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THE APPLICATION OF ZOO-FISH TECHNIQUE FOR ANALYSIS OF CHROMOSOMAL REARRANGEMENTS IN THE *EQUIDAE* FAMILY*

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Abstract

Genome analysis is necessary to trace evolutionary rearrangements and relationships between species. Initially, to this end, the tools of classical cytogenetics were used but along with the development of molecular cytogenetics methods it became possible to analyse the genome more thoroughly. One of the widely used methods is fluorescence *in situ* hybridization (FISH) and its different types. Zoo-FISH, or cross-species chromosome painting, which uses painting probes specific for whole chromosomes, enables detecting homologous synteny blocks, the occurrence of which is evidence that species share a common ancestry and are related. Zoo-FISH technique is complemented by FISH with probes specific to chromosome arms or repetitive sequences (telomeres, centromeres), which provide additional information about karyotype organization, as well as karyotype polymorphism and conservation. Another method used is FISH with gene-specific probes, which enable the localization of single loci, thus making it possible to determine linkages between genes and verify data obtained after using painting probes in Zoo-FISH technique. Because of its diverse karyotype and rapid karyotypic evolution, the *Equidae* family is an ideal object of study using a number of methods based on *in situ* hybridization, which, in turn, enables information to be obtained at many levels of DNA organization.

Key words: *Equidae*, FISH, Zoo-FISH

Genome analysis occupies a special place in evolutionary biology and research on phylogenetic relations between organisms because small changes in a nucleotide sequence (for example, point mutations) as well as changes affecting larger areas of chromosomes, including changes in morphology and number, may underlie evolutionary processes (Faraut, 2008). Initially, the studies focused on the analysis of basic characteristics of karyotype: the number of chromosomes, their size, morphology and typical structural elements which can be detected using classical cytogenetic methods (Mäkinen and Gustavsson, 1982). Along with the development of cytogenetics and structural genomics, more detailed comparative studies of genomes became possible: the localization of specific nucleotide sequences on chromosomes,

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the creation of interspecies comparative maps and more detailed analysis of similarities and differences in the organization of eukaryotic genomes (Dobigny et al., 2004; Murphy et al., 2005; Davis et al., 2009). Homologous regions were discovered in different species, which suggests evolutionary conservatism of these parts of genetic material (Richard et al., 2003; Murphy et al., 2005). The conservatism can be present on different levels of organization: from a nucleotide sequence of smaller or larger DNA fragments (for example, a specified gene) to the order of a group of specific coding sequences or/and noncoding sequences on a chromosome (Santani et al., 2002; Faraut, 2008). Homologous genomic segments are evidence that the groups of organisms originate from the common ancestor. Their number, size and location can show possible ways of karyotype evolution. The reconstruction of chromosomal rearrangements, which took place during speciation, can lead to the reconstruction of the karyotype of the last common ancestor, as well as allow for evaluation of the degree of species kinship (Ferguson-Smith and Trifonov, 2007). Zoo-FISH, or comparative/cross-species chromosome painting, has been playing for years a key role in the analysis of large chromosomal region homology (Hameister et al., 1997; Nash et al., 2001; Ropiquet et al., 2010). The technique is based on hybridization of molecular probes, which are peculiar to a specific chromosome (or its part) of one species to the genetic material of the second species (Chowdhary and Raudsepp, 2001). The number, size and structure of chromosomes forming a genome of eukaryotic organisms is varied. The variety is also observed among mammals, whose karyotype can comprise from 6 (*Muntjakus muntiac*) to 102 (*Tympanoctomys barrerae*) chromosomes (Kemkemer et al., 2009). A different structure of a set of chromosomes can be found even among specimens of the same species (Graphodatsky et al., 2000; Richard et al., 2003, Ropiquet et al., 2010). Despite significant differences, which sometimes occur in the organization of a karyotype, organisms from distinct systematic groups can contain conservative genomic regions, so called homologous synteny blocks (Murphy et al., 2005). Synteny describes co-localization of genes on one chromosome (Passarge et al., 1999). Homologous synteny blocks are chromosomal segments present in genomes of two or more species, in which corresponding (homologous) DNA sequences are located (Hardison, 2003; Ng et al., 2009). Their occurrence is evidence of the common origin (Ferguson-Smith and Trifonov, 2007). The order of sequences in a synteny block can be totally or partially preserved. However, minor rearrangements leading to a new arrangement of genes in a synteny block are not a scarce phenomenon (Pevzner and Tesler, 2003; Ng et al., 2009). Conservative genomic segments can embrace smaller or larger areas of chromosomes, likewise arms or the whole structure of a chromosome (Chowdhary and Raudsepp, 2001; Murphy et al., 2004). One of the main tools used for their detection and identification is Zoo-FISH technique. Zoo-FISH is one of the principal methods which makes it possible to evaluate similarities and differences in the organization of a genome (Figure 1). Its birth is dated back to the early 1990s, when the results of the first cross-species karyotype analysis, using fluorescent *in situ* hybridization, appeared (Jauch et al., 1992; Scherthan et al., 1994).

The application of molecular probes enabled a better understanding of the genome architecture of different species to a degree unattainable for traditional cytogenetic

techniques. Zoo-FISH has become a leading tool in, for example, the evaluation of the level of chromosome evolutionary conservatism. The analysis using painting probes allows comparing karyotype organization of organisms from different phylogenetic groups, not only belonging to the same family but also those less related. However, apart from a few exceptions, it is not possible to carry out comparative research on species, whose evolutionary lines became separated more than 105 million years ago (Glas et al., 1999; Ferguson-Smith and Trifonov, 2007). The technique of comparative chromosome painting has also other limitations. There is no possibility of detecting intrachromosomal rearrangements, such as inversions or interstitial insertions, as well as determining the orientation of homologous DNA segments. The resolution of fluorescent *in situ* hybridization, defined as the smallest chromosomal fragment that can be detected with this method, is the main limitation of Zoo-FISH. It depends on the type of the molecular probe used. Painting probes are used in the chromosome painting technique, which means that the whole structure, arms or other large regions of a chromosome are covered with the probe. It is estimated that they provide information about the presence or lack of homologous synteny blocks, which embrace areas no smaller than 7–10 millions of base pairs (Scherthan et al., 1994; Chowdhary and Raudsepp, 2001). The appearance of a fluorescent signal, which is present when a probe hybridizes with the analysed chromosomal preparation, is evidence of a homology. However, this does not mean that there is complete compatibility between a molecular probe and the analysed material. Differences concerning the area of DNA smaller than the resolution of the method used are not detectable. It has been repeatedly stated that homology between compared genomes, demonstrated by the chromosome painting technique, does not have to be reflected in the same arrangement of specified genes (Sun et al., 1999; Szczerbal et al., 2007). Cross-species chromosome painting allows for a global comparative analysis of genomes, that is carries information about the occurrence or lack of homologous synteny blocks. However, it is not possible to say if the order of specified sequences of DNA inside these segments is fully preserved.

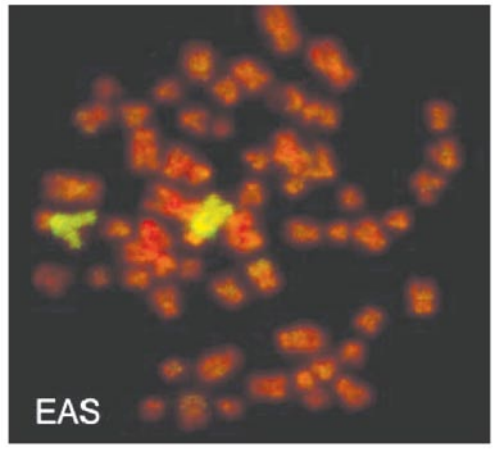

Figure 1. Interspecies hybridization (Zoo-FISH) with a probe specific for 4th pair of horse chromosomes on a donkey metaphase

Other techniques of analysis, which complement each other, are used in the comparative studies of karyotypes. Apart from classic Zoo-FISH technique with painting probes, it is possible to use probes specific for smaller regions of a genome – subchromosomal or specified loci (Raudsepp and Chowdhary, 1999; Musilova et al., 2007). The construction of interspecies cytogenetic maps has been intensively developing as one of the directions of comparative genomics. Many of them are created based on the usage of FISH technique with gene-specific probes as well as the analysis of hybrid cell genome (Radiation Hybrid Maps – RH) (Raudsepp et al., 2004). An important place in the comparative research on genomes is occupied by genome sequencing and the analysis of DNA with the use of bioinformatic methods (Murphy et al., 2004; Kemkemer et al., 2009; Ng et al., 2009). Thanks to these methods, it is possible to detect smaller regions of homology between species and identify intrachromosomal rearrangements or changes in defined loci at the DNA sequence level (Helou et al., 2001; Nie et al., 2003; Richard et al., 2003; Perelman et al., 2005; Graphodatsky et al., 2000, 2008; Musilova et al., 2007, 2009).

The *Equidae* family is a perfect example illustrating the application and usefulness of comparative genome analysis, including Zoo-FISH technique, to trace the evolution of genomes. In the order Perissodactyla, the *Equidae* show a wide variety of morphology and number of chromosomes (Ryder et al., 1978; Power, 1984). Such a large degree of diversity occurring during a short time of evolution suggests rapid karyotype changes (Bush et al., 1977; Wichman et al., 1991). The number of possible rearrangements between the horse and donkey karyotypes was determined to be at least 20 (Raudsepp et al., 2001).

Because of the large scale of changes which had occurred during the *Equidae* evolution, classical cytogenetic techniques were insufficient to investigate them deeply enough. That is why Zoo-FISH technique with probes specific for chromosomes (whole chromosome paints – WCP) or large fragments as well as FISH method with gene-specific probes have been used on a large scale to date.

A comparison of the horse and donkey genomes is of great interest to researchers; although the genomes differ by only one chromosome pair (the horse: $2n = 64$, the donkey: $2n = 62$), they are separated by numerous fusions and rearrangements (Raudsepp et al., 1999, 2001, 2002; Myka et al., 2003 b; Yang et al., 2004). To detect homologies as well as possible rearrangements between *Equus caballus* and *Equus asinus*, Zoo-FISH technique was carried out; painting probes from horse chromosomes (ECA1-13, X and Y) were applied on donkey metaphase spreads (Raudsepp and Chowdhary, 1999). In 2004, Yang et al. performed Zoo-FISH using whole chromosome paints, specific for all autosomes and the X chromosome of horse, on donkey metaphase chromosomes, which enabled us to verify and extend the previous results (Yang et al., 2004). In order to understand the evolution of the *Equidae* karyotype, genomes of the domestic horse and Przewalski's horse (Myka et al., 2003 a; Yang et al., 2003), the horse and zebra (Yang et al., 2003) and even the zebra and rhinoceros (Trifonov et al., 2003), were compared. However, not only whole chromosome painting probes but also probes specific for chromosome arms have been used so far. Chromosome arm painting probes were obtained from horse chromosomes and a few Hartmann's zebra chromosomes and applied on Grevyi's

zebra metaphases to study karyotypic relationships between these species (Musilova et al., 2007). Also Zoo-FISH with probes specific for telomeres, centromeres and NORs, which contain highly repeated sequences, can be very helpful for the relationship analysis. The analysis of the distribution of these sequences in the genomes of members of the *Equidae* family can provide additional supplementary information about potential rearrangements which could have taken place. A good example of the application of this type of probes is Zoo-FISH with centromere and NOR specific probes from domestic horse genome hybridized to donkey metaphase spreads (Bugno-Poniewierska et al., 2009). Thanks to that, the confirmation of centromere repositioning (CR) in the donkey karyotype was possible.

The comparative analyses carried out using Zoo-FISH technique, directly or indirectly allowed for the determination of homologous regions between members of the *Equidae* family (Raudsepp et al., 1996; Raudsepp and Chowdhary, 1999; Raudsepp and Chowdhary, 2001; Yang et al., 2004; Myka et al., 2003a; Yang et al., 2003; Musilova et al., 2007). Despite the fact that Zoo-FISH method is indispensable for establishment of chromosomal correspondence between species, it imposes some restrictions. It allows determining the similarity and organization of large segments or chromosomes but it does not provide any information about the arrangement of particular genes, which, in spite of the shown homology of a specific region, can be different in various species (Prakash et al., 1997; Milenkovic et al., 2002; Bugno-Poniewierska et al., 2010). To this end, gene-specific probes widely used for the creation of physical maps, are applied (Chowdhary et al., 1996; Hu et al., 1997; Prakash et al., 1997; Raudsepp et al., 1997; Thomas et al., 1999; Raudsepp et al., 2001; Gomez-Fabre et al., 2002; Milenkovic et al., 2002; Dranchak et al., 2006; Bugno et al., 2007; Brinkmeyer-Langford et al., 2008; Bugno et al., 2009; Bugno-Poniewierska et al., 2010), which allows for the comparative analysis of genomes at a higher molecular level. The creation of the donkey physical map with the use of probes obtained from the horse genome is a good example of the application of FISH technique with gene-specific probes (Bugno et al., 2007; Bugno et al., 2009; Bugno-Poniewierska et al., 2010).

The usage of Zoo-FISH technique, supplemented with FISH with gene-specific probes, opens new possibilities in the comparative analysis of genomes, bringing us closer to the understanding of complex changes which occur during evolution and, as a result, its mechanisms. The application of these methods in the karyotype analysis of members of the *Equidae* family just confirms their usefulness and great importance.

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Zastosowanie techniki Zoo-FISH do analizy rearanżacji chromosomowych w rodzinie *Equidae*

STRESZCZENIE

W celu prześledzenia rearanżacji ewolucyjnych i pokrewieństwa między gatunkami niezbędna jest analiza genomu. Początkowo stosowano w tym celu m.in. narzędzia cytogenetyki klasycznej, lecz wraz z rozwojem metod cytogenetyki molekularnej możliwa stała się jego dogłębniejsza analiza. Jedną z metod wykorzystywaną szeroko w tym celu jest fluorescencyjna hybrydyzacja *in situ* (FISH – Fluorescence *in situ* Hybridization) i jej różne odmiany. Zoo-FISH, czyli porównawcza międzygatunkowa hybrydyzacja, z wykorzystaniem sond malujących specyficznych dla całych chromosomów, umożliwia wykrycie homologicznych bloków syntenii, których występowanie jest dowodem na wspólne pochodzenie i pokrewieństwo gatunków. Uzupełnieniem Zoo-FISH jest FISH z sondami specyficznymi do ramion chromosomowych lub sekwencji powtarzalnych (telomery, centromery), dostarczającymi dodatkowych informacji o organizacji oraz polimorfizmie i konserwatyzmie kariotypu. Stosuje się również FISH z sondami genowo specyficznymi, które umożliwiają lokalizację pojedynczych *loci*, co pozwala ustalić sprzężenia pomiędzy genami, a także zweryfikować dane uzyskane po zastosowaniu sond malujących w technice Zoo-FISH. Rodzina *Equidae*, ze względu na zróżnicowany kariotyp i jego szybką ewolucję, jest doskonałym obiektem do badań z wykorzystaniem wachlarza metod opartych na hybrydyzacji *in situ*, co z kolei pozwala na uzyskanie informacji na wielu poziomach organizacji DNA.