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Karyological characterization of the endemic iberian rock lizard, *Iberolacerta monticola* (Squamata, Lacertidae): insights into sex chromosome evolution

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Cytogenetic and Genome Research Volume 142, Issue 1, pages 28–39, December 2013
Accepted 24 June 2013, Published online 28 November 2013, Issue release date December 2013

This is the peer-reviewed but unedited manuscript version of the following article:

Rojo V, Giovannotti M, Naveira H, Nisi Cerioni P, González-Tizón A, Caputo Barucchi V, Galán P, Olmo E, Martínez-Lage A, Karyological characterization of the endemic iberian rock lizard, *Iberolacerta monticola* (Squamata, Lacertidae): insights into sex chromosome evolution. Cytogenet Genome Res 2014;142:28-39 (DOI: 10.1159/000356049).

The final, published version is available at http://www.karger.com/?doi=10.1159/000356049.

Abstract

Rock lizards of the genus *Iberolacerta* constitute a promising model to examine the process of sex chromosome evolution, as these closely related taxa exhibit remarkable diversity in the degree of sex chromosome differentiation with no clear phylogenetic segregation, ranging from cryptic to highly heteromorphic ZW chromosomes and even multiple chromosome systems ($Z_1Z_1Z_2Z_2/Z_1Z_2W$). To gain a deeper insight into the patterns of karyotype and sex chromosome evolution, we performed a cytogenetic analysis based on conventional staining, banding techniques and fluorescence in situ hybridization in the species *I. monticola*, for which previous cytogenetic investigations did not detect differentiated sex chromosomes. The karyotype is composed of 2n = 36 acrocentric chromosomes. NORs and the major ribosomal genes were located in the subtelomeric region of chromosome pair 6. Hybridization signals of the telomeric sequences $(TTAGGG)_n$ were visualized at the telomeres of all chromosomes and interstitially in 5 chromosome pairs. C-banding showed constitutive heterochromatin at the centromeres of all chromosomes, as well as clear pericentromeric and light telomeric C-bands in several chromosome pairs. These results highlight some chromosomal markers which can be useful to identify species-specific diagnostic characters, although they may not accurately reflect the phylogenetic relationships among the taxa. In addition, C-banding revealed the presence of a heteromorphic ZW sex chromosome pair, where W is smaller than Z and almost completely heterochromatic. This finding sheds light on sex chromosome evolution in the genus *Iberolacerta* and suggests that further comparative cytogenetic analyses are needed to understand the processes underlying the origin, differentiation and plasticity of sex chromosome systems in lacertid lizards.

Keywords: Chromosome banding; Comparative cytogenetics; FISH; Rock lizards; Sex chromosomes

The genus *Iberolacerta* is a group of rock lizards (family Lacertidae) mainly distributed in the highland areas of Western Europe. According to recent taxonomic revisions [Mayer and Arribas, 2003; Arribas and Carranza, 2004; Arribas and Odierna, 2004; Carranza et al., 2004; Crochet et al., 2004; Arribas et al., 2006], the genus *Iberolacerta* comprises 8 species, which can be subdivided into 3 main units: (1) *I. horvathi* (Méhely, 1904), occurring in the Eastern Alps and the north of the Dinaric Chains; (2) the subgenus *Pyrenesaura* (Arribas, 1999), which includes the 3 species found in the Pyrenees Mountains, namely *I. aranica* (Arribas, 1993), *I. aurelioi* (Arribas, 1994) and *I. bonnali* (Lantz, 1927); and (3) the 4 species included in the 'Iberian group', i.e. *I. cyreni* (Müller and Hellmich, 1937), *I. martinezricai* (Arribas, 1996), *I. galani* (Arribas, Carranza and Odierna, 2006), and *I. monticola* (Boulenger, 1905), with disjunct distributions in central and northern mountain ranges of the Iberian Peninsula.

The phylogeny of this genus has been under continual revision, but the evolutionary relationships among some taxa still remain unresolved [Mayer and Arribas, 2003; Carranza et al., 2004; Arribas et al., 2006]. Within the Iberian group, data from mitochondrial and nuclear genes suggest that *I. cyreni* split earlier, between 6 and 7.5 mya, while the speciation events within the clade formed by *I. martinezricai*, *I. galani* and *I. monticola* occurred considerably later, at the beginning of the Pleistocene (roughly 2.5 mya). Recent molecular analyses support the hypothesis that *I. monticola* was the first lineage to diverge from the common branch, shortly before the separation of *I. martinezricai* and *I. galani,* approximately 1.8 mya (see www.karger.com/doi/10.1159/000356049 for online suppl. fig. 1) [Remón et al., 2013].

Karyological studies based on conventional staining and banding techniques have proven useful for establishing phylogenetic relationships and delimiting species and subspecies boundaries in the genus *Iberolacerta*, as well as in several other lacertid groups [e.g. Olmo et al., 1993; Odierna et al., 1996; in den Bosch et al., 2003; Kupriyanova and Melashchenko, 2011]. Previous cytogenetic surveys of the *Iberolacerta* species [Capula et al., 1989; Odierna et al., 1996; Arribas and Odierna, 2004; Arribas et al., 2006] showed a common diploid number of 2n = 36 and a similar karyotypic macrostructure, with all chromosomes acrocentric. Only the karyotypes of the 3 *Iberolacerta* species from the Pyrenees differ from this formula, with reduced diploid numbers that range from 2n = 24 to 26 in males and from 23 to 26 in females and numerous biarmed chromosomes, which probably evolved from the ancestral acrocentric complement through a series of Robertsonian fusions (online suppl. fig. 1) [Odierna et al., 1996].

Interestingly, C-banding analyses uncovered high levels of diversity regarding the sex chromosome system. A ZW sex chromosome pair, in which the W chromosome is smaller than the Z and highly heterochromatic, has been described in *I. horvathi*, *I. cyreni* and *I. galani* [Capula et al., 1989; Odierna et al., 1996; Arribas et al., 2006]. In contrast, the sex chromosomes of *I. aranica*, *I. martinezricai* and *I. monticola* are reported to be homomorphic and indistinguishable by differences in size, morphology or heterochromatinization [Odierna et al., 1996; Arribas and Odierna, 2004]. More significant differences are present in the Pyrenean species *I. bonnali* and *I. aurelioi*, with multiple $Z_1Z_1Z_2Z_2/Z_1Z_2W$ sex chromosome systems where the W chromosome is biarmed and the Z_1 and Z_2 counterparts are uniarmed (online suppl. fig. 1) [Odierna et al., 1996]. The presence of ZW-derived multiple sex chromosome systems is a particularly uncommon feature within lizards, so far reported for only 2 other species of lacertids, namely *Zootoca vivipara* and *Podarcis taurica* (Chromorep: A reptile chromosomes database, http://193.206.118.100/professori/chromorep.pdf).

The heterogeneous situation concerning sex chromosomes in the genus *Iberolacerta* is illustrative for the wide diversity of sex chromosomes found in the family Lacertidae. Female heterogamety is considered to be universal within this family. Even so, sex chromosomes at different stages of differentiation are frequently found between closely related species and even between populations of the same species, suggesting that sex

chromosomes can have multiple and independent origins in related lacertid taxa [e.g. Olmo et al., 1987; Odierna et al., 1993, 2001; in den Bosch et al., 2003].

Typically, sex chromosomes are thought to evolve after suppression of recombination through increasing stages of differentiation, from a primitive form, in which nascent sex chromosomes differ only in a limited region and are otherwise indistinguishable, to an advanced state, in which sex chromosomes are highly heteromorphic [Charlesworth et al., 2005; recently reviewed in Charlesworth and Mank, 2010]. Reports on lacertid karyotypes, mainly accomplished through conventional banding techniques, suggest that lacertid sex chromosomes have evolved primarily via heterochromatinization followed by degeneration of the female-specific W chromosome, although this is probably not the only mechanism operating in this family [Olmo et al., 1986, 1987; Ezaz et al., 2009]. Chromosomal rearrangements, such as inversions or translocations, can be also involved in the primary differentiation of lizard sex chromosomes [for a review, see Olmo et al., 1987; Ezaz et al., 2009], implying that even newly evolved sex chromosomes can be heteromorphic [Charlesworth and Mank, 2010]. In this regard, comparative cytogenetic analyses within the genus *Iberolacerta* can provide valuable insights into the processes underlying the origin, differentiation and evolutionary transitions of sex chromosomes.

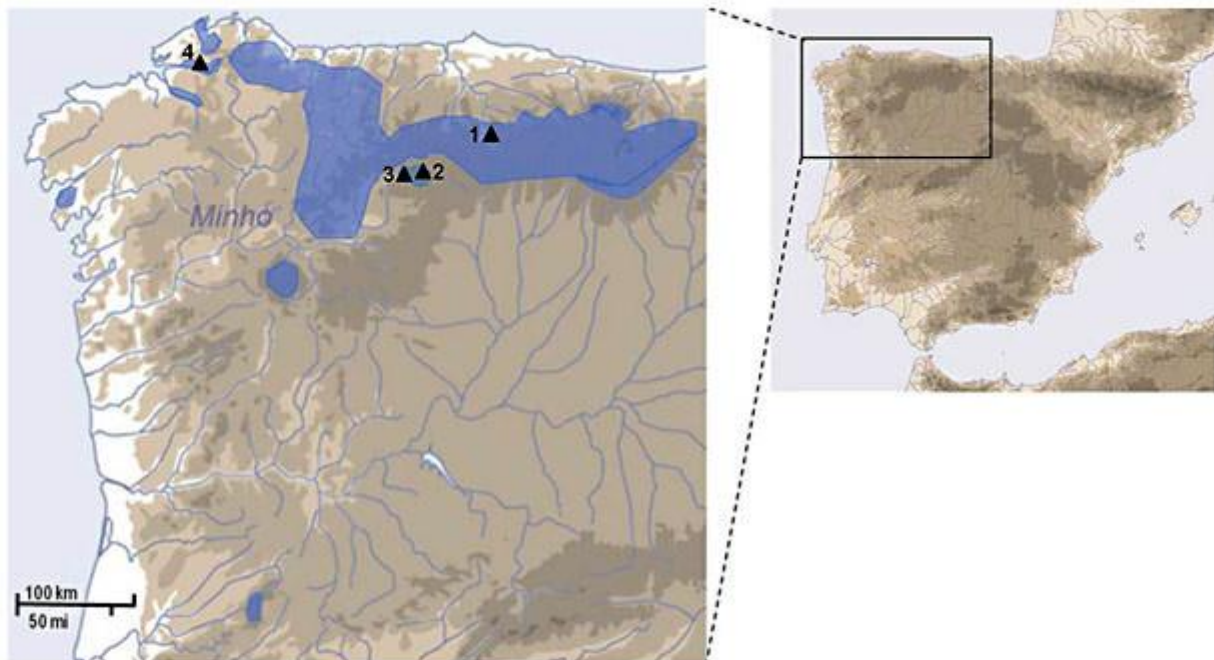

Fig. 1. Map of the Iberian Peninsula showing the current distribution area of *I. monticola* (blue areas). Numbers represent localities sampled in the present study: (1) Puerto de Vegarada, (2) Villabandín, (3) Salientes, and (4) Eume. See text for further details.

In this study, we focus on one of the *Iberolacerta* species, *I. monticola*, for which previous cytogenetic investigations did not detect differentiated sex chromosomes. This species is distributed across a wide area in the north of the Iberian Peninsula, along the Cantabrian Mountain range, where it inhabits mainly rocky habitats at middle-high altitudes [Mayer and Arribas, 2003; Carranza et al., 2004; Crochet et al., 2004]. Apart from this continuous area, there are several other isolated populations in the Serra da Estrela Mountains, in Portugal, and in Galicia, at the north-west corner of Spain (fig. 1). Some populations in this last region are found at areas of exceptionally low altitudes, most of them associated to Atlantic forests in shady fluvial gorges [Galán, 1999; Galán et al., 2007].

Material and Methods

Specimens

One adult male and one adult female of *I. monticola* were collected from each of the following localities: (1) Puerto de Vegarada (43.04N, -5.46E), (2) Villabandín (42.90N, -6.14E), (3) Salientes (42.85N, -6.31E), and (4) the fluvial valley of the river Eume (43.41N, -8.07E) (fig. 1). Permissions for fieldwork and ethics approval of experimental procedures were issued by the competent authorities Xunta de Galicia and Junta de Castilla-León, in Spain, in accordance with the Spanish legislation (Royal Decree 1201/2005 and Law 32/2007, on the protection of animals used for experimentation and other scientific purposes).

Phenotypic sex was determined on the basis of external morphology and then confirmed via visual inspection of gonads upon dissection.

Cell Culture and Chromosome Preparations

Metaphase chromosome spreads were prepared according to previously described protocols [Giovannotti et al., 2009a]. Fibroblast cell lines were cultured in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mML-glutamine (all from Gibco). Cultures were incubated at 30°C in a humidified atmosphere of 5% CO_2 in air. When exponential cell growth was observed around the primary explants (usually after 2-3 weeks of culture), the cells were trypsinized and subcultivated at a 1:2 split ratio. Following this first passage, the cell lines were grown until 70-80% of overall confluence was reached. Six hours prior to harvesting, 0.1 µg/ml colcemid (Roche) was added to the cultures followed by 30 min of hypotonic treatment in 0.075 M KCl at 30°C and fixation in 3:1 methanol:glacial acetic acid. Fifteen microlitres of the cell suspension were dropped onto glass slides and air-dried.

Chromosome Analysis

Conventional chromosome staining was performed using a 5% Giemsa solution at pH 7. C-banding was carried out according to Sumner [1972]. C-banded chromosomes were independently stained with 10% Giemsa solution at pH 7 for 10 min and sequentially with both fluorochromes chromomycin A_3 (CMA_3), and 4′,6-diamidino-2-phenylindole (DAPI) [Schweizer, 1976; Schmid et al., 1983]. Silver-staining of nucleolar organizer regions (Ag-NORs) was performed as described by Howell and Black [1980].

Chromosomal locations of the 18S-5.8S-28S rRNA genes were determined by FISH as described in González-Tizón et al. [2000], with slight modifications, using the DNA probe p*Dm* 238 from *Drosophila melanogaster* [Roiha et al., 1981], labeled by nick translation with digoxigenin-11-dUTP (Roche).

Briefly, the slides were dehydrated by serial ethanol washes [twice for 2 min in 70% (v/v) ethanol, twice for 2 min in 90% ethanol and once for 5 min in 100% ethanol], air dried and aged at 65°C for 30 min. Subsequently, they were incubated in DNase-free RNase (100 µg/ml in 2× SSC) at 37°C for 30 min and washed in 2× SSC for 10 min. One hundred nanograms of labeled probe (2.5 µl) were made up to 30 µl with hybridization buffer (50% formamide, 2× SSC and 10% dextran sulphate), denatured at 75°C for 15 min, chilled on ice, placed onto each slide, covered with a coverslip, and finally sealed with rubber cement. Chromosome denaturation was performed in a slide PCR (MJ Research, MJ 100) as follows: 75°C for 7 min, 55°C for 2 min, 50°C for 30 s, 45°C for 1 min, 42°C for 2 min, 40°C for 5 min, 38°C for 5 min, and 37°C for 5 min. Hybridization took place at 37°C overnight in a humid chamber. Posthybridization washes consisted of two 5-min incubations in 2× SSC at 37°C and at room temperature, respectively, followed by a 5-min incubation in washing solution composed of 0.1 M Tris, 0.15 M NaCl and 0.05% Tween-20 at room temperature. Signal detection included 3 consecutive incubation steps, at 37°C for 30 min each, with: (i) mouse anti-digoxigenin antibody (Roche), (ii) fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (Sigma-Aldrich) and (iii) FITC-conjugated goat anti-rabbit IgG (Sigma-Aldrich). After each

incubation step, slides were washed 3 times for 5 min with washing solution at room temperature. Chromosomes were counterstained with 1.5 µg/ml propidium iodide in the anti-fade medium Vectashield (Vector Laboratories).

Chromosome mapping of the $(TTAGGG)_n$ sites was carried out with a Cy3-labeled pan-telomeric DNA probe (Cambio) following the manufacturer's instructions. The slides were mounted using the anti-fade medium Vectashield (Vector Laboratories), containing 1.5 µg/ml DAPI.

Images were captured using an epifluorescence microscope Nikon Microphot-FXA equipped with a Nikon DS-Qi1Mc digital camera and processed with the NIS-Elements D 3.10 software.

Results

Karyotypes, Heterochromatin Distribution and Fluorochrome Staining

All analyzed specimens of *I. monticola* showed a karyotype composed of 2n = 36 acrocentric chromosomes of gradually decreasing size (fig. 2).

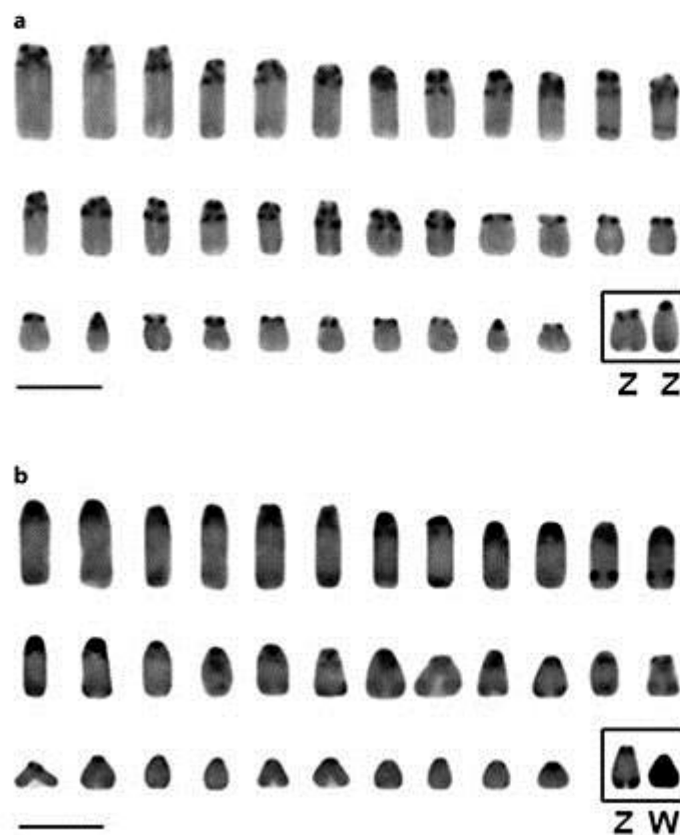

Fig. 2. C-banded karyotypes of male (a) and female (b) *I. monticola* from the population of Eume. Sex chromosome pairs ZZ and ZW (inset). Scale bars = 5 µm.

C-banding evidenced constitutive heterochromatin at the centromeres of all chromosomes and interstitially at the pericentromeric regions of the 10 larger chromosome pairs (figs. 3, 4). These conspicuous heterochromatic blocks were uniformly stained with both DAPI and CMA_3, and hence, they do not seem to

contain particularly AT- or GC-rich repetitive DNA families (figs. 3c-f, 4). Faint C-positive bands were also found at the ends of several chromosome pairs (tentatively, in the 12 larger chromosome pairs) and resulted only positively stained by CMA_3, indicating that this telomeric heterochromatin was composed of GC-rich sequences. In addition, CMA_3 staining produced an intense fluorescent signal in the subterminal region of a large chromosome pair, probably correlated with NOR-associated heterochromatin (figs. 3e, f, 4c, d).

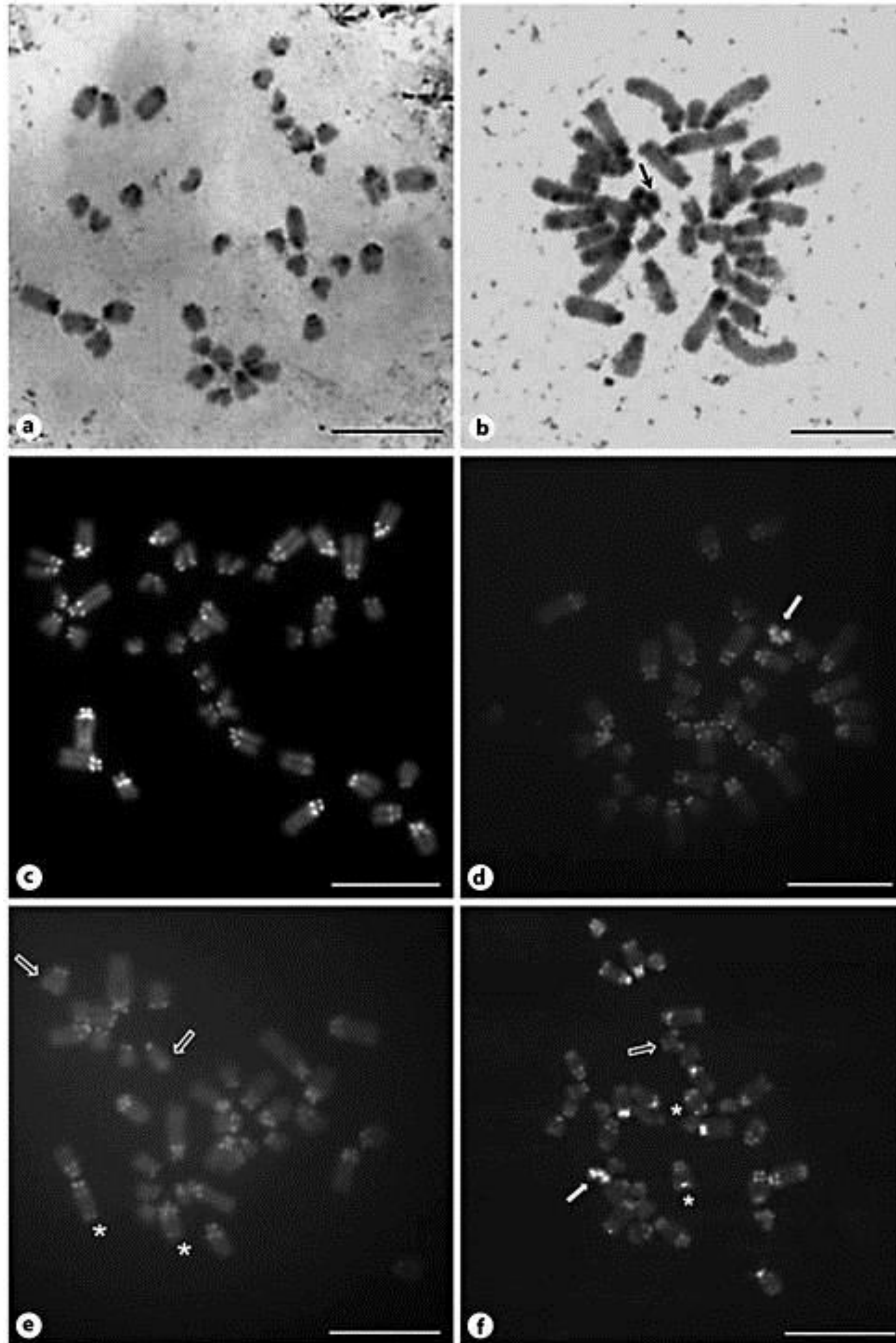

Fig. 3. Metaphase plates of male (a, c, e) and female (b, d, f) *I. monticola* from Eume, C-banded and stained with Giemsa (a, b), DAPI (c, d) and CMA_3 (e, f). Asterisks in e and f indicate CMA_3-positive signals associated with NORs. Empty and filled arrows point to Z and W sex chromosomes, respectively. Scale bars = 10 µm.

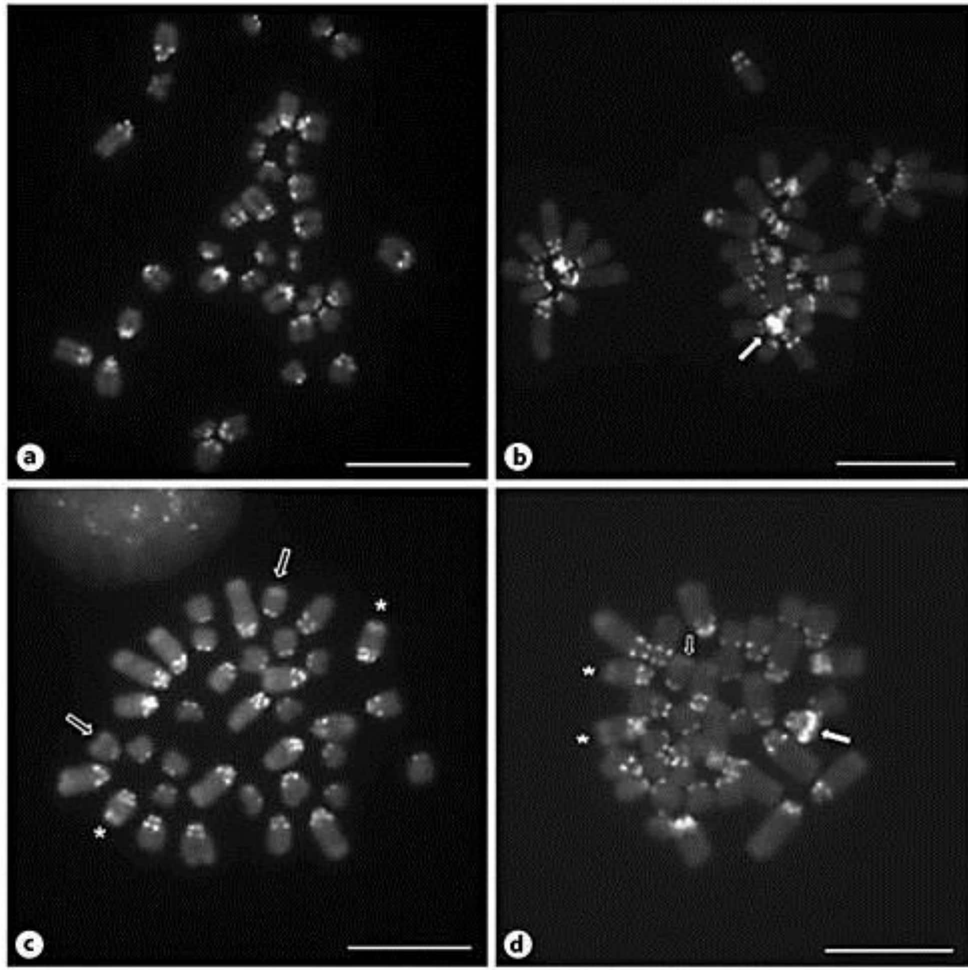

Fig. 4. Metaphase plates of male (a, c) and female (b, d) *I. monticola* from Puerto de Vegarada, C-banded and stained with DAPI (a, b) and CMA_3 (c, d). Asterisks in c and d indicate CMA_3-positive signals associated with NORs. Empty and filled arrows point to Z and W sex chromosomes, respectively. Scale bars = 10 μm.

The differences in the pattern of heterochromatin distribution between sexes clearly revealed the presence of a cytologically differentiated ZW sex chromosome pair. The W chromosome was easily recognizable in female metaphases, being one of the smallest chromosomes of the karyotype (fig. 2b) and almost completely heterochromatic, with only a small euchromatic region located in an interstitial position (fig. 3b). The heterochromatin of the W chromosome was intensely stained with both DAPI and CMA_3 (figs. 3d, f, 4b, d). C-banding also allowed the identification of the Z chromosome, present in 2 copies in males and in a single copy in females. This element was as large as the chromosomes of the 9th or 10th pair and differed only slightly from the autosomes in bearing a brighter, CMA_3-positive, telomeric C-band (figs. 2a, 3e, 4c).

Chromosomal Mapping of the 18S-5.8S-28S rRNA Genes

Ag-NOR banding agreed with CMA_3 evidence and showed active NORs on the secondary constriction in the subtelomeric regions of chromosome pair 6 (figs. 2, 5a, b).

Fluorescent hybridization signals of the 18S-5.8S-28S rRNA genes were also coincident with Ag-NOR bands and did not reveal more inactive loci (fig. 5c, d).

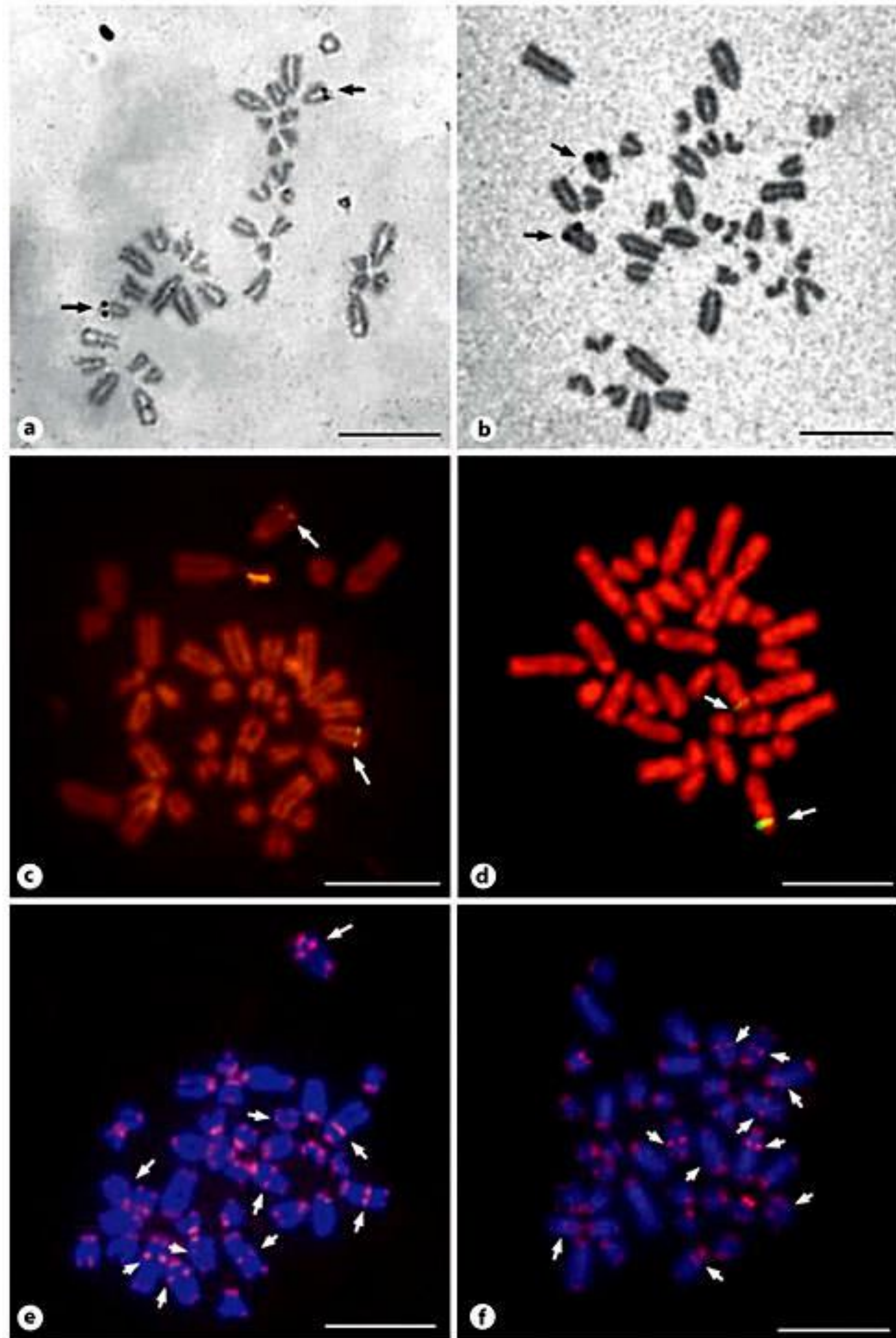

Fig. 5. Chromosomal localization of the 18S-5.8S-28S rRNA genes and $(TTAGGG)_n$ telomeric sequences in male (a, c, e) and female (b, d, f) *I. monticola*. Ag-NOR bands (a, b) and FISH signals (c, d) of the 18S-5.8S-28S rRNA genes (arrows). e, f Hybridization patterns of the telomeric probe $(TTAGGG)_n$. Arrows point to interstitial telomeric sites. Scale bars = 10 μm.

Chromosomal Location of the $(TTAGGG)_n$ Sites

FISH with a telomeric probe $(TTAGGG)_n$ produced discrete fluorescent signals at the telomeres of all chromosomes (fig. 5e, f). Additionally, bright hybridization signals were detected at interstitial sites (so-called interstitial telomeric sites, ITSs) in 5 large chromosome pairs in all the metaphase spreads examined. None of these ITSs were located on either the sex chromosomes or the NOR-bearing pairs.

Discussion

Chromosome Number and Karyotypes

In accordance with previously published results [Odierna et al., 1996], the karyotypes obtained from males and females of *I. monticola* showed a diploid chromosome complement of 2n = 36 acrocentric elements, which is common to all the species assigned to the 'Iberian group' of the genus *Iberolacerta*, namely *I. galani*, *I. martinezricai*, *I. cyreni*, and the said *I. monticola.*

In contrast with chromosome morphology, the pattern of heterochromatin distribution is not so conservative between these taxa [Odierna et al., 1996], and each species displays its own heterochromatin profile. In general, all the *Iberolacerta* species - with the only exception of *I. bonnali* - show prominent C-bands at the centromeres of almost all the acrocentric chromosome pairs. The presence of centromeric heterochromatin is a widespread character in lacertids [Olmo et al., 1986, 1993; Odierna et al., 1996], and it has been suggested that it may play a role in centromere structure and function [e.g. Capriglione et al., 1998].

However, the composition of the highly repetitive DNA sequences that constitute this centromeric heterochromatin is not necessarily conserved between the different *Iberolacerta* species, as indicated by the fact that these DAPI-positive C-bands are also brightly stained by CMA_3 in *I. monticola* and *I. galani* [Arribas et al., 2006], but are CMA_3-negative in *I. martinezricai* [Arribas and Odierna, 2004].

Moreover, the C-banding technique revealed the presence of additional DAPI- and CMA_3-positive heterochromatin in the pericentromeric regions of the 10 larger chromosome pairs. These interstitial heterochromatic regions have not been previously detected by C-banding in any of the *Iberolacerta* species, although they are probably correlated with the pericentromeric bands generated on the 6 larger chromosome pairs of *I. monticola* after the digestion of heterochromatin with the endonuclease *Alu*I [Odierna et al., 1996]. This *Alu*I banding pattern shows the variation in sequence composition between the *Alu*I-sensitive heterochromatin located at the centromeres and the pericentromeric *Alu*I-resistant heterochromatin present at least on 6 chromosome pairs.

Even though satellite DNAs in constitutive heterochromatin are usually composed of AT-rich elements [e.g. King and Cummings, 1997; Plohl et al., 2008], the faint C-bands revealed at the telomeres in the 12 larger chromosome pairs of *I. monticola* were only visible after CMA_3 staining, and therefore, a high GC content can be postulated. GC-rich satellites have been reported for some animal species [Meneveri et al., 1995; Malykh et al., 2001; Barragán et al., 2002; Petrović et al., 2009], and in Squamate reptiles, a telomeric GC-rich satellite has been described for the skink *Eumeces schneideri* [Giovannoti et al., 2009b]. The compartmentalization of GC-rich elements in telomeric heterochromatin could be related to the hypothesized role of short guanine stretches in telomere maintenance and stability [Muniyappa et al., 2000], as well as in promoting chromosome rearrangements through recombination between satellite and telomeric sequences [e.g. Hartmann and Scherthan, 2004].

The presence of telomeric heterochromatin blocks in some chromosome pairs of *I. monticola* and in all chromosomes of *I. galani* [Arribas et al., 2006] constitutes a cytogenetic marker that further discriminates the karyotypes of both species from *I. martinezricai*, where all chromosomes are devoid of telomeric C-bands [Arribas and Odierna, 2004].

On the whole, C-banding data gathered so far in the genus *Iberolacerta* reveal extensive heterogeneity in the amount and distribution of the heterochromatic fraction, even between species so closely related as *I. martinezricai*, *I. monticola* and *I. galani*. However, the karyological affinities unveiled between *I. monticola* and *I. galani* are not consistent with molecular data [Arribas et al., 2006; Remón et al., 2013], which indicate that *I. monticola* is the sister taxon to the clade formed by *I. galani* and *I. martinezricai* (online suppl. fig. 1). In the light of the phylogeny, it seems likely that the C-banding patterns

of *I. monticola* and *I. galani* represent the ancestral condition for this lineage; thus, the particular differences in heterochromatin distribution and composition reported for *I. martinezricai* constitute a derived character that, similarly to other cytogenetic traits (e.g. NOR location, see below) or osteological autapomorphies distinctive of this taxon [Arribas and Odierna, 2004], could have become fixed after the species divergence, due to a random genetic drift in small populations. In conclusion, our findings support the idea that, even if C-banding patterns in lacertid lizards can be useful to identify species' diagnostic characters, they may not accurately reflect the phylogenetic relationships among taxa [Olmo et al., 1986].

Ribosomal Loci

As previously reported in *I. monticola* [Odierna et al., 1996], silver-staining documented a single NOR site in a subtelomeric position of chromosome pair 6. Such NOR location at the telomeres of a large chromosome pair (L-type after Olmo et al. [1993]) appears to be ubiquitous among lacertids [Olmo et al., 1993], and it is also the plesiomorphic condition for the genus *Iberolacerta*, where only *I. cyreni* and *I. martinezricai* differ in showing a NOR in an interstitial position on a medium-sized chromosome pair (M-type after Olmo et al. [1993]) [Odierna et al., 1996; Arribas and Odierna, 2004].

FISH with the 28S-5.8S-18S rDNA probe, carried out for the first time in this genus, confirmed the presence of the ribosomal clusters at the sites identified by silver-staining and did not show additional transcriptionally inactive loci. In addition, the bright CMA_3 signal associated with the NOR site highlighted the GC-richness in rDNA base composition, as reported for a wide variety of organisms [e.g. Sumner, 1990 and references therein].

Telomeric Repeats

Hybridization signals of the $(TTAGGG)_n$ probe were located at the telomeres of all chromosomes and at interstitial positions on 5 large chromosome pairs.

ITSs have been observed in many vertebrate species [e.g. Meyne et al., 1990; Lee et al., 1993; Nanda and Schmid, 1994; Garagna et al., 1997; Ventura et al., 2006], including several families of Squamate reptiles [Meyne et al., 1990; Schmid et al., 1994; Pellegrino et al., 1999; Bertolotto et al., 2001; Srikulnath et al., 2009]. They usually consist of large arrays of telomeric-like repeats commonly located in pericentromeric regions, within or at the margins of constitutive heterochromatin.

A large body of evidence indicates that ITSs may be remnants of chromosomal rearrangements that occurred during chromosome evolution [for a review, see Lin and Yan, 2008; Ruiz-Herrera et al., 2008]. Likewise, the ITSs detected in *I. monticola* could be the result of chromosome reorganization events, such as tandem fusions of ancestral acrocentric chromosomes, paracentric inversions involving the telomeric sequences or pericentric inversions in ancestral sub-/metacentric chromosomes. The high intensity of the ITS signals, generally larger than those detected at the telomeric ends, suggests that the retained $(TTAGGG)_n$ sequences have also been amplified. In this regard, it is interesting to point out that karyotype evolution in lacertids is thought to be characterized by a progressive translocation of microchromosomes to macrochromosomes [Olmo et al., 1986; Odierna et al., 1987]. In fact, the basic diploid number of *Iberolacerta* (2n = 36) differs from the common lacertid karyotype in that it lacks a pair of microchromosomes [Olmo et al., 1993]. Moreover, ITSs have been associated with fragile sites and recombination hotspots [recently reviewed in Bolzán, 2012] that may confer greater flexibility for karyotype change by providing potential new sites for telomere formation [Meyne et al., 1990].

However, the presence of ITSs in the karyotype is not always related to structural chromosome changes. Preexisting ITSs, including the short stretches of telomeric hexamers that are presumably inserted during the repair of double strand breaks [Nergadze et al., 2004, 2007], could be subsequently spread and expanded at different intrachromosomal regions by common mechanisms of repetitive DNA amplification, such as

unequal crossing-over or sequence conversion [Wiley et al., 1992; Vermeesch et al., 1996; Garagna et al., 1997; Nanda et al., 2008]. For instance, a process of heterochromatin association and unequal exchange has been proposed to explain the dispersion and amplification of ITSs embedded within heterochromatin to new chromosomal locations in lemur and rodent species [Go et al., 2000; Rovatsos et al., 2011].

Therefore, further studies of the occurrence of ITSs and comparative karyological analyses, such as chromosome painting, between lacertids and closely related lizard families are required to elucidate the origin of these nontelomeric sites and clarify their association with karyotype evolution in this lineage.

Sex Chromosomes

Populations of *I. monticola* from the locality of Puerto de Vegarada, in the Cantabrian Mountain range, were first reported to lack differentiated sex chromosomes [Odierna et al., 1996]. In the present study, however, a heteromorphic ZW chromosome pair was consistently identified in the female specimens analyzed from this same population. The discrepancy between those observations and our results could be just due to experimental artifacts. For instance, the higher degree of chromosome condensation in metaphase spreads obtained by scraping techniques from tissues (former work) in comparison with chromosomes obtained from cell cultures (present study) could hamper the detection of the small-sized W chromosome by C-banding.

The presence of a cytologically distinguishable ZZ/ZW system was also confirmed in specimens from 2 other Cantabrian populations, as well as from the population of Eume, at the northwesternmost edge of the species' range. All 4 studied populations are currently isolated, and according to recent molecular analysis [Remón et al., 2013], their independent evolution began roughly between 1.5 and 0.9 mya, possibly as a consequence of climatic fluctuations during the Pleistocene. Even so, the sex chromosome pairs of any of these populations are highly similar in terms of relative size and in the amount and distribution of heterochromatin, albeit they could exhibit some differentiation at finer scales hardly evidenced by C-banding and fluorochrome staining. Therefore, a closer examination with more sensitive cytogenetic methods would be required to investigate the presence of subtle differences in DNA content of sex chromosomes between genetically divergent populations of *I. monticola*.

Likewise, the sex chromosome pair detected in *I. monticola* closely resembles that of other *Iberolacerta* species for which sex chromosomes have been described, i.e. *I. horvathi*, *I. cyreni* and *I. galani* [Capula et al., 1989; Odierna et al., 1996; Arribas et al., 2006]. All of them possess a highly heteromorphic ZW pair, in which the W chromosome is smaller than the Z and completely or almost completely heterochromatic. Nevertheless, greater similarities are found between *I. monticola* and *I. galani*. In particular, the presence of a bright telomeric heterochromatic block in the Z chromosome is a feature that appears to be exclusive of both species. Even if the nature of the sequences responsible for the heteromorphism in the sex chromosome pair is not known, reverse fluorochrome staining revealed at least certain differences in molecular composition, since heterochromatin in the Z chromosome resulted only positive after CMA_3 staining (similarly to the weak C-bands at the ends of some autosomal pairs), while W chromosome heterochromatin was completely stained with both CMA_3 and DAPI.

In general, the properties of sex chromosomes in *I. monticola* and the remaining *Iberolacerta* species may be concordant with the evolutionary model proposed for other lacertids [Olmo et al., 1987; Odierna et al., 1993]: the initial step of sex chromosome differentiation would be the accumulation of repetitive sequences on either homologue, leading to the formation of 2 heterochromatic areas, a proximal and a distal, as observed in the W chromosome of *I. monticola*. This may subsequently be followed by structural rearrangements, such as deletions of heterochromatic regions not involved in sex determination, originating a heteromorphic sex chromosome pair in which the W is distinctly smaller than the Z. In this context, it would be of interest to verify whether the W chromosome of *I. galani*, reported to be totally imbibed with

heterochromatin [Arribas et al., 2006], certainly lacks the intercalary euchromatic region observed in the W chromosome of *I. monticola* and thus represents a more advanced stage of sex chromosome differentiation.

Despite the common features of the ZW pair of these *Iberolacerta* species, it is likely that not all of the sex chromosome systems in this genus followed the same evolutionary pathway: multiple sex chromosome systems ($Z_1Z_1Z_2Z_2$ male and Z_1Z_2W female), with W chromosomes at different degrees of heterochromatinization, have been found in *I. bonnali* and *I. aurelioi* [Odierna et al., 1996]. In addition, homomorphic and cytologically undetectable sex chromosomes are presumably present in *I. aranica* and *I. martinezricai* (online suppl. fig. 1) [Odierna et al., 1996; Arribas and Odierna, 2004]. Moreover, variation in the degree of sex chromosome differentiation is found among species that diverged no more than 2.5 mya *(I. monticola*, *I. galani* and *I. martinezricai).*

Such interspecific variability in the stage of degeneration of the W chromosomes, with no clear phylogenetic correlation, is representative of the remarkable heterogeneity of sex chromosome systems reported for lacertid lizards (Chromorep: A reptile chromosomes database) [Olmo et al., 1986, 1987; Odierna et al., 1993], which suggests that in this family, as in many reptile lineages, sex chromosomes can have multiple independent origins even in closely related taxa [e.g. Ezaz et al., 2009].

Thus, considering that degradation of W chromosome and dosage compensation would evolve more slowly in ZW taxa, as compared with XY taxa [Naurin et al., 2010], and bearing in mind the advanced state of degeneration of the W chromosome in the basal *Iberolacerta* species, *I. horvathi* [Capula et al., 1989], it seems probable that the presence of a heteromorphic ZZ/ZW pair is the ancestral condition for this genus. Accordingly, it could be hypothesized that the seemingly undifferentiated sex chromosomes in *I. martinezricai* and *I. aranica* might represent neo-sex chromosomes resulting from recent turnover events (e.g. the appearance of a new sex-determining gene on an autosome or the transposition of a sex-determining gene to a new chromosomal location), which would have replaced the preexisting heteromorphic ZW pair. Nonetheless, the putative absence of heteromorphic sex chromosomes in both species should be further investigated in detail.

Future comparative cytogenetic analyses, along with the application of high-resolution molecular cytogenetic techniques, will therefore be necessary to deepen the knowledge about the degree and patterns of sex chromosome differentiation and the transitions between simple ZW and multiple Z_1Z_2W systems in the genus *Iberolacerta*, which ultimately would shed light on the mechanisms underlying sex chromosome evolution and the plasticity of sex determination systems in lacertid lizards.

Acknowledgements

This research was supported by grants REN2003-02931/GLO (Ministerio de Ciencia y Tecnología, Spain), PGIDIT03RFO10301PR and PGIDIT06RFO10301PR (Xunta de Galicia, Spain), awarded to H.N., and by a grant PRIN 2009 'Ricerche di citogenetica molecolare sui sistemi di determinazione del sesso nei rettili squamati (sottordine Sauria)' provided by the Ministry of Education, University and Research, Italy, to V.C.B. The funding to V.R. was provided by a 'FPU' fellowship from the Ministerio de Educación, Cultura y Deporte, Spain.

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