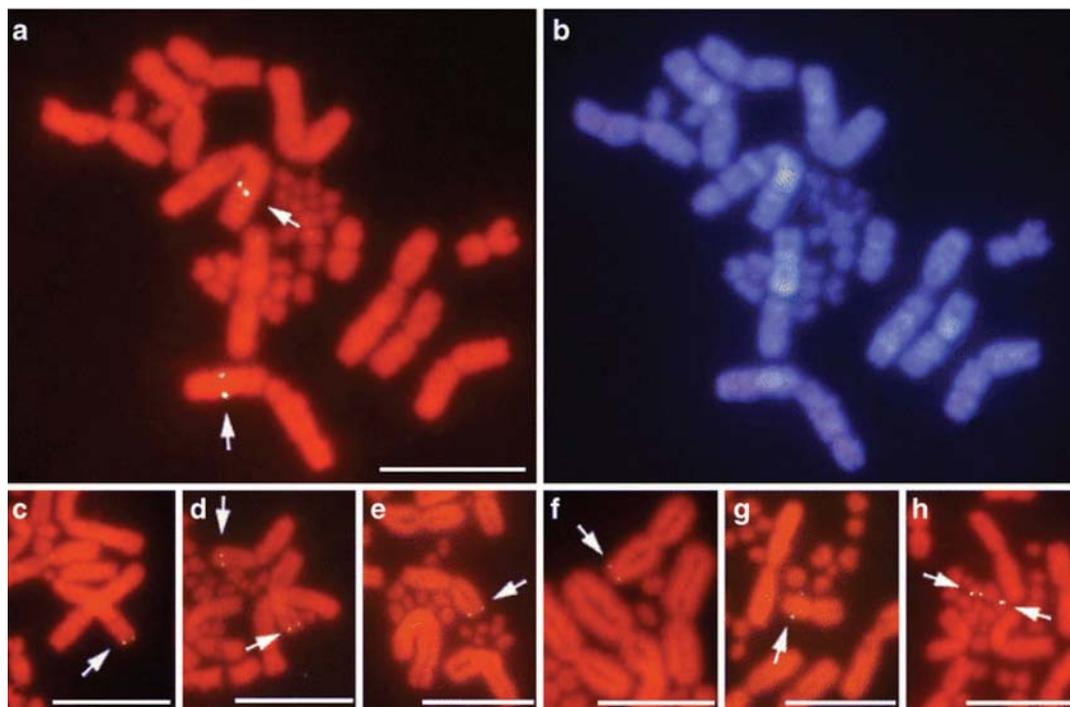
Fig. 2 Chromosomal localization of cDNA fragments of functional genes in *L. reevesii rubritaeniata*. *ESR1* (a), *BRD2* (c), *TLOC1* (d), *ENPP2* (e), *SOX5* (f), *MYST2* (g), and *TRIM37* (h) are localized to chromosomes 1, 2, 3, 4, 5, 6, and a pair of microchromosomes, respectively. (b) Fluorescent Hoechst stained pattern of the PI-stained metaphase spread shown in a, which represents the same banding pattern as G-banded chromosomes. Arrows indicate the hybridization signals. Scale bars indicate 10 μm (reprinted after modification with permission from Srikulnath *et al.*, 2009a). See color figure on the journal website.

The Japanese wrinkled frog (*Rana rugosa*) has a karyotype of 2n = 2x = 26 that comprises metacentric-submetacentric chromosome whose size variation sequentially forms a continuous distribution from the largest to the smallest chromosome. It is exceedingly difficult to clarify each chromosome by conventional chromosome staining. Notably, the seventh largest sub-metacentric is sex chromosome showing morphological variation both the ZZ/ZW- and XX/XY-individual sex chromosome among population. Hence, the replication R-banding enables the accurate autosome, and determines the localization of chromosome mapping on R-banded chromosomes in the frog (Uno *et al.*, 2008) (Fig. 1). This aspect authorizes rapid and efficient construction of high-

density and high-resolution cytogenetic maps in other vertebrates such as reptile, bird and mammalian species (Matsuda and Chapman, 1995; Kawai *et al.*, 2007; Nishida-Umehara *et al.* 2007; Srikulnath *et al.*, 2009a).

However, replication R-banded chromosomes might not be clearly seen in some animal species. Timing of cell cycle and properties of each cell would hardly anticipate in the process of replication R-banding. The size variation of heterochromatin might also affect the precision of chromosome mapping procedure. Thus, the other approach in which the chromosomal signal displayed against chromosome length on the X-axis is alternatively proposed. This procedure is expressed as the

fractional length (FL) of the total chromosome relative to a fixed reference point such as short arm terminal (pter) designated as FLpter (Lichter *et al.*, 1990). In human chromosome, there are highly variable polymorphic heterochromatin regions on chromosome 1, 9, 16 and Y. The size variation of the heterochromatin does not affect the precision of the mapping procedure. FLpter have been also applied to turkey and Pekin duck chromosomes for constructing high-resolution cytogenetic maps (Griffin *et al.*, 2008; Skinner *et al.*, 2009). FLpter could, therefore, be used to assist the localization of chromosome map position. It will be the best, if much less variation of the FLpter values is observed when more extended chromosomes are examined.

Chromosomal localization of repetitive DNA sequence through FISH

　　　　Repetitive DNA sequences widely distributed in eukaryotic genomes as one of the major components of chromosomes are a large portion of the DNA content of the cells, and the variation in the genome size of different eukaryotes is often attributed to differentiate these sequences (Brenner *et al.*, 1993). They are also important for structural and functional organization of the genome (Schueler *et al.*, 2001). Physical chromosome mapping of repetitive DNA sequence can provide a better landscape of the genome for insufficient detail chromosome map in animal species (map-poor animal species). This idiosyncrasy is a good chromosome marker for investigating the process of karyotypic evolution and sex chromosome identification, and for comparing the genomic structure of vertebrate species. Repetitive DNA sequences are principally classified into two groups according to genome organization and chromosomal distribution (Singer, 1982). One is the interspersed type of repeated sequences distributed throughout the genome, and the other is the site-specific type of repeated sequences. Site-specific repetitive sequences are mostly satellite DNA (satDNA) comprising highly repetitive sequences. They are clustered in the interstitial chromosomal

and heterochromatic regions of chromosomes such as centromeres, pericentromeric region, and telomeres. SatDNA from several vertebrate species such as reptile, bird and rodent have been extensively characterized by nucleotide sequence analysis, FISH mapping and filter hybridization (Yamada *et al.*, 2002; Kawagoshi *et al.*, 2008; Matsubara *et al.*, 2008). Notably, the karyotypes of turtle and bird are usually composed of macro-and microchromosome. Microchromosome-specific centromeric repetitive DNA sequences have been cloned from the lesser rhea (*Pterocnemia pennata*), greater rhea (*Rhea Americana*) and the Chinese soft-shelled turtle (*Pelodiscus sinensis*) (Yamada *et al.*, 2002; 2005), suggesting that homogenization of the centromeric repetitive sequences has not occurred between macro- and microchromosomes owing to their structural differences (Kawagoshi *et al.*, 2008). Interestingly, the other kind of repetitive DNA sequence is the telomeric (TTAGGG)*n* sequences which are widely conserved among vertebrates (Meyne *et al.*, 1990). This repeated sequence has been detected not only in telomeric sites but also in interstitial and centromeric chromosomal region in a variety of vertebrate species (Nanda and Schmid, 1994; Srikulnath *et al.*, 2009b) (Fig. 3). Although the origin of non-telomeric sites named interstitial sites (ITSs) has not been investigated in detail, it might be relics of chromosomal rearrangement as fusion or inversion occurring in the course of genome evolution (Nanda and Schmid, 1994).

　　　　SatDNA is not only found in the heterochromatic regions of chromosomes but also distributed abundantly on the sex-specific (Y or W) chromosomes. In avian species, isolation of W chromosome-specific repetitive sequences and their molecular and cytogenetic characterization provides important information on the process of sex chromosome differentiation (Itoh and Mizuno, 2002). The novel family of repetitive sequences from Galliformes species is an interspersed-type repetitive sequence amplified site-specifically on the W chromosome (Yamada *et al.*, 2006). This family of
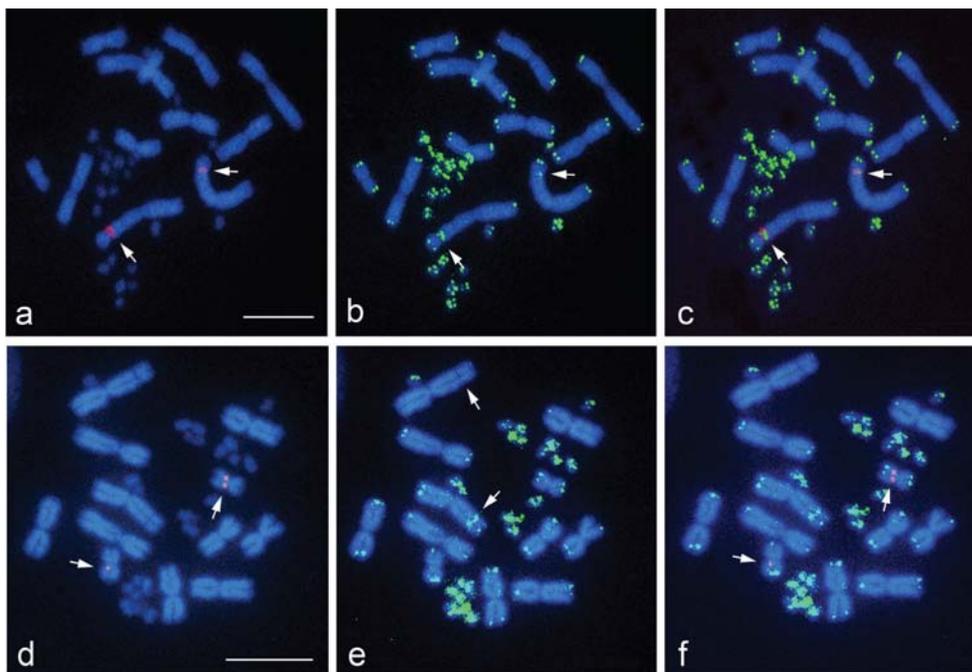
Fig. 3 Chromosomal localization of the 18S-28S and 5S rRNA genes and (TTAGGG)*n* sequences in female *L. reevesii rubritaeniata*. a − c Hybridization patterns of the 18S-28S rRNA genes (red) (a) and (TTAGGG)*n* sequences (green) (b) on DAPI-stained chromosomes, and their co-hybridization pattern (c). d − f Hybridization patterns of the 5S rRNA genes (red) (d) and (TTAGGG)*n* sequences (green) (e), and their co-hybridization pattern (f). Arrows indicate FISH signals of the 18S-28S rRNA genes (a, c), the 5S rRNA genes (d, f), and interstitial telomeric sites (ITSs) (b, c, e, f). Scale bars represent 10 µm (reprinted after modification with permission from Srikulnath *et al.*, 2009b). See color figure on the journal website.

repetitive sequences is highly conserved in neognathous birds but not in palaeognathous birds. The W-specific repetitive sequences are highly diverged between different species as rapidly evolved molecules; therefore, they are good molecular cytogenetic markers for estimating phylogenetic relationships in birds (Yamada *et al.*, 2006).

In addition to satDNA, there are gene families of repetitive elements that encode gene products such as histone and rRNA genes. In higher eukaryotes, rRNA genes are classified into two distinct gene families. The major family encoding 18S, 5.8S and 28S rRNA genes generally locate at the nucleolar organizing region (NOR), whereas the minor family encodes 5S rRNA gene. Both gene families are tandemly arrayed repeats and are considered to have evolved in a concerted manner (Liao, 1999). They are essentially species-specific providing a useful karyotypic marker as Ag-NOR banding and FISH mapping in several vertebrate species. FISH enables us to visualize all 18S-28S rRNA and 5S rRNA genes, whereas silver staining detects only the transcriptionally active nucleolar organizer regions (Silva *et al.*, 2008). For example, the 18S-28S rRNA genes of iguanian lizard locate on a pair of microchromosomes or chromosome 2 (Porter *et al.*, 1991), whereas those genes of butterfly lizard (*Leiolepis reevesii rubritaeniata*) are localized onto chromosome 1q (Srikulnath *et al.*, 2009b). With this information, a specific linkage group of both

lizard karyotypes can be further allocated.

Construction of chromosome mapping with single copy DNA sequence

Chromosome mapping with single copy DNA sequence is the most effectual application of FISH which makes it possible to determine homologous chromosomal regions between species belonging to different orders or classes, and provides valuable information about the chromosomal rearrangement, genome architecture and genome evolution. Using single copy DNA sequence as a probe, it can be categorized into two most common types used for mapping studies: (1) the unique sequence DNA probe and (2) the single copy genomic DNA probe (Beatty *et al.*, 2002). The unique sequence DNA probe is conventionally referred to the functional genes or cDNA clones. Physical and genetic linkage maps of chicken and human revealed that the highly conserved linkage homology is retained between them (Groenen *et al.*, 2000; Schmid *et al.*, 2005), although the two lineages (diapsids and synapsids) were diverged from the common ancestor of amniotes around 310 MYA (Kumar and Hedges, 1998). Comparative chromosome maps of functional genes between both species, therefore, gave us new insight into the evolution of vertebrate genomes (Burt *et al.*, 1999; Nanda *et al.*, 1999; Burt, 2002). However, the chromosome mappings of several taxa have not been clarified since the functional genes have not yet been developed as DNA probes, e.g., map-poor species of reptiles.

Orthologs are homologous genes between different species which have been evolved from common ancestral gene and maintained the same function throughout the course of evolution. Partial sequencing of a large number of cDNAs to establish expressed sequence tags (ESTs) library reinforces gene discovery using the EST database (dbEST). These ESTs provide a ready source of single copy DNA probe to construct comparative chromosome maps between species. Interestingly, completion of the chicken whole genome sequencing established in 2004 provided a new breakthrough and perspectives in comparative genomics between Reptilia and Aves (International Chicken Genome Sequencing Consortium, 2004), and enabled us to compare genome structures between the two taxa at the molecular level by constructing chromosome maps of functional genes for reptilian species. A large-scaled chromosome mapping of reptiles has been firstly performed on the Chinese soft-shelled turtle (*P. sinensis*) and the Japanese four-striped rat snake (*Elaphe quadrivirgata*). Meanwhile, the conserved chromosome synteny among Aves, Testudines and Squamata have been established (Matsuda *et al.*, 2005; Matsubara *et al.*, 2006). However, the construction of EST library might not be empirical to build up chromosome maps because it requires labor, time and cost; therefore, direct molecular cDNA cloning by RT-PCR method is a beneficially alternative approach. The functional genes are systematically selected from map-rich animal species, and primer designs are contrived based on the nucleotide sequence data available in Genbank (www.ncbi.nlm.nih.gov).

The variety of sex chromosomal origins in the lineages of Synapsida has been revealed by comparative mapping of the reptilian homologues of the chicken Z-linked genes, suggesting that the chicken Z-linked genes are associated to the Z and W chromosomes of Hokou gecko (*Gekko hokouensis*) (Kawai *et al.*, 2009), whereas this genetic linkage has been found in other reptilian autosomes (Kawai *et al.*, 2007). Therefore, the sex chromosomes of Reptilia, Aves and Mammalia are independently differentiated in each lineage from autosomal pairs of the common ancestor of amniotes (Kawai *et al.*, 2007; 2009). The highly conserved linkage homology of chicken Z-linked genes, however, was identified on chromosome 2p of the butterfly lizard (*L. reevesii rubritaeniata*) and that of the Japanese four-striped rat snake (*E. quadrivirgata*) (Srikulnath *et al.*, 2009a; 2009b). This occurrence was conducted in prerequisite to prove the highly conserved synteny between two species by constructing comparative chromosome

map, which supply information of multiple chromosomal rearrangement in the lineage of snakes and lizards (Srikulnath *et al.*, 2009a; 2009b) (Fig. 2 and 4).

Although, comparative mapping of functional genes is a perfectly ideal approach to identify homologous chromosomal regions between different species, this technique might not be accomplished to provide chromosome evolution details in map-poor animal vertebrates because it imposes excessively skillful techniques to map small cDNA clones onto chromosome. Moreover, the visualization of functional gene probes is also dependent on its intron/exon structure. Another approach is a screening of genomic BAC or PAC library by locating the functional genes at a corresponding genomic clone which can be subsequently mapped by FISH method. A comparative chromosome map between the Australian dragon lizard (*Pogona vitticeps*) and snake and chicken sex chromosome has been determined by screening a *P. vitticeps* genomic BAC library, and physically mapped onto chromosomes by FISH (Ezaz *et al.*, 2009). *DMRT1* gene was identified as a single gene on chicken and *G. hokouensis* Z chromosome, as well as other reptilian autosomes; but, it was found on chromosome 16 and 18 of Tuatara (*Sphenodon punctatus*) (O'Meally *et al.*, 2010). These results collectively suggest that the
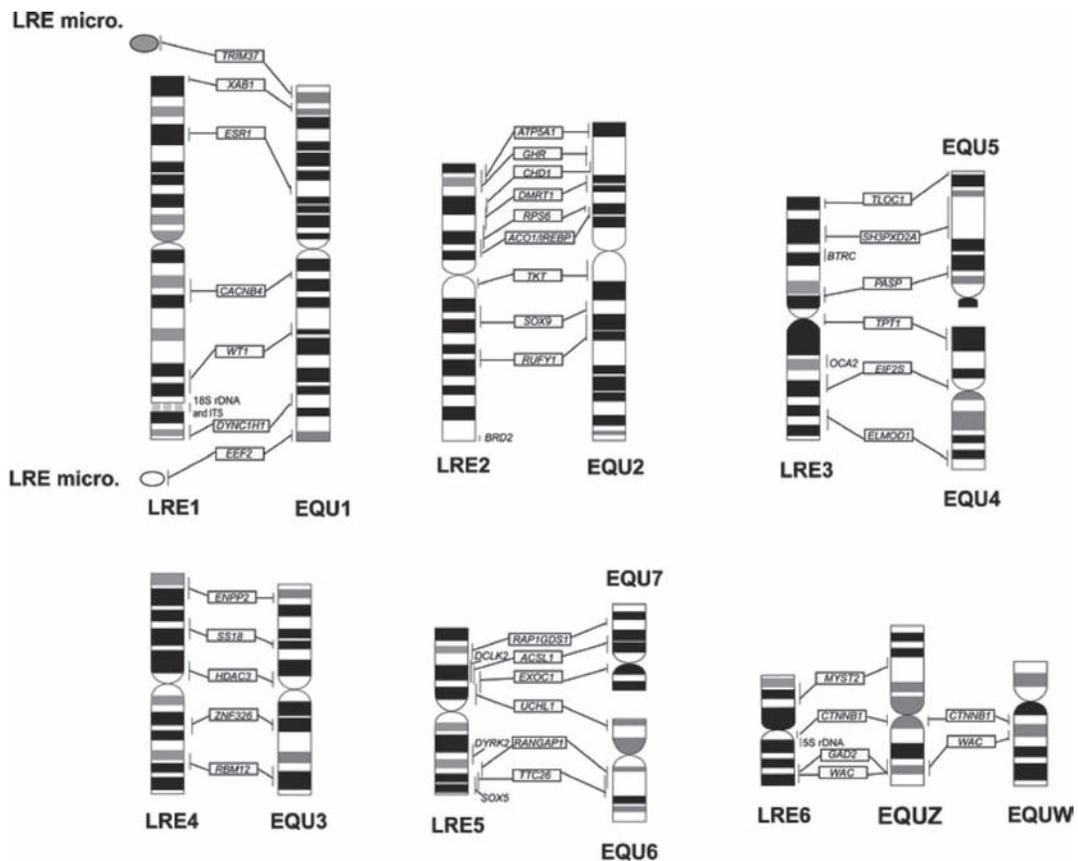
Fig. 4 Comparative cytogenetic maps between *L. reevesii rubritaeniata* macrochromosomes and *E. quadrivirgata* macrochromosomes which were constructed with 43 functional genes. EQU5, EQU7, and EQUZ are inverted to facilitate comparison of the gene order with LRE3p, LRE5p, and LRE6, respectively (reprinted after modification with permission from Srikulnath *et al.*, 2009a).

localization of FISH signal on different chromosome sites probably occurs because of a gene family or a pseudogene.

Several BAC clones were selected from the Wageningen chicken BAC library (Crooijmans *et al.*, 2000) based on the position of markers on the chicken consensus linkage map (Groenen *et al.*, 2000) to construct comparative chromosome mapping between chicken and turkey, and chicken and Pekin duck. These maps provide indications of chromosomal rearrangement among these species macrochromosomes and conserved synteny among all microchromosome analysis (Griffin *et al.*, 2008; Skinner *et al.*, 2009). In Bovidae, the cytogenetic map of river buffalo (*Bubalus bubalis*), an economically important species for clinical, breeding and evolution purposes, has also been constructed. Caprine and bovine BAC clones were selected to perform FISH mapping, and compare their integrated cytogenetic map with the corresponding cattle and human homologues to find the conserved chromosome segments and complex rearrangements differentiating river buffalo (and cattle) and human chromosomes (Di Meo *et al.*, 2008).

Identification of chromosome homology by comparative chromosome painting

Advancement in chromosome flow-sorting and microdissection become a breakthrough for analyzing chromosome evolution in vertebrates. They allow chromosomal species-specific DNA probes prepared from flow-sorted and microdissected chromosomes to hybridize *in situ* onto specific chromosomes of distantly related species, detecting homologous genomic regions between them (Ferguson-Smith *et al.*, 2005). Thus, comparative chromosome painting, named ZOO-FISH, has possibly not only enhanced the robust method of choice for genome comparisons at the cytogenetic level but this approach also provides indications for delineating the process of the chromosomal rearrangements that have occurred during the evolution of species (Wienberg, 2004). The cross species chromosome

painting has been predominantly performed in mammals (Chowdhary and Raudsepp, 2001). Especially in rodent species, chromosome-specific painting probes from the laboratory mouse (*Mus musculus*) have been developed by flow-sorting of chromosomes (Rabbitts *et al.*, 1995). The comparative studies of karyotypes using mouse chromosome paints were conducted for several species of the Rodentia such as *Apodemus sylvaticus* (Matsubara *et al.*, 2004) and four *Akodon* species (Ventura *et al.*, 2009). In Indian spiny mouse (*Mus platythrix*), twenty-seven segments homologous to mouse chromosomes were identified, and subsequently speculated that tandem fusions are major events in the process of chromosomal rearrangement (Matsubara *et al.*, 2003) (Fig. 5). The ZOO-FISH studies have been extensively applied to some avian species with chicken probes of chromosome 1-9 and Z (Griffin *et al.*, 1999; Nishida-Umehara *et al.*, 2007). Several novel chromosomal rearrangements are identified to deduce the process of chromosome evolution in Galliformes, and the ancestral karyotype of the Galliformes is acquired (Shibusawa *et al.*, 2004).

Comparative genomic hybridization as a tool to determine sex specific region

Comparative genomic hybridization (CGH) method effectively allows a genome-wide screening of changes in all type of DNA sequence (gains and losses) by a single hybridization of whole genomic DNA, and makes mapping of these changes to normal chromosomes. CGH has been initially developed to recognize molecular differences between genome of normal and tumor cells at the cytogenetic level (Kallioniemi *et al.*, 1992). Whole genomes of tumor cells and normal cells are jointly used as probes to hybridize on normal metaphase chromosome. By comparison of signal intensities along hybridized chromosomes, the relative copy number changes of chromosomal regions within the tumor genome could be identified. Even though CGH could not be used to detect the balanced chromosomal rearrangement (inversions or reciprocal translocations), this technique
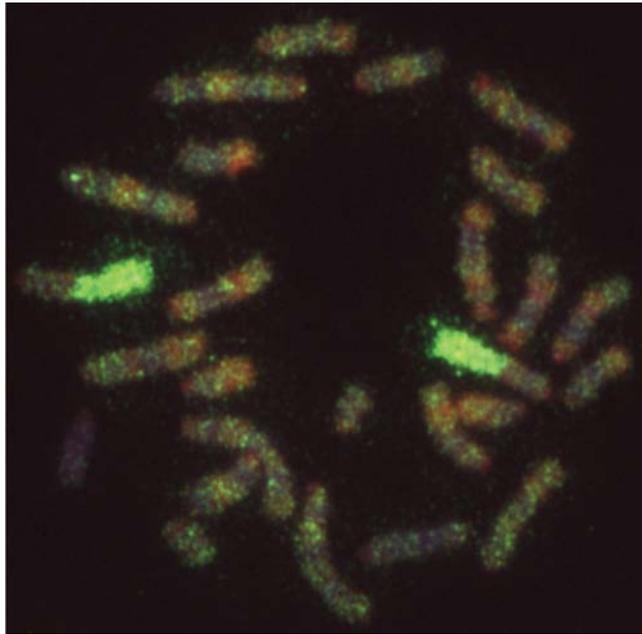
Fig. 5   Cross-species chromosome hybridization with mouse (*Mus musculus*, MMU) chromosome-specific paints. Hybridization of MMU3 probes to *Mus platythrix* chromosomes (courtesy of Chizuko Nishida, Hokkaido University, Japan). See color figure on the journal website.

has been successfully adapted to identify molecularly differentiated sex-specific regions in animal species (Traut *et al.*, 2001).

In vertebrates, there are apparently two distinct strategies to trigger gonad differentiation: genetic sex determination (GSD) and environmental sex determination (ESD; known as commonly temperature dependent sex determination, TSD). GSD mechanisms have originally occurred through allelic variation at a single locus. The sex-determining gene is subsequently borne on a pair of homomorphic sex chromosomes, and extensively complete differentiated sex chromosomes that are obviously seen under the microscope. Hence, sex chromosome identification appears to be genetic marker for understanding karyotypic evolution in vertebrates which is a topic of speculation among cytogeneticists and evolutionary biologists. Chromosome banding like G-, C- and NOR-banding helps to detect sex chromosomes that are not able to clarify by gross chromosome structure. However, homomorphic sex

chromosomes have generally posed a problem when the morphological feature of chromosomes and chromosome bandings are not detected. CGH has been capably performed to label sex chromosome in an XX/XY turtle, *Chelodina longicollis* (Ezaz *et al.*, 2006), an XX/XY guppy fish, *Poecilia reticulate* (Traut and Winking, 2001), and a ZZ/ZW lizard, *P. vitticeps* (Ezaz *et al.*, 2005). Total DNA from females labeled with one fluor as f-probes and whole DNA from males labeled with another fluor as m-probe were simultaneously hybridized and competed for binding sites on the chromosome. Autosomes gave a balanced signal, whereas Y and Z chromosomes were preferentially labeled by m-probe, and X and W chromosomes by f-probes. A ZZ/ZW Chinese soft shell turtle (*P. sinensis*) was identified by chromosome banding and CGH analysis (Kawai *et al.*, 2007). The 18S-28S rRNA genes were co-localized with the female-specific C-positive heterochromatin that located partially on W chromosome. These findings can discriminate the heterochromatin of the Z chromosome

and autosomes by CGH (Fig. 6).

However, sex chromosomes of some species could not be identified using CGH. The platyfish (*Xiphophorus maculatus*) that has an XX/XY sex determination system, has a molecular marker, *XIR*, locates on the distal end of Y chromosome (Nanda *et al.*, 2000). The platyfish Y chromosome that is morphologically indistinguishable from the X chromosome, however, failed to identify sex specific regions, suggesting that its sex chromosomes is less differentiated (Traut and Winking, 2001). Likewise, the minute sex specific regions of two Ryukyu spiny rats (*Tokudaia osimensis* and *T. tokunoshimensis*) which have X0 sex chromosome constitution could

not be identified by CGH (Kobayashi *et al.*, 2007), although the *Tspy* and *ZFY* Y-linked genes in human and mouse located on distal part of the long arm of the X chromosome in both species (Arakawa *et al.*, 2002). Increaseingly, the fluorescence intensity for each chromosome of two Ryukyu spiny rats was measured, and compared them between male and female chromosomes. The findings indicated that the male- and female-derived gains and losses are detected in the heterochromatic and/or telomeric regions of several chromosomes, suggesting that the variation of chromosomal distribution of gains and losses might be caused by polymorphism of the copy numbers of repeated DNA sequences. Interestingly,
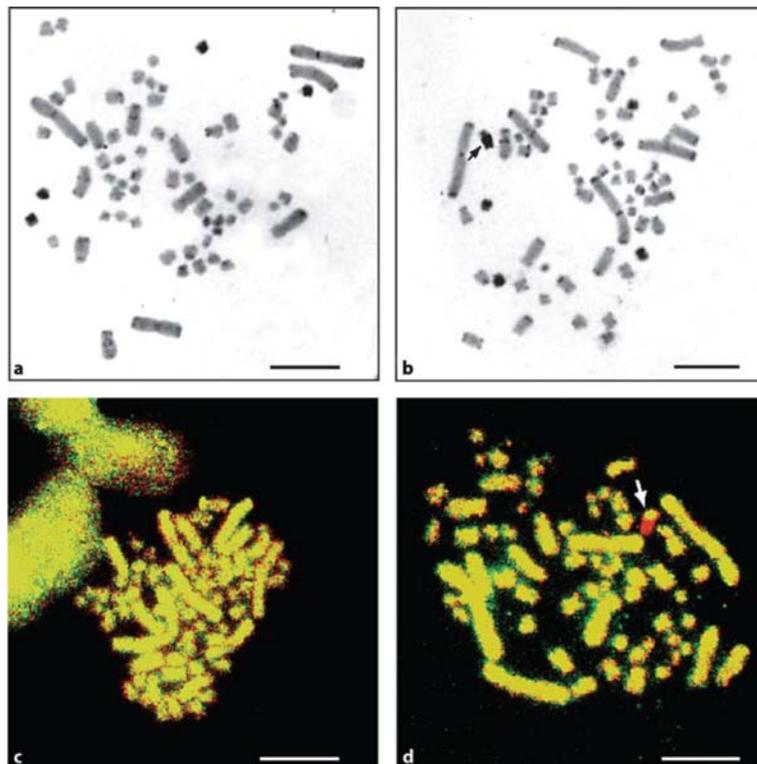
Fig. 6 C-banded metaphase chromosome spreads of male (a) and female (b) *P. sinensis* and the patterns of comparative genomic hybridization with FITC-labeled male genomic DNA and Texas Red-labeled female genomic DNA to chromosomes of male (c) and female (d) *P. sinensis*. (b) The arrow indicates the female-specific chromosome with a large and intense C-positive band. (d) The arrow indicates the chromosome with the female-specific region painted with Texas Red-labeled female-driven DNA. Scale bars indicate 10 μm (reprinted after modification with permission from Kawai *et al.*, 2007). See color figure on the journal website.

the lower limit of detectability of CGH techniques is 2 Mb for amplifications and 10 Mb for deletions (Kallioniemi *et al.*, 1992; Piper *et al.*, 1995). Therefore, the sex-specific region, where the key of sex determination lies, must be larger than 10 Mb to successfully examine by CGH method.

Identification of genome homology by genomic *in situ* hybridization

The analysis of genome in its species has been performed using CGH to detect changes in any DNA sequence involved sex-specific region. The genomic relationships between different species, however, can be done by alternative procedure. Schwarzacher *et al.* (1989) successfully modified FISH protocol called genomic *in situ* hybridization (GISH) to study the organization of parental genomes in an intergeneric *Hordeum* X *Secale* hybrid. Whole genomic DNA of a parental species was hybridized by simultaneous or subsequent reaction to chromosomes of a hybrid, where it enabled discrimination of parental genomes. GISH has become an energetic tool for analyzing interspecific and intergeneric hybrids and allopolyploid species as well as intergenomic exchanges on chromosome. The genomes of North American unisexual salamanders in the genus *Ambystoma* were identified using GISH analysis. *A. laterale*-2 *jeffersonianum* (LJJ) triploid and its tetraploid derivative, *A. laterale*-3 *jeffersonianum* (LJJJ), could be identified giving ten different patterns of intergenomic exchanges between L and J genomes from 18 isolated populations (Bi *et al.*, 2008).

A relatively new strategy of GISH is a tool in phylogenetic studies. Comparing the genome of Nile tilapia, *Oreochromis niloticus*, with those of other cichlids from Africa and South America, total genomic DNA of *O. niloticus* was hybridized to other cichlids chromosome with blocking DNA (Valente *et al.*, 2009). The signal intensity and statistical analysis were subsequently estimated with genetic distance among species to plot graph. The pericentromeric heterochromatin of Nile tilapia was

species-specific and that the sequence of majority of the long arm of the largest chromosome pair conserved only within tilapiine group.

Even though the GISH methodology could not provide as precise data as those obtained through hybridization of individual chromosomes or gene marker, it allows the gross comparison of related species without the need to isolate specific chromosomal segment. However, comparative gene mapping and chromosome painting are still necessary to confirm a striking feature of karyotypic evolution in vertebrate species.

Figuration of the process of karyotypic evolution in vertebrates

Molecular cytogenetic studies on chromosome homology between different species have been comprehensively conducted in several vertebrates. The delineation of the process of chromosomal rearrangement can be realized by joining the pieces of data concomitantly like jigsaw puzzle through various FISH approaches. In three *Tokudaia* species, the diploid chromosome number of *T. muenninki* is 2n = 44 with the XX/XY type of sex chromosomes, whereas the diploid numbers of *T. osimensis* and *T. tokunoshimensis* are 2n = 25 and 2n = 45, respectively (Tsuchiya *et al.*, 1989). The divergent time between two species might roughly have 2 MYA (Suzuki *et al.*, 1999). Thus, the remarkable difference of the chromosome number between *T. osimensis* and *T. tokunoshimensis* indicated that frequent chromosomal rearrangements had occurred between the two species in less than 2 million years since they diverged from the common ancestor. Comparative chromosome painting between *T. tokunoshimensis* and *T. osimensis* with chromosome-specific DNA probes of the laboratory mouse (*Mus musculus*) was extensively conducted to examine the chromosome homology and deduced a possible ancestral karyotype of *Tokudaia* species (Nakamura *et al.*, 2007). These results collectively suggested that the proposed ancestral karyotype with the diploid number of 2n = 48, XX/XY was similar to the

karyotype of *T. tokunoshimensis*, and the karyotype of *T. osimensis* would have been established through at least 14 chromosomal changes, mainly centric fusion and tandem fusion, from the ancestral karyotype (Nakamura *et al.*, 2007). Accordingly, both of them have a unique X0/X0 sex determining system without a Y chromosome or a *Sry* gene (Sutou *et al.*, 2001), whereas the sex-specific chromosomal region could not be identified by CGH analysis, suggesting that it is very minute in X0 species of *Tokudaia* (Kobayashi *et al.*, 2007). Comparison of the karyotypes of the two species indicated a difference in the morphology of the X chromosomes; the single X chromosomes of *T. osimensis* and *T. tokunoshimensis* are submetacentric and subtelocentric, respectively. However, the homology of the *T. osimensis* and *T. tokunoshimensis* X chromosomes with the mouse X chromosome has been revealed by comparative chromosome painting with a mouse X probe (Arakawa *et al.*, 2002; Kobayashi *et al.*, 2007). Analysis of the G-banding patterns and locations of centromeres on X chromosomes between the two species implied that the pericentric inversion event might have occurred in the X chromosome of either species. By contrast, comparative functional gene mapping with cDNA clones of the X-linked genes on the chromosomes of the two species showed that the gene orders of the X chromosomes are conserved in the two species, while the position of the centromere on the X chromosome is different (Kobayashi *et al.*, 2008). Therefore, the rearrangement occurred in either of the X chromosomes after the two species diverged from a common ancestor should explicitly be centromere repositioning rather than pericentric inversion (Kobayashi *et al.*, 2008).

CONCLUSION

Summarizing several FISH strategies to delineate karyotypic evolution in vertebrates, there is no one-step technical approach that permits a direct identification of all chromosomes. Repetitive DNA sequences used as FISH probes could identify some chromosomes in the genome. These results are narrow information on the chromosomal localization of the genome, and are still far from the goal of identifying individual chromosomes. Nevertheless, the obtained data enable us to illustrate a differential distribution of different repeats, and might finally indicate specific chromosomes. Moreover, the telomeric (TTAGGG)*n* sequences have been revealed not only in the telomeric sites but also in the interstitial and centromeric chromosomal regions that might deduced the process of chromosomal rearrangement as fusion or inversion. On the other hand, cross-species chromosome hybridization with chromosome-specific probes (ZOO-FISH) is a robust method to provide conserved syntenic chromosomal regions between distantly related species. This approach is amenable to depict the process of karyotypic evolution and the ancestral karyotype. Though ZOO-FISH is available for chromosomal comparisons between different species within the same order or class, it fails to carry out across species of different classes. The intrachromosomal rearrangements in homologous segments identified between different species are also incapable to detect them using comparative chromosome painting. By contrast, comparative functional gene mapping is an efficacious tool to compare the chromosomal architecture and genome composition between distantly related species. The nucleotide sequence and location of orthologous genes can be searched in the highly advanced gene map of map-rich animal species like human and chicken. Hence, it absolutely works on rapid expansion from comparative chromosome painting data by physically mapping gene with known position on the genetic map to assign linkage groups into particular chromosomes in map-poor animal species. Then, the evidences are discussed to find the course of chromosome evolution between different species. Similarly, construction of chromosome maps with the defined functional genes can be accomplished through the direct comparison of chromosome homology between different species. Furthermore, CGH and GISH performed by genome

comparison can determine sex-specific region and genome homology in vertebrates. Therefore, all approaches are useful to identify chromosome in map-poor animal species to clarify the phylogenetic hierarchy of genome evolution in vertebrates.

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