Gene Reports 11 (2018) 235–238

Contents lists available at ScienceDirect

Gene Reports

journal homepage: www.elsevier.com/locate/genrep

# Cytogenetic identity: A new parameter for estimating whole-genome differences

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ARTICLE INFO

*Keywords:*
Whole comparative genomic hybridization
Satellite DNA
Cytogenetic identity
Species
Chromosomes

ABSTRACT

In this paper, we introduce a novel parameter, called Cytogenetic Identity, to describe differences and similarities between genomes. Using Whole Comparative Genomic Hybridization plus Digital Image Analysis, we present a new methodology that employs the whole genome, including highly repeated DNA sequences, to provide a general picture about the differences between individuals of the same or different species. The proposed approach has a great potential in many different fields of research, like evolution, ecology, phylogenesis, etc. In the present study, we applied Cytogenetic Identity to establish a quantitative degree of divergence between different goat breeds. Advantages as well as disadvantages of the new parameter are discussed.

## 1. Introduction

Currently observed biodiversity has been shaped by millions of years of evolution and, more recently, by human intervention. Biodiversity can be described by analyzing differences occurring within and between species. The ability to establish resemblances and differences between individuals of the same and/or different species is of a paramount importance for researchers in many different fields. From the evolutionary interest in determining the degree of divergence of species, to the necessity of precisely describing the polymorphisms that are responsible for diseases, or to manage threatened and endangered species.

Initially, species were compared by morphological criteria; subsequently, with the development of molecular biology, degree of divergence was based mainly on protein and DNA analysis. In particular, DNA sequencing of a 658 bp fragment of the mitochondrial gene cytochrome *c* oxidase I (COI) allowed a DNA "barcoding" system (Hebert et al. 2003). In the near future, next generation sequencing (NGS) may allow a taxonomy based on entire genome analysis (Ellegren 2008).

However, since DNA analysis is focused on coding sequences, even whole sequencing studies will miss non-coding regions, like chromosome centromeres. Centromeres are the loci responsible for the correct segregation of chromosomes during mitosis and meiosis, and they are contained within regions of highly repetitive sequences, called satellite DNA. Centromeres are stably inherited: however, being non-coding, their DNA sequences are not under evolutionary constraint and show a higher mutation rate. Therefore, centromeric sequences, as well as other highly repeated DNAs, are of particular interest for studies on evolution, since they can depict mutation accumulation proportional to the divergence rate. Indeed, centromere evolution has been proposed to be a key factor in speciation (Henikoff et al. 2001).

In this paper, we propose a new tool to compare genomes of individuals of the same or different species, named Cytogenetic Identity (CI). This new parameter allows to include the whole genomes of the involved individuals in the comparison study, providing a general picture. We define and estimate Cytogenetic Identity between individuals as a reflection of the degree of divergence they have experienced throughout evolution. Our results provide a novel insight in the differences between genomes that, although less precise than molecular, bears into account all the genome sequences, including those with a higher mutation rate.

To estimate Cytogenetic Identity, we use Whole-Comparative Genomic Hybridization (W-CGH), a technique that allows detecting in a single Fluorescent in situ Hybridization (FISH) protocol all the chromosome differences between too compared genomes (Pita et al. 2003). The technique has proven its reliability in different species (Pita et al. 2008, 2009) and, in this new approach complemented with Digital Image Analysis (DIA), it allow us to obtain quantitative information.

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https://doi.org/10.1016/j.genrep.2018.04.005
Received 26 September 2017; Accepted 13 April 2018
Available online 14 April 2018

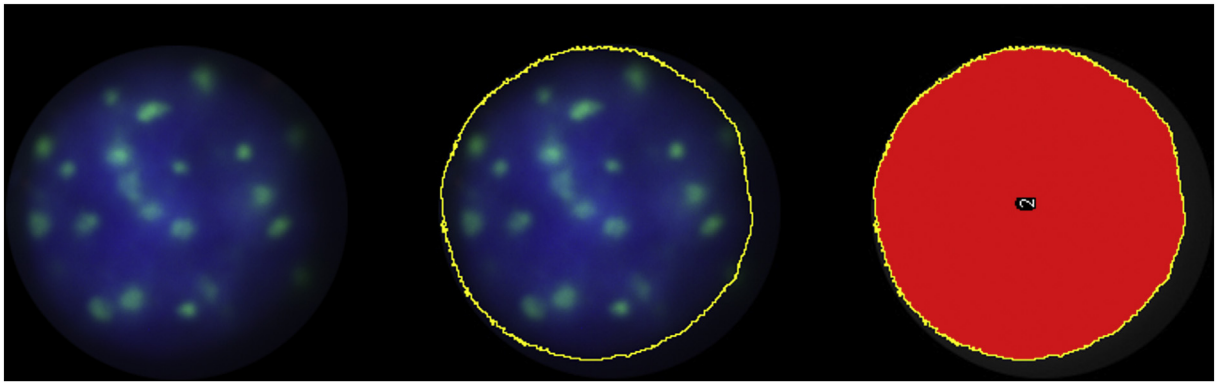

**Fig. 1.** From left to right: Example of a nucleus to be measured, selection of the region of interest using the DAPI unspecific counterstaining and mask of the region under which ImageJ software is automatically measuring Green and Red Grey levels.

This new use permits the estimation of Cytogenetic Identity offering useful application in the comparison of closely related individuals. In this paper, we analyzed individuals belonging to different goat breeds, as well as closely related species, since they provide us with a wide range of breeds to explore the accuracy of the approach.

## 2. Materials and methods

### 2.1. Samples

Blood samples were collected from *Capra hircus* (Sarda breed, Maltese breed and Murciana breed), *Ovis aries* and *Bos taurus*. DNA and metaphase chromosomes were obtained with standard methods. ARRIVE guidelines have been followed.

### 2.2. Probe labeling

DNA labeling with direct fluorochromes was performed using a Nick Translation kit (Enzo Life Sciences). One μg of each DNA was independently labeled with 0.3 mM Green-496 dUTP or Orange-552 dUTP (Enzo Life Sciences). Probe fragments were checked on 1% agarose gel to be similar in size and in the range of 600–2000 bp. Probes were then precipitated overnight with ethanol, centrifuged at full speed and supernatant was discarded. After complete air drying, probes were dissolved in hybridization buffer containing 50% (vol/vol) formamide, 10% (w/vol) dextran sulfate in 2× SSC, at pH 7, to a final concentration of 20 ng/μl.

### 2.3. W-CGH (whole-comparative genomic hybridization)

Comparative genomic hybridization of any two probes was performed as follows: slides were dehydrated in an ethanol series (70%, 85% and 100%), for 3 min each, at −20 °C. After air drying, slides were denatured in 2× SSC, with 70% formamide, for 2 min at 73 °C, dehydrated and dried again. Mixed probes were prepared adding equimolar concentration of the two labeled genomes to be compared (one probe labeled with Green-496 dUTP and the other probe labeled with Orange-552 dUTP) to a final volume of 15 μl. Mixed probes were denatured for 10 min at 73 °C, chilled on ice for 5 min and applied to the slide. Slides were incubated on wet chamber for 16 h at 37 °C for hybridization. After hybridization, slides were washed in in 2× SSC, with 50% formamide, for 15 min at 42 °C, and in 2× SSC for 8 min at 37 °C. Finally, slides were mounted with anti-fade solution (Vectashield, Vector Laboratories) and counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (100 ng/μl).

For each two species, breeds or individuals compared, the same set of two slides was prepared. It consisted of one slide with Sarda-breed cells hybridized with a mixed probe composed of Sarda genome labeled with Green-498 dUTP and an *Alien* genome labeled with Orange-552 dUTP. As a control, a second slide with Sarda-breed cells was hybridized with the inverted mixed probe, i.e. Sarda genome labeled in Red and the *Alien* genome labeled in Green.

### 2.4. Image capturing and digital image analysis (DIA)

Slides were analyzed using a DIA platform based on a Leica DMRB fluorescence microscope (Leica Microsystems) with three independent filters for Green-496 dUTP (Green fluorescence), Orange-552 dUTP (Red fluorescence) and DAPI (Blue fluorescence) detection (I3, Y3, and DAPI, respectively). Images were captured as three independent .tiff files (Green channel, Red channel and Blue channel) employing Leica DFC 350 FX (Leica Microsystems) running in Adobe Photoshop software (Adobe Systems Incorporated). Several images (at least 20) were captured to analyze interphases nuclei for quantitative results, as well as mitotic metaphases for qualitative description of the hybridization.

Adobe Photoshop software was also used to merge the Green, Red and Blue channels to create an RGB image after background subtraction. Also a larger image with several nuclei (5–15) was created assembling all the nuclei images of the same slide to facilitate DIA. Mitotic metaphase images were not employed for DIA since interphase nuclei provide a more homogeneous and individualized material to capture more detailed fluorescence information.

DIA of FISH images was performed employing ImageJ software (https://imagej.nih.gov/ij/). For each RGB image, Blue channel (unspecific DAPI counterstaining) was used to select the area to be measured on each cell (Fig. 1). Green and Red Fluorescence on each nucleus was measured, under that area, as Sum of Grey in the range of 0 to 255 (for Green and Red, independently). Area of each cell as the number of pixels was also recorded. Results were exported to Excel to estimate cytogenetic identity (CI) from Sum of Grey and Pixels area.

### 2.5. Cytogenetic identity (CI)

Several parameters were calculated using Sum of Grey (of Green and Red independently) and the Area of each cell (number of pixels). First, Average Grey (in the range of 0–255) was estimated, for each single cell, as the division of the Sum of Grey (for Green and Red independently) and its Area. Afterwards, Average Grey 2 (for Green and Red independently) was estimated as the mean value of all the Average Grey values of the cells on each experiment, and the same was done for the converse experiment (i.e. Sarda-G/*Alien*-R and Sarda-R/*Alien*-G).

Then, we calculated a Sarda Average Grey (SAG) and an *Alien* Average Grey (AAG). For example, to estimate SAG we employed the Average Grey 2 of Green from the experiment Sarda-G/*Alien*-R and the Average Grey 2 of Red from Sarda-R/*Alien*-G. In this same experiment, for AAG we used the Average Grey 2 of G from Sarda-R/*Alien*-G and the