Dottorato di Ricerca in Biologia Ambientale ed Evoluzionistica. Curriculum: Biologia Animale.

Anno Accademico 2020/2021 – XXXVI Ciclo. Lorenzo Attili Tutor: prof. Riccardo Castiglia

Title: A new hybrid zone between Robertsonian races of House Mouse, *Mus domesticus*: focus on chromosomal and molecular aspects.

Context: The house mouse, *Mus domesticus*, started the colonization of the Mediterranean basin from Middle East around 10 000 ya and settled in the western Mediterranean area during the Bronze Age (~ 3 000 ya) (Piálek et al 2005). Here the house mouse has undergone an extraordinary chromosomal diversification. The standard and most common karyotype is composed of 40 telocentric chromosomes, however, this species shows numerous populations, called chromosomal races (more than 100, Grize et al 2019), with a different diploid number. This is due to a high occurrence of Robertsonian (Rb) fusions, a mutation that involves the joining of two telocentric non-homologous chromosomes at the centromere to form a metacentric one. As consequence of several Rb fusions, the different chromosomal races may show a reduced diploid number, ranging from 2n=40 up to a minimum of 2n=22 (Piálek et al 2005).

The Rb races have been found in a scattered distribution within the Mediterranean basin, usually located in restricted and isolated regions. Nevertheless, there are several examples of areas where different Rb races or a single Rb race and the standard populations make contact and interbreed (Gündüz et al 2010). Those are called "hybrid zones", usually characterized by hybrid specimens with different chromosomal structures (Giménez et al 2017, Hauffe et al 2012). Several hybrid zones have been investigated in house mouse, especially focusing on distribution of Rb fusions within them. Presently, about 20 hybrid zones, which occur as discrete contacts between chromosomal races, have been described, excluding ten additional polymorphic areas considered unconfirmed zones (Tapisso et al 2020, Hauffe et al 2012). The spatial progression from one race to another usually reveals chromosomal clines. Those clines can be coincident or staggered within the hybrid zone, usually corresponding respectively to a primary or a secondary contact between populations (Gündüz et al 2010). In limited cases, there are documented specimens in homozygous conditions within a staggered hybrid zone that are different from either of the hybridizing races. If homozygous type undergoes a range expansion it can be considered as a new race, this process is called "zonal raciation" (Piálek et al 2005).

Hybrids, which show chromosomal heterozygosity, can be distinguished in "simple heterozygotes" or "complex heterozygotes". The firsts form trivalent configurations, a short chain of three chromosomes, in meiosis while the latter form chains or rings of four or more chromosomes (Gündüz et al 2010). Complex configurations are produced when hybridization occurs between races that differ by metacentrics with "monobrachial homology", i.e., when two different metacentrics share the same homologous arm, for example: Rb(1.18) and Rb(2.18). Multivalents cause errors of segregation leading to gametic aneuploidy and high rate of germ cell death (Hauffe et al 2012). In addition, the presence of metacentric chromosomes, either in a homozygous or a heterozygous condition, disturbs recombination events leading to a reduction in number of chiasmata during diakinesis (Capilla et al 2014). These meiotic aberrations influence the fertility of

hybrid specimens: while simple heterozygotes show a little reduction of fertility, in complex heterozygotes the effects are stronger up to complete sterility (Hauffe et al 2012). Nevertheless, recent studies found that, despite multiple defects, hybrids with complex chain configurations remain fertile, since a large number of cells progress to the later stages of meiosis (Grize et al 2019, Ribagorda et al 2019).

House mouse chromosomal hybrid zones show an extraordinary diversity (Hauffe et al 2012), providing a suitable model to study how chromosomal speciation occurs on mammals (Giménez et al 2017, Franchini et al 2020). Chromosomal changes can be involved in speciation via two mechanisms: "hybrid dysfunction" and "suppressed recombination". The low fertility of specimens and the absence of recombination in heterozygous configuration cause a reduction of gene flow, allowing the build-up of incompatibilities between populations (Giménez et al 2017, Franchini et al 2010). At the same time, reproductive isolation could be attributable also to genic factors. In this regard, recent attention has focused on the Prdm9 gene, the only known speciation-associated gene described for mammals (Mihola et al 2009). Prdm9 is a meiosis-specific gene, located within the proximal centromeric regions of mouse chromosome 17. It codes for a meiotic-specific histone (H3) methyltransferase with a C-terminal tandem repeat zinc finger (ZnF) domain, which mediates sequence-specific bindings to DNA. The number of zinc fingers encoded appears to directly affect hybrid sterility (*Mus domesticus* x *Mus musculus*), because interallelic PRDM9 incompatibilities trigger a failure in recognition of DNA-binding sites (Mihola et al 2009).
Aim: In this context, Rb populations of house mouse represent "natural laboratories for evolutionary studies" (Franchini et al 2010, Franchini et al 2020) since they provide to potentially investigate the role of both chromosomal and genic mechanisms in the establishment of reproductive isolation (Capilla et al 2014). Furthermore, the main purpose of the project is to evaluate concurrently chromosomal and genetic (Prdm9) variation across a hybrid zone. The comparison between these two aspects may help to better understand their role and how they combine in reproductive isolation between natural populations, where barriers to gene flow are acting.

Project: A new hybrid zone will be explored in central Italy. The area is characterized by a Robertsonian system (a group of geographically proximal Rb populations with an apparently common origin) of four metacentrics races: Ancarano IACR (2n=24), Campobasso ICBO (2n=22), Cittaducale ICDE (2n=22) and Colfiorito ICOL (2n=34) (Castiglia et al 2005). The peculiarity of this system is the low sharing of metacentrics. Only few metacentrics are in common between the races. All the others differ, suggesting a past isolation between populations (Castiglia et al 2005). The contact zones between IACR and ICDE races and between ICDE and standard populations have been deeply studied, showing a strong reduction of gene flow in both cases due to chromosomal incompatibilities (Franchini et al 2008, Franchini et al 2010).

The project under discussion would focus on a new contact zone between ICDE and ICBO races. Since the closer sites known between the races are distant about 100 Km (between the provinces of L'Aquila and Chieti, Abruzzi region), a systematic trapping across the contact area could identify the presence of heterozygous specimens. Both ICBO and ICDE races have a 2n=22 karyotype, however, they share only one metacentric: Rb(9.16). In a F1 hybrid is expected a meiotic configuration of a single chromosomes ring, involving 16 metacentrics, and three bivalents. This

complex configuration is the result of a high level of "monobrachial homology" between the races. The case study that will be examined provides a good opportunity to explore both meiotic abnormalities and nucleotide variation of Prdm9 gene, in order to understand how chromosomal rearrangements may impact the genetic structure of populations and their diversification in a natural context. The study of Prdm9 may have two important implications: first of all it can contribute, together with chromosomal heterozygosity, to the low recombination rates usually observed in Robertsonian mice. Secondly, being a high variable marker, it represents a useful tool to shed light on the degree of genetic divergence and evolutionary relationships among populations (Vara et al 2019).

In particular, the specific purposes and steps of the project are the following:

I. To describe the structure of the supposed hybrid zone, detecting the level of reproductive isolation through the analysis of distribution of hybrid individuals and the pattern of chromosomal clines, evaluating also possible influences of geographical and ecological barriers within the contact area.

   Methods:

   A linear transect crossing the contact area (~100 Km, North-west to South-est direction, between the provinces of L'Aquila and Chieti, Abruzzi region), will be mapped out. About 10 sample sites (~10 Km distant) will be randomly selected in order to perform a systematic sampling. Human dwellings and human-modified habitats (farms, cultivated fields, and barns) represent typical trapping sites for house mouse in a mountain setting. Mice are captured with baited live traps (e.g. Sherman or Longworth) set for one to three nights and checked every 8/12 hours. Sampling will be followed by a karyotype analysis of trapped specimens in laboratory. Karyotypes are prepared from bone marrow cells, following standard protocols, namely the 'air-drying' procedure and G-banding of chromosomes. Then metaphases are observed under a microscope for the count and detection of chromosomes. Using these cytogenetics techniques, the identity of Rb chromosomes in each specimen will be figured out, thereby, the chromosomal heterozygous condition of mice within the area is recorded (a similar approach was followed by Tapisso et al 2020 on Madeira island). The sample size for these analyses will include about 100 animals.

II. To assess the meiotic behavior of the multivalent configurations and the resulting degree of fertility in heterozygous specimens.

   Methods:

   As explained above, chromosome reorganizations in heterokaryotypes have strong influence on the progress of synapsis and crossing over during meiosis. The analysis of meiotic progress in spermatocytes will be performed through the immunochemical identification of specific proteins involved in chromosomes association during prophase-1 and microscope observation. Recently, both Berrios et al (2018) and Ribagorda et al (2019) employed this method to investigate meiosis in hybrids formed by crossing mice from Vulcano and Lipari island (Aeolian archipelago) populations (both 2n=26). This technique allows the observation of synapsis dynamics during meiosis, with a focus on heterologous synapsis, chromosome recombination and inactivation events. By comparing the same events in homozygous

specimens, the magnitude of meiotic defects in hybrids can be evaluated. These analyses will concern about 10 laboratory reared mice obtained through oriented crossings between individuals of different races. They will be performed in the laboratory of Jesus Page (Departamento de Biología Celular, Facultad de Ciencias, Universidad Autónoma de Madrid, Spain).

III. To identify the extent of genetic diversity between the races and within the hybrid zone, using Prdm9 as molecular marker.

Methods:

Genomic DNA will be extracted from tissue samples preserved in ethanol. The last exon of Prdm9, which includes the ZnF array, will be amplified by PCR using the primers: ZFA-L_F (forward) and ZFA-L_R (reverse) (See in Vara et al 2019). This fragment exhibits both length polymorphism (number of ZnF repeats) and amino acid variation. PCR products will be separated on 1% agarose gels to discern the ZnF array length (in homozygous or heterozygous condition) for a subsequent sequencing. PRDM9 alleles will be classified using the number of ZnF repeats and the extent of amino acid variation in the highly variable positions. Sequences analyses will concern the evaluation of allelic diversity, building phylogenetic trees and networks, but also measures of population differentiation (e.g. Fst) (following Vara et al 2019). Genetic variability may be related to chromosomal features, by matching chromosomal and genetic clines (e.g. difference in Znf repeats with the presence of some metacentrics). To obtain robust and accurate results about the genetic structure and diversification within the hybrid zone at least 80 mice (already sampled for the chromosomal characterization) will be examined.

Program:

ACTIVITIES	1st year						2nd year						3rd year					
Develop a sampling strategy and figure out the sample sites																		
Collection of samples																		
Analysis of karyotypes																		
Analysis of meiosis																		
Prdm9 sequencing																		
Variability analysis of Prdm9 sequences																		
Participate to a conference																		
Write papers																		
Write the PhD thesis																		

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