RESEARCH ARTICLE

Isolation and Characterization of Two Satellite DNAs in some Iberian Rock Lizards (Squamata, Lacertidae)

JOURNAL OF EXPERIMENTAL ZOOLOGY
PART B
MOLECULAR AND DEVELOPMENTAL EVOLUTION

MASSIMO GIOVANNOTTI[1], VERÓNICA ROJO[2], PAOLA NISI CERIONI[1], ANA GONZÁLEZ-TIZÓN[2], ANDRÉS MARTÍNEZ-LAGE[2], ANDREA SPLENDIANI[1], HORACIO NAVEIRA[2], PAOLO RUGGERI[1], ÓSCAR ARRIBAS[3], ETTORE OLMO[1], AND VINCENZO CAPUTO BARUCCHI[1,4]*

[1]Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Ancona, Italy
[2]Departamento de Biología Celular y Molecular, Universidade da Coruña, La Coruña, Spain
[3]Avda. Francisco Cambó 23, Barcelona, Spain
[4]Consiglio Nazionale delle Ricerche, Istituto di Scienze Marine Sezione Pesca Marittima, Ancona, Italy

ABSTRACT

Satellite DNAs represent a large portion of all high eukaryotic genomes. They consist of numerous very similar repeated sequences, tandemly arranged in large clusters up to 100 million base pairs in length, usually located in the heterochromatic parts of chromosomes. The biological significance of satDNAs is still under discussion, but most of their proposed functions are related to heterochromatin and/or centromere formation and function. Because information about the structure of reptilian satDNA is far from exhaustive, we present a molecular and cytogenetic characterization of two satDNA families in four lacertid species. Two families of tandemly repeated DNAs, namely *Taq*I and *Hin*dIII satDNAs, have been cloned and sequenced from four species belonging to the genus *Iberolacerta*. These satDNAs are characterized by a monomer length of 171–188 and 170–172 bp, and by an AT content of 60.5% and 58.1%, respectively. FISH experiments with *Taq*I satDNA probe produced bright signals in pericentromeric regions of a subset of chromosomes whereas all the centromeres were marked by *Hin*dIII probe. The results obtained in this study suggest that chromosome location and abundance of satDNAs influence the evolution of these elements, with centromeric families evolving tenfold faster than interstitial/pericentromeric ones. Such different rates render different satellites useful for phylogenetic investigation at different taxonomic ranks. *J. Exp. Zool. (Mol. Dev. Evol.) 9999B: 1–14, 2013.* © 2013 Wiley Periodicals, Inc.

How to cite this article: Giovannotti M, Rojo V, Nisi Cerioni P, González-Tizón A, Martínez-Lage A, Splendiani A, Naveira H, Ruggeri P, Arribas Ó, Olmo E, Caputo Barucchi V. 2013. Isolation and characterization of two satellite DNAs in some Iberian rock lizards (Squamata, Lacertidae). J. Exp. Zool. (Mol. Dev. Evol.) 9999B:1–14.

J. Exp. Zool. (Mol. Dev. Evol.) 9999B:1–14, 2013

Satellite DNAs (satDNAs) form a substantial part of eukaryotic genomes and consist of tandemly repeated DNA sequences typically arranged in large clusters of hundreds or thousands of copies usually located in the heterochromatic regions of chromosomes, mainly in the regions close to the centromeres and telomeres. The biological significance of satDNAs remains intriguing and challenging. The sequence conservation of some satellites over long evolutionary times, the presence of differentially expressed transcripts in several species and interactions with centromeric-specific proteins (e.g., the histone H3 variant CENH3) suggest a biological role for some satellites, although this is not fully understood (see Plohl et al., 2008; Plohl, 2010).

A satDNA family could arise in a phylogenetically short period by explosive amplification (Bachmann and Sperlich, '93) and afterwards its repeats could follow a gradual mode of sequence evolution during a long evolutionary time (Bachmann and Sperlich, '93). The processes by which satDNA families arise are not well known. A set of molecular-exchange mechanisms has been proposed to account for its origin by amplification of a tandem array of multi-copy sequences. These mechanisms include unequal crossing-over (Smith, '76), transposition (Miller et al., 2000), or extrachromosomal rolling-circle replication and reintegration of tandem arrays into the genome (Feliciello et al., 2006). A recently originated tandem array is initially homogeneous in sequence because of the multi-copy amplification of the same repeat. In the course of time, random mutations would accumulate and the repeats would diverge. However, the nonallelic repeats of a satDNA family do not evolve independently, but concertedly leading to near homogeneity for species-specific mutations (Bachmann and Sperlich, '93; Rudd et al., 2006). This phenomenon, known as concerted evolution, is achieved by a number of genomic mechanisms, mainly unequal crossing-over, biased gene conversion, slippage replication, and amplification by rolling-circle (Dover, '82; Walsh, '87; Charlesworth et al., '94). However, the rates of sequence change (homogenization and fixation) vary for each satDNA family or even for the same satDNA family within different lineages. Levels of sequence variation among repeats would depend on factors such as mutation rate, inter- and intrachromosomal recombination rates, copy number, array size and structure, chromosomal distribution, chromosomal structure, population size, divergence time, and reproductive mode; it is also subject to random genetic drift and possibly natural selection (Strachan et al., '85; Stephan and Cho, '94; Luchetti et al., 2003; Navajas-Pérez et al., 2005; Dawe and Henikoff, 2006; Kuhn et al., 2007). The relative importance of each factor remains controversial.

In this context, very little information exists on satDNA array size, composition and long-range organization, especially in reptiles (see Giovannotti et al., 2009). An exception is represented by Lacertidae, a species rich family of squamate reptiles, widespread in the Palaearctic region (Sindaco and Jeremcenko, 2008). This family comprises the subfamilies Gallotiinae and Lacertinae, with the latter comprising two monophyletic tribes, the Eremiadini of Africa and arid southwest and central Asia, and the Lacertini of Europe (Arnold et al., 2007). So far, five satDNA families have been described for the genome of the Lacertinae subfamily: the pLCS (190 bp in length) is shared by the genera *Algyroides*, *Teira*, *Lacerta*, and *Podarcis* (Capriglione et al., '89, '91; Capriglione, 2000); the pLHS (140 bp) is specific for *Podarcis* only (Capriglione et al., '94; Capriglione, 2000); the pGPS (185 bp) is present in the genome of *Podarcis* and in species belonging to the genera *Archaeolacerta*, *Algyroides*, *Lacerta*, and *Zootoca* (Capriglione et al., '98), so that its appearance would precede the divergence within the Lacertinae subfamily; the CLsat family is described for the Caucasian genus *Darevskia* (145–147 bp, Ciobanu et al., 2003; Grechko et al., 2006); the Agi160 is restricted to the genus *Lacerta* (138–184 bp, Ciobanu et al., 2004; Grechko et al., 2005). These satDNA families revealed several common features, such as the same range of monomer lengths (140–190 bp), AT content (tendency toward AT enrichment 50–65%) and homopolymeric (A_{3-4} and T_{3-4}) stretches (Capriglione et al., '91; Ciobanu et al., 2001, 2004). All these features were also found in other nonreptilian satDNAs (see King and Cummings, '97).

The genus *Iberolacerta* (see Arribas, '99) has a disjunct range in mountain areas of western Europe: a portion comprises central Portugal, central and northern Spain and Pyrenees; another part embraces western Alps and northern Dinaric chain. Until recently the rock-lizard populations endemic to the Iberian Peninsula were considered to represent a single species, *Lacerta monticola* Boulenger, 1905 (see Salvador, '85), that has recently been split into the following taxa: *Iberolacerta aranica*, *I. aurelioi*, and *I. bonnali* restricted to the Pyrenees and *I. cyreni*, *I. galani*, *I. martinezricai*, and *I. monticola*, in the central-western parts of Iberian Peninsula (see Arribas et al., 2006). An additional species is represented by the east-Alpine and Dynaric species *I. horvathi*. This classification was based on (i) morphological (biometry, scalation), ostelogical, and karyological data; (ii) on the use of

Grant sponsor: Ministry of Education, University and Research (Italy) ("Ricerche di citogenetica molecolare sui sistemi di determinazione del sesso nei rettili squamati, sottordine Sauria"); grant number: PRIN2009/20093HYH97; grant sponsor: Ministerio de Ciencia y Tecnología, Spain; grant number: REN2003-02931/GLO; grant sponsor: Xunta de Galicia, Spain; grant numbers: PGIDIT03RFO10301PR, PGIDIT06RFO10301PR; grant sponsor: "FPU" fellowship; grant number: AP2008-02686; grant sponsor: Ministerio de Educación (Spain) (Beca de Formación de Profesorado Universitario).

Conflicts of interest: None.

*Correspondence to: Vincenzo Caputo Barucchi, Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Ancona, Italy. E-mail: v.caputo@univpm.it

Received 8 April 2013; Revised 29 July 2013; Accepted 31 July 2013

DOI: 10.1002/jez.b.22530

Published online XX Month Year in Wiley Online Library (wileyonlinelibrary.com).

molecular tools, namely nuclear (c-mos) and mitochondrial DNA (12S and cytochrome *b*), and (iii) on the construction of phylogenetic trees ranking the different allopatric populations based on the degree of genetic divergence, with *I. horvathi* as the most basal species (for a revision see Arribas et al., 2006). Another conceptual framework influencing the species subdivision of these largely allopatric lizards is the phylogenetic species concept, according to which species are segments of a phylogenetic lineage beyond nodes, irrespective of the degree of reproductive isolation (for a criticism see Mace, 2004). Considering the well-known usefulness of satDNAs in facing phylogenetic issues (i.e., Martinsen et al., 2009), the aim of the present paper was to isolate and characterise satDNA in some lacertid species in order to (i) increase the knowledge of this genomic elements in an important amniote group for which data on occurrence, genomic distribution, and evolutionary rates are limited to a handful of species; (ii) use the satDNAs isolated to verify the robustness of the proposed phylogenetic reconstruction for some *Iberolacerta* taxa on the light of independent molecular markers.

MATERIALS AND METHODS

Samples

Two males and two females of *Iberolacerta monticola* (from Fragas do Eume, A Capela, Galicia, Spain) and two males and two females of *I. galani* (from A Ponte, Pena Trevinca, A Veiga, Galicia, Spain) were used to make metaphase chromosomes and to extract genomic DNA. In addition, genomic DNA was extracted from seven ethanol preserved specimens of *I. cyreni* from three different Iberian locations (Navacerrada, Sierra de Guadarrama, Segovia-Madrid, Spain; Pico Zapatero, Sierra de la Paramera, Ávila; Puerto de Peña Negra, Sierra de Villafranca, Ávila, Spain) and one of *I. martinezricai* (Puerto El Portillo, Salamanca, Spain). Permissions for field work and experimental procedures were issued by the competent Spanish authorities: Xunta de Galicia (for *I. monticola* and *I. galani*) (permission number 79/2008) and Junta de Castilla y León (for *I. cyreni* and *I. martinezricai*) (permission numbers: 20051630007003/2005, 20061630024599/2006, 2007167004130/2007, 20081630020386/2008, 20092390004760/2009). Finally, genomic DNA of *Lacerta bilineata*, *Podarcis muralis*, *P. siculus*, and *Timon lepidus*, was extracted from ethanol preserved tissues of voucher specimens belonging to one of the authors (Vincenzo Caputo Barucchi).

Isolation and Characterization of Satellite DNAs

Genomic DNA was extracted from whole blood, using standard protocols with proteinase K digestion followed by phenol/chloroform extraction (see Sambrook et al., '89). Fifteen restriction endonucleases (*Alu*I, *Apa*I, *Ava*II, *Bam*HI, *Bcn*I, *Bgl*I, *Bgl*II, *Dra*I, *Eco*RV, *Hin*dIII, *Msp*I, *Rsa*I, *Sma*I, *Taq*I, *Xba*I) (Fermentas International, Inc., Burlington, ON, USA) were screened and about 8 μg of *I. monticola* and *I. galani* purified genomic DNA were utilized for each digestion. Electrophoresis on 2% agarose gel of the digested DNA revealed a band of about 170 bp for *Hin*dIII and 190 bp for *Taq*I, corresponding to the monomeric unit of repetitive DNA (Fig. 1A), whereas no clear bands were produced by the remaining 13 endonucleases. The 170 and 190 bp fragments were excised from agarose gel, purified with Pure Link Quick Gel Extraction Kit (Invitrogen, Carlsabad, CA, USA) and cloned in the pCR®-blunt vector with Zero Blunt PCR Cloning Kit (Invitrogen) following the manufacturer's recommendations. Ten clones of each *I. monticola* satellite DNAs (*Hin*dIII and *Taq*I satDNAs henceforth) and 13 (*Hin*dIII) and 16 (*Taq*I) of *I. galani* satDNAs were sequenced on an ABI PRISM 3730XL (Applied Biosystems, Foster City, CA, USA) automatic sequencer.

Digoxigenin-labeled probes were produced by PCR amplification of single clones and used in Southern hybridization experiments to verify that the elements isolated were tandemly arranged, as expected for satDNAs. In these experiments, *Hin*dIII and *Taq*I digested genomic DNAs from *I. monticola* and other lizards (*I. cyreni*, *I. galani*, *I. martinezricai*, *Lacerta bilineata*, *Podarcis muralis*, *P. siculus*, *Timon lepidus*) were used in order to assess the presence of these repetitive elements in other genera of this family. The hybridization with the digoxigenin-labeled satDNA probes was performed at 50°C overnight with the Sure Blot CHEMI Hybridization and Detection Kit (EMD Millipore Co., Billerica, MA, USA) following the manufacturer's recommendations. The hybridization was detected with the same kit.

The genomic abundance of satDNAs was estimated by quantitative dot blot analysis. Dilutions of genomic DNA and clones containing *Hin*dIII and *Taq*I satDNAs used as a standard were blotted onto a nylon membrane with BIO-DOT® microfiltration apparatus (Bio-Rad Laboratories, Hercules, CA, USA), following manufacturer's recommendations. In order to avoid errors due to the differences in the hybridization kinetics, sonicated salmon sperm DNA was used as a carrier and added to each sample up to a final amount of 0.5 μg DNA/sample (see Cafasso et al., 2003). Hybridization was performed overnight at 45°C. The same clones as those used as a standard were employed to produce digoxigenin-labeled probes. The detection protocol was carried out with the same protocol as the one used for Southern hybridization.

From the sequences of the monomeres of *I. monticola* and *I. galani*, *Hin*dIII and *Taq*I satDNAs two pairs of primers (*Hin*dIII-F: 5′-TGAGTGTTTTACAGTTGAAAAGCT-3′; *Hin*dIII-R: 5′-CATTGTGTTATTTGAGCGCAA-3′; *Taq*I-F: 5′-ATTCTGACCCTGGGGGTTAG-3′; *Taq*I-R: 5′-CATATTTAAAGAAATCAGGCCTCG-3′) were designed and used for isolation of these satellites from the genomes of the other two *Iberolacerta* species. PCR products from the amplification of *Iberolacerta* genomic DNAs with above primers were run on 2% agarose gel, the band corresponding to the amplified monomers excised from the gel, purified with Pure Link Quick Gel Extraction Kit (Invitrogen) and cloned in the pCR®-blunt vector with Zero Blunt PCR Cloning Kit (Invitrogen)

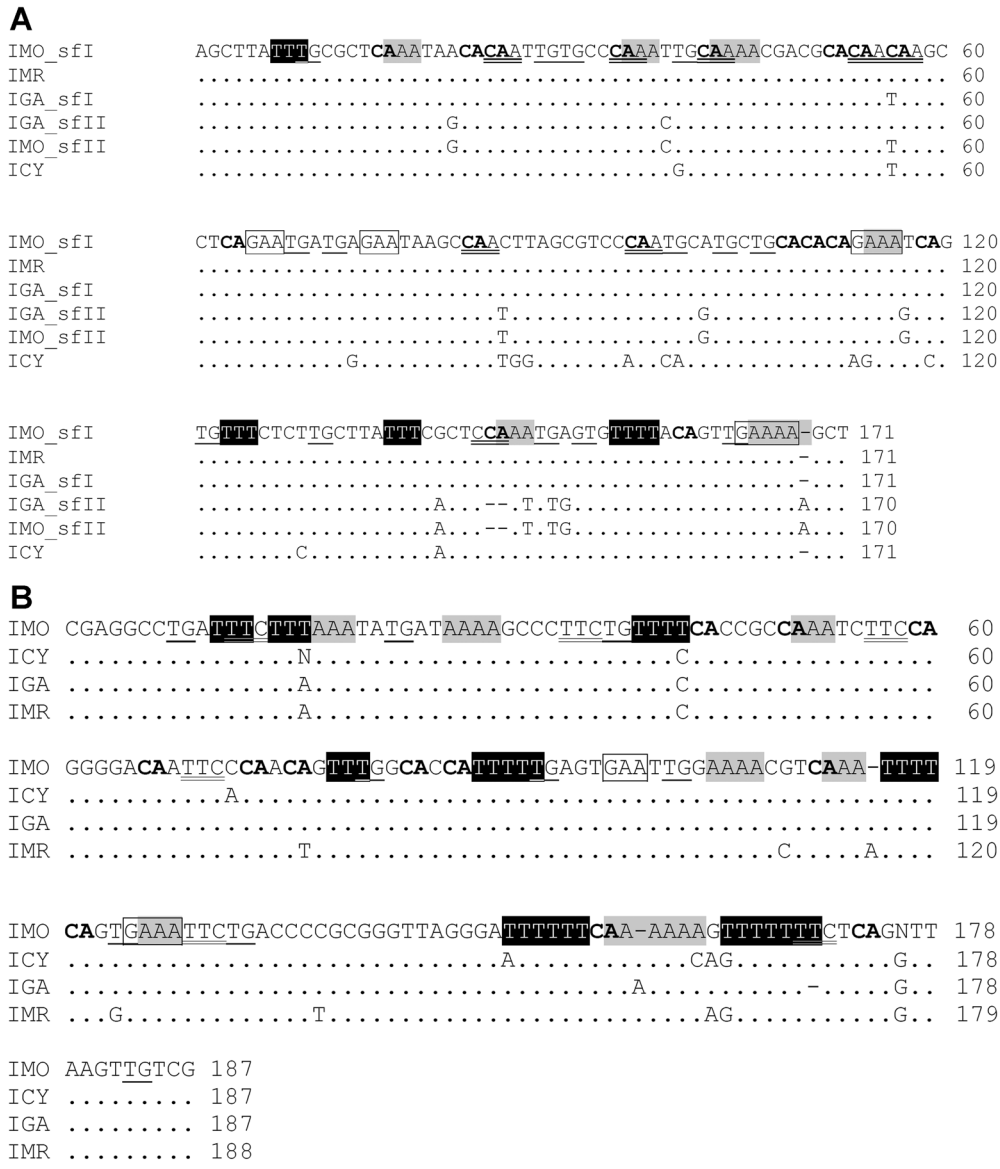

Figure 1. Comparisons of consensus sequences of *Hind*III (a) and *Taq*I (b) between the four *Iberolacerta* species analysed. Repeated motifs are highlighted. sfI: HindIII satDNA subfamily I; sfII: HindIII satDNA subfamily II. Dots refer to nucleotide identity and dashes indicate indels.

following manufacturer's recommendations. Clones of *Hind*III and *Taq*I satDNAs were sequenced on an ABI PRISM 3730XL (Applied Biosystems) automatic sequencer. These sequences were then aligned in CLUSTAL W (Larkin et al., 2007), using default parameters. The visual inspection of sequence alignments was carried out to check for the presence of shared nucleotide changes, which could serve as diagnostic positions to define subsets (subfamilies) within each satDNA family. A GenBank search was performed in order to compare *Hind*III and *Taq*I satDNAs with other satDNAs in the database.

Maximum parsimony (MP), neighbor joining (NJ), maximum likelihood (ML), and Bayesian analyses (BA) were used to infer the phylogenetic relationships among sequences of each satDNA. MP consensus trees (50% majority rule) were constructed with PAUP* version 4.0b10 (Swofford, 2002) using the heuristic search method with 1,000 random-addition-sequence replicates, tree-bisection-reconnection (TBR) branch swapping and holding 100 trees at each cycle of the stepwise-addition procedure. To increase the number of informative characters, gaps were coded as binary (presence/absence) characters.

NJ analyses were performed in MEGA version 5 (Tamura et al., 2011). The NJ trees were based on distances obtained by the maximum composite likelihood method, with pairwise deletion and 1,000 bootstrap replicates. ML analyses were conducted in MetaPIGA v.2.1.3 (http://www.metapiga.org) (Helaers and Milinkovitch, 2010) using the metapopulation genetic algorithm (metaGA) with probability consensus pruning among four populations of four individuals each. The best-fitting nucleotide substitution models [Jukes–Cantor (JC) for *Hin*dIII satDNA and Hasegawa–Kishino–Yano plus Gamma (HKY + G) for *Taq*I satDNA] were selected based on the Likelihood Ratio Test implemented in this software. Branch support values that approximate the posterior probability distribution of the corresponding branches were estimated by performing a minimum of 100 replicated metaGA searches that were stopped when the mean relative error (MRE) among 10 consecutive consensus trees remained below 5%. BA were carried out using the software MrBayes v.3.2.1 (Ronquist and Huelsenbeck, 2003). As in the MP analyses, gaps were coded as binary characters and included as a separate data partition in the matrix. A binary model (lset coding = variable) was applied to the coded gaps, whereas the previously selected models of sequence evolution, JC and HKY + G, were applied to the DNA partitions of *Hin*dIII and *Taq*I satDNAs, respectively. The analyses included two separate concurrent Monte Carlo Markov Chain (MCMC) runs, each composed of four chains (one cold, three heated). Each Markov chain was started from a random tree and run for up to 10^6 generations, sampling every 500 generations. Stationarity was assessed using the software Tracer v.1.5 (Rambaut and Drummond, 2009). Samples obtained during the first 25% generations were discarded as burn-in, and the remaining data were used to generate a majority-rule consensus tree where the percentage of samples recovering any particular clade of the consensus tree represented the clade's posterior probability.

Intraspecific nucleotide diversity (π) was estimated using DnaSP v. 5 (Librado and Rozas, 2009). Net average genetic distances between groups were calculated under the appropriate substitution model for each satDNA family (see above) with MEGA v. 5. Rates of *Hin*dIII and *Taq*I satDNAs evolution were determined according to the divergence times estimated for the four *Iberolacerta* species here investigated by Arribas et al. (2006).

The occurrence of genetic differentiation between the four species analyzed was assessed with the analysis of molecular variance (AMOVA) (Excoffier et al., '92) calculating Φ-statistics. This test was performed at two hierarchical levels to test how satDNAs sequence variability was distributed within species and among species, for both *Hin*dIII and *Taq*I satDNAs. The test was based on pair wise genetic distances between clones and performed as implemented in ARLEQUIN 2.000 (Schneider et al., 2000), using 1,000 permutations.

The repeats of the analyzed species were compared using satDNA Analyzer version 1.2 (Navajas-Pérez et al., 2007). This program allows the discrimination between shared and nonshared polymorphic sites. The program identifies polymorphic sites shared between two species when the same polymorphism is found in both species. When this occurs, we assume that these are ancestral sites that appeared before the split between the two species (Navajas-Pérez et al., 2005). By contrast, nonshared polymorphic sites are autapomorphies, representing different transitional stages in the process of intraspecific sequence homogenization and interspecific divergence. Under the assumption that concerted evolution is a time dependent process, the expected stages of transition during the spread of a variant repeat unit toward its fixation can be defined according to the model of Strachan et al. ('85). This is a method of partitioning the variation by analyzing the patterns of variation at each nucleotide site considered independently among all the repeats of a repetitive family when comparing a pair of species (Strachan et al., '85; Navajas-Pérez et al., 2007). This method examines the distribution of nucleotide sites among six stages (Classes I–VI) in the spread of variant repeats through the family and the species. Briefly, the Class I site represents complete homogeneity across all repeat units sampled from a pair of species, whereas Classes II, III, and IV represent intermediate stages in which one of the species shows a polymorphism. The frequency of the new nucleotide variant at the site considered is low in Class II and intermediate in Class III, while Class IV represents sites in which a mutation has replaced the progenitor base in most members of the repetitive family in the other species. Class V represents diagnostic sites in which a new variant is fully homogenized and fixed in all the members of one of the species while the other species retains the progenitor nucleotide. A Class VI site represents an additional step over the stage of Class V (new variants appear in some of the members of the repetitive family at a site fully divergent between the two species). The statistical significance (*P*-value) of the variation in the relative proportions of Strachan transitions stages among different interspecific comparisons was evaluated using chi-square heterogeneity tests that were performed in the interactive online calculator available at http://www.quantpsy.org/chisq/chisq.htm (Preacher, 2001).

Chromosome Analysis

For metaphase preparations, about 50 μl of blood were taken from *I. monticola* and *I. galani* individuals with a sterile heparinized syringe and cultured in CO_2 incubators using the culture conditions indicated by Ezaz et al. (2005). Metaphase preparations were obtained by exposing cell cultures to 75 ng/ml of Demecolcine (Sigma-Aldrich Co., St Louis, MO, USA) for 4 hr before harvesting (Ezaz et al., 2005). Cells were hypotonized in KCl 0.75 M for 30 min at 37°C, prefixed by adding several drops of freshly prepared methanol:acetic acid fixative (3:1), then fixed through three changes of fixative. Suspensions of fixed cells were dropped onto microscope slides and air dried at room temperature.

Fluorescence in situ hybridization (FISH) experiments were performed on metaphase preparations using (i) a telomeric probe

(TTAGGG)n produced by PCR according to Ijdo et al. ('91), and (ii) the probes obtained by PCR amplification of *Taq*I and *Hin*dIII satDNA clones. Telomeric and *Taq*I probes were also used in two-color FISH experiments. The probes were labeled by PCR either with biotin-16-dUTP (Roche) or digoxigenin-11-dUTP (Roche Diagnostics GmbH, Mannheim, Germany). Slide pretreatment, denaturation, hybridization, post-hybridization washes, and detection were performed according to Schwarzacher and Heslop-Harrison (2000). The *Hin*dIII satDNA and telomeric probes were evidenced with fluorescein iso-thyocianate (FITC) and tetramethyl rhodamine iso-thyocianate (TRITC), respectively. Chromosomes were observed with a Nikon Eclipse 800 epifluorescence microscope and the images were captured and processed with a Leica CytoVision version 7.2 system.

In order to define the relationships between satDNAs and the constitutive heterochromatin, C-banding was performed on metaphase plates following Sumner ('72). The relations between AT-rich heterochromatic regions and satDNAs were determined by staining C-banded metaphases with 4′,6-diamidino-2-phenylindole (DAPI) (Schweizer, '76).

RESULTS

Isolation and Characterization of Satellite DNAs

The digestion of *I. monticola* and *I. galani* genomic DNA with *Hin*dIII and *Taq*I restriction enzymes revealed bands corresponding to a monomer of a repetitive element of about 170 and 190 bp, respectively (not shown). PCR amplification using primers designed by aligning *I. monticola* and *I. galani* sequences of both satDNAs was successful in individuals representing the other two lineages of *Iberolacerta* recognized as distinct species (*I. martinezricai*, *I. cyreni*). The length of the 45 clones sequenced for *Hin*dIII ranged between 170 and 172 bp, whereas the length of the 42 clones sequenced for *Taq*I ranged between 171 and 188 bp (Table 1). Sequences of both satDNAs were deposited in GenBank (*Hin*dIII accession numbers: from KF453637 to KF453681; *Taq*I accession numbers: from KF453682 to KF453723). When *Hin*dIII and *Taq*I satDNA sequences were subjected to a BLASTN search, no significant similarities with sequences deposited in databases were found.

Southern blot analysis revealed hybridization of both satDNA probes onto *Iberolacerta monticola* digested genomic DNA with a ladder-like pattern, indicating the tandem arrangement of repeating units which is typical of satDNAs. A strong hybridization signal was also produced on the other three *Iberolacerta* species whit both *Hin*dIII and *Taq*I probes; this latter probe also produced a clear signal on the other lizards tested, whereas no signal appeared when *Hin*dIII probe was hybridized on representatives of the genera *Lacerta*, *Podarcis*, and *Timon* (not shown).

Quantitative dot blot analysis revealed that *Hin*dIII satDNA represents around 10% of *I. monticola* and *I. galani*, and 5% of *I. cyreni* and *I. martinezricai* genomes. *Taq*I satDNA represents 5% of *I. cyreni*, *I. galani*, and *I. monticola* genomes, and 2.5% in *I. martinezricai* (data not shown). The estimation of the number of repeats was not possible because the genome size of these lizards is not known.

The consensus sequences of the two satDNAs were very similar in the four *Iberolacerta* species, with an AT average content of 58.4% for *Hin*dIII and 60.3% for *Taq*I, indicating an enrichment in AT (Table 1). Both satellites repeats are characterized by the occurrence of short motifs such A and T stretches and dinucleotides steps TG and CA, with more numerous and longer A (T) stretches in *Taq*I satDNA (Fig. 1), as expected from its higher AT content. Within *Hin*dIII satDNA, two monomer variants or subfamilies (I and II) were detected in *I. galani* and *I. monticola* (Fig. 1A). The consensus sequences of subfamily I in both species were virtually identical to the consensus of *I. martinezricai*, whereas subfamily II showed several (nine) randomly distributed diagnostic nucleotide substitutions, as well as three exclusive indels located in the terminal region of the monomer. Both

Table 1. Summary of repeat features and π values.

	*Hin*dIII				*Taq*I			
Species	*n*	%AT	Repeat length	π	*n*	%AT	Repeat length	Nucleotide diversity (π)
I. cyreni	11	57.0	171	0.0055 ± 0.0022	9	60.2	186–187	0.0384 ± 0.0058
I. galani	13	58.9	170–171	0.0358 ± 0.0033	16	60.1	186–187	0.0475 ± 0.0070
I. galani (sfI)	6	59.4	171	0.0175 ± 0.0031				
I. galani (sfI)	7	58.5	170	0.0101 ± 0.0020				
I. monticola	10	59.0	170–171	0.0187 ± 0.0035	10	60.8	171–188	0.0569 ± 0.0062
I. monticola (sfI)	9	59.0	171	0.0062 ± 0.0019				
I. monticola (sfI)	1	58.8	170	–				
I. martinezricai	10	58.7	171–172	0.0105 ± 0.0052	7	60.1	187–188	0.0428 ± 0.0114

Number of monomeric repeats sequenced (*n*), nucleotide composition of repeats (AT), length of repeats (expressed in base pairs), and nucleotide diversity (π) ± SE for both satDNAs for each *Iberolacerta* species investigated. sfI: HindIII satDNA subfamily I; sfII: HindIII satDNA subfamily II

monomer variants were present in similar proportions in the sequence data set of *I. galani*, but only one out of ten sequences in *I. monticola* belonged to subfamily II (Table 1).

The phylogenetic tree obtained from the Bayesian analysis of *Hin*dIII satDNA is shown in Figure 2. The four different phylogenetic analyses (NJ, MP, ML, and BA) yielded very similar topologies, with some minor incongruences. Two major clades were recovered with maximum support, one harbouring *I. cyreni* clones and the other the sequences of the remaining three *Iberolacerta* species. Within this second cluster, monomers of subfamily II constitute a well-supported clade sister to that formed by sequences belonging to subfamily I, [with the exception of two clones from *I. galani* (IGA_32 and IGA_39) that share some private nucleotide substitutions]. Within subfamily I, relationships between most monomers were poorly resolved and they were not grouped according to the species of origin.

The Bayesian tree constructed using the sequences of *Taq*I satDNA was largely unresolved, regardless of the phylogenetic method employed, showing that this satellite cannot discriminate effectively the four *Iberolacerta* species here investigated (Fig. 3).

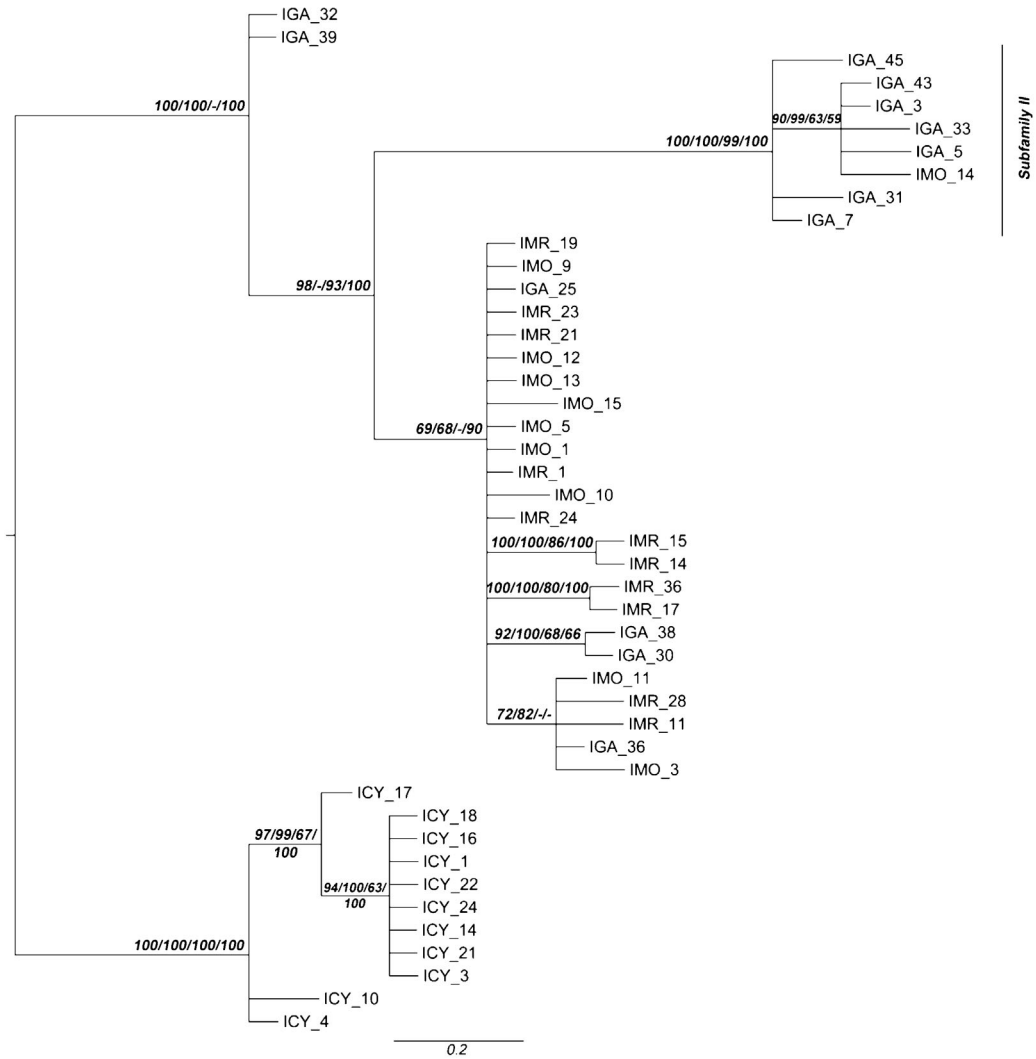

Figure 2. Bayesian phylogenetic tree depicting the the phylogenetic relationships between the 45 monomeric units of *Hin*dIII satDNA sequenced. Support values obtained by four different methods of analysis are shown at each node; from left to right: Bayes posterior probability (100×), metaGA branch support values (100×), NJ-bootstrap (%), and equally MP trees (%). A hyphen was inserted whenever a particular method did not support the Bayesian topology. Numbers after the species names are experimental number for clone identification. ICY: *Iberolacerta cyreni*; IGA: *Iberolacerta galani*; IMO: *Iberolacerta monticola*; IMR: *Iberolacerta martinezricai*.

Even though several well-supported subclusters including conspecific monomers were recognized, the number of diagnostic mutations shared by these sequences was too low to be considered species-specific *Taq*I satDNA subfamilies (not shown).

The π values indicated that intraspecific sequence heterogeneity is higher for *Taq*I satDNA (from 3.84% in *I. cyreni* to 5.69% in *I. monticola*) than for *Hin*dIII satDNA (from 0.55% in *I. cyreni* to 3.58% in *I. galani*) (Table 1). Interspecific mean net distances are low and similar for both satellites when *I. cyreni* is excluded from the analysis of *Hin*dIII satDNA (from 0.04% between *I. monticola* subfamily I and *I. martinezricai* to 5.60% between *I. galani* subfamily II and *I. martinezricai* for *Hin*dIII, and from 0.90% between *I. galani* and *I. martinezricai* to 1.30% between *I. monticola* and *I. galani* for *Taq*I satDNA) (Tables 2 and 3). Pair wise comparisons of *Hin*dIII satDNA involving *I. cyreni* and the other *Iberolacerta* analyzed, showed distance values substantially higher, between 8.40% and 13.90% (Table 2).

In addition, higher levels of sequence divergence were obtained in the comparisons between subfamilies I and II of *Hin*dIII satDNA in *I. galani* (4.5%) than in the comparisons between monomeric repeats belonging to subfamily I in different species (from 0.04% to 0.4%) (Table 2).

The evolutionary rate of these two satellites was then calculated based on sequence divergence between *I. cyreni* and the other three species, that were considered as a single taxonomic unit not being discriminated by either satellite. The values found are 1.2% for *Hin*dIII and 0.14% for *Taq*I, indicating an evolutionary rate almost 10-fold faster for the former.

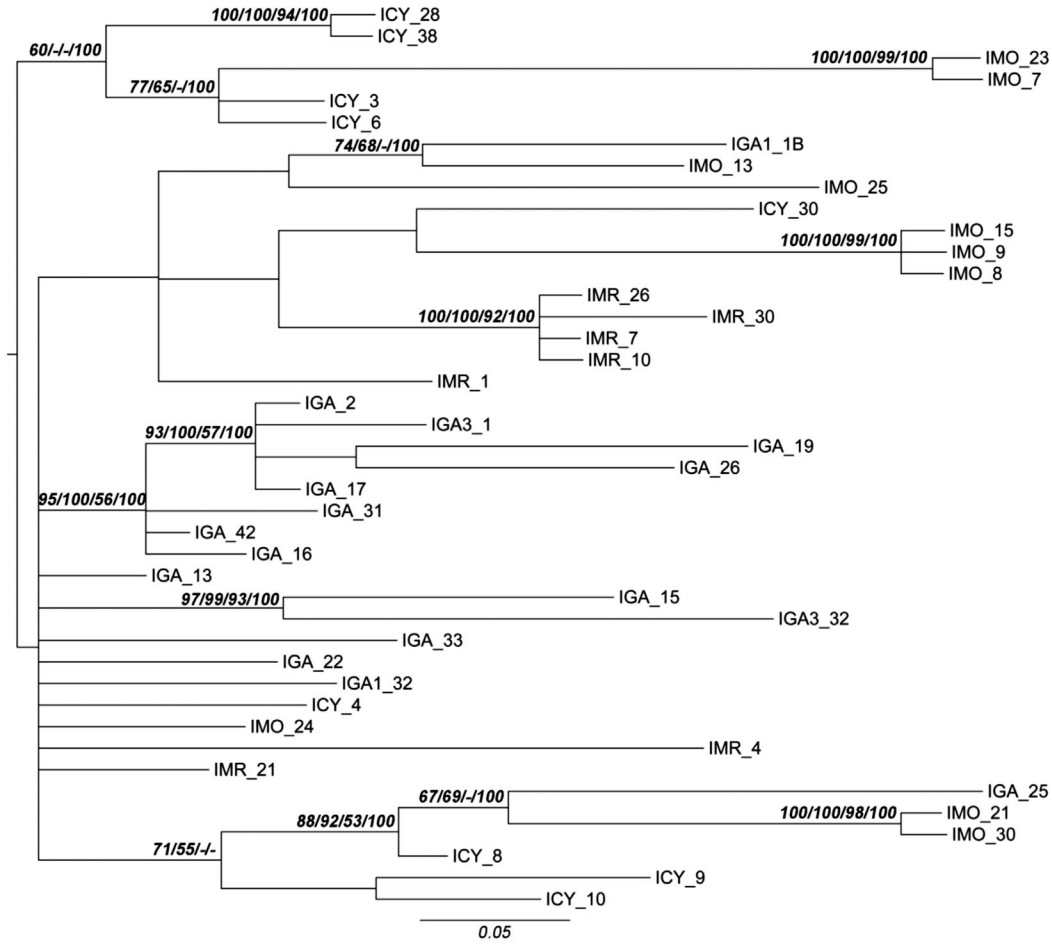

Figure 3. Bayesian phylogenetic tree depicting the the phylogenetic relationships between the 42 monomeric units of *Taq*I satDNA sequenced. Support values obtained by four different methods of analysis are shown at each node; from left to right: Bayes posterior probability (100×), metaGA branch support values (100×), NJ-bootstrap (%) and equally MP trees (%). A hyphen was inserted whenever a particular method did not support the Bayesian topology. Numbers after the species names are experimental number for clone identification. ICY: *Iberolacerta cyreni*; IGA: *Iberolacerta galani*; IMO: *Iberolacerta monticola*; IMR: *Iberolacerta martinezricai*.

Table 2. Interspecific and intersubfamily comparative analysis of *Hin*dIII repeats

Species comparison	SP (%)	Strachan sites II–III (%)	Strachan sites IV–VI (%)	Genetic distance
*Hin*dIII				
I. cyreni versus *I. galani* (sfI)	0 (0%)	4 (2.3%)	15 (8.8%)	0.0838 ± 0.0232
I. cyreni versus *I. galani* (sfII)	0 (0%)	5 (2.9%)	21 (12.3%)	0.1388 ± 0.0326
I. cyreni versus *I. monticola* (sfI)	0 (0%)	5 (2.9%)	16 (9.4%)	0.1025 ± 0.0265
I. cyreni versus *I. martinezricai*	0 (0%)	5 (2.9%)	15 (8.8%)	0.0996 ± 0.0258
I. galani (sfI) versus *I. monticola* (sfI)	1 (0.59%)	7 (4.1%)	1 (0.59%)	0.0038 ± 0.0025
I. galani (sfI) versus *I. martinezricai*	2 (1.2%)	6 (3.5%)	1 (0.59%)	0.0034 ± 0.0026
I. monticola (sfI) versus *I. martinezricai*	2 (1.2%)	4 (2.3%)	0 (0%)	0.0004 ± 0.0005
I. galani (sfII) versus *I. monticola* (sfI)	0 (0%)	8 (4.7%)	9 (5.3%)	0.0545 ± 0.0190
I. galani (sfII) versus *I. martinezricai*	0 (0%)	8 (4.7%)	9 (5.3%)	0.0555 ± 0.0192
I. galani (sfI) versus *I. galani* (sfII)	0 (0%)	6 (3.5%)	10 (5.8%)	0.0447 ± 0.0160

The table reports number and percentage of shared polymorphic sites (SP); variable nucleotide sites classified according to Strachan et al. ('85); net genetic distances (Jukes–Cantor method) in pair wise comparisons of species. sfI: *Hin*dIII satDNA subfamily I; sfII: *Hin*dIII satDNA subfamily II.

The poor phylogenetic differentiation of these species based on the sequences of the satDNAs here isolated was confirmed by AMOVA analysis. When this test was performed on the *Hin*dIII sequences, most of the percentage of the molecular variation was distributed among species (69.60%; Φ_{ST} 0.69596, $P < 0.0001$) whereas the percentage of variation within species was much lower, but still significant (30.40%; Φ_{ST} 0.69596, $P < 0.001$) (Table 4). The variance among species became much lower (32.07%; Φ_{ST} 0.32072, $P < 0.001$) and the one within populations became the preponderant variance component (67.93%; Φ_{ST} 0.32072, $P < 0.001$) when the sequences of *I. cyreni* were excluded from the analysis (Table 4). This result can be explained by the fact that *I. cyreni* was recovered as a distinct cluster with a high support in the phylogeny based on *Hin*dIII sequences, whereas the other three cannot be discriminated by this molecular marker. The AMOVA test carried out on *Taq*I satDNA sequences produced results very similar to those obtained with *Hin*dIII sequences after excluding *I. cyreni*, with a preponderant variance component distributed within species (82.69%; Φ_{ST} 0.17314, $P < 0.001$), confirming that this satDNA cannot effectively discriminate between these *Iberolacerta* species (Table 4). These results emerged also by analyzing the pattern of variation at each nucleotide position considered independently among all *Hin*dIII repeats (Table 2). Indeed, when comparing *I. cyreni* with the other species, a high percentage of Strachan sites belonging to the categories IV, V, and VI were found (average = 9.9%), while 5.1% of sites per repeat were Strachan transition stages (II + III), and no shared polymorphic sites were observed. Conversely, for *Taq*I satDNA sites of the classes IV–VI were very few (average = 0.5%) in all the comparison, while 20.7% of the sites represented Strachan stages II–III and an average of 4.1% were polymorphic sites (Table 3). According to the chi-square heterogeneity test, these differences in the relative proportions of Strachan transition stages between *Hin*dIII and *Taq*I satDNAs are highly significant ($P < 0.001$).

The relatively high degree of genetic differentiation detected in the analysis of sequence divergence between *Hin*dIII subfamily II

Table 3. Interspecific comparative analysis of *Taq*I repeats.

Species comparison	SP (%)	Strachan sites II–III (%)	Strachan sites IV–VI (%)	Genetic distance
*Taq*I				
I. cyreni versus *I. galani*	8 (4.3%)	51 (27.3%)	3 (1.6%)	0.0099 ± 0.0040
I. cyreni versus *I. monticola*	9 (4.8%)	25 (13.4%)	1 (0.5%)	0.0113 ± 0.0040
I. cyreni versus *I. martinezricai*	7 (3.7%)	25 (13.4%)	2 (1.1%)	0.0109 ± 0.0039
I. galani versus *I. monticola*	10 (5.3%)	43 (23%)	1 (0.5%)	0.0130 ± 0.0057
I. galani versus *I. martinezricai*	5 (2.7%)	57 (30.5%)	3 (1.6%)	0.0089 ± 0.0037
I. monticola versus *I. martinezricai*	7 (3.7%)	31 (16.6%)	2 (1.1%)	0.0114 ± 0.0040

The table reports number and percentage of shared polymorphic sites (SP); variable nucleotide sites classified according to Strachan et al. ('85); net genetic distances (maximum composite likelihood method) in pair wise comparisons of species.

Table 4. AMOVA analysis.

Source of variation	Variance components	Percentage of variation
Among species	4.21275	69.60
	1.07719	32.07
	1.14218	17.31
Within species	1.84035	30.40
	2.28152	67.93
	5.45487	82.69

The test was carried on *Hin*dIII satDNA sequences including the four species selected for this study (first line of each hierarchical level), and removing *Iberolacerta cyreni* from the analysis (second line of each hierarchical level). The test on *Taq*I satDNA sequences included all four of the *Iberolacerta* investigated (third line of each hierarchical level). Φ-statistics were highly significant in all comparisons ($P < 0.001$).

and subfamilies I from *I. galani*, *I. monticola*, and *I. martinezricai* was also evident in the comparisons of Strachan transition stages among these groups (Table 2). No shared polymorphisms were found and the number of sites falling in classes IV and V (between 5% and 6%) was significantly larger ($P < 0.001$) than the average frequency of these "differentiated sites" in the comparisons among subfamilies I in different species.

Chromosome Analysis

FISH experiments with *Hin*dIII satDNA probe on metaphase chromosomes of *I. galani* and *I. monticola* revealed that this repetitive element is widespread in the genome of these species, occurring at centromeres of all the 36 chromosomes of the diploid complement (Fig. 4A,B), with no differences between males and females. The occurrence of "bouquet" figures where chromosomes are linked together at the level of centromeres seems to indicate that this satDNA is involved in the interchromosome connection during mitosis (Fig. 4B). FISH with *Taq*I satDNA probe produced bright signals in interstitial position in a subset of 18 chromosomes in *I. galani* and 20 in *I. monticola*. No differences between males and females were detected with this probe either (Fig. 4C,D). Results of FISH experiments are consistent with the genomic abundance of *Hin*dIII and *Taq*I satDNAs as showed by quantitative dot blot analysis for these two species, with the former around twofold more abundant than the latter.

FISH with a telomeric probe (TTAGGG)n produced a fluorescent signal at telomeres of all the chromosomes. Besides telomeric signals, also interstitial telomeric sites (ITS) were marked in about five chromosome pairs. When a two-color FISH with both telomeric and *Taq*I satellite probes were performed, the fluorescent signals of ITS resulted distally located to the satellite ones (Fig. 4D).

C-banding, performed in order to assess the relationships between the isolated satellites and constitutive heterochromatin, revealed that in *Iberolacerta* the chromosomal distribution of *Hin*dIII satDNA overlaps the centromeric heterochromatic blocks, whereas *Taq*I probe colocalizes with pericentromeric heterochromatin (Fig. 4E,F).

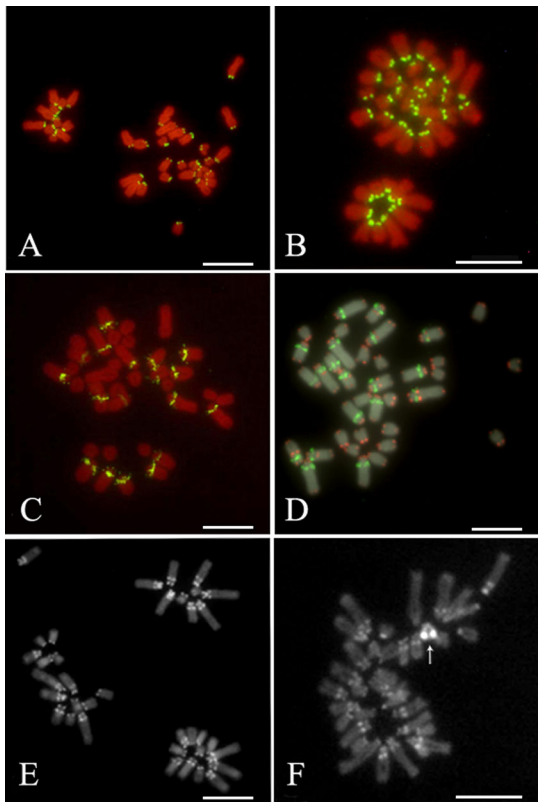

Figure 4. FISH with *Hin*dIII probe onto metaphases from females of *Iberolacerta galani* (**A**) and *I. monticola* (**B**). FISH with *Taq*I probe onto a metaphase of *I. galani* female (**C**). Two-color FISH with telomeric (red) and *Taq*I (green) probes on a metaphase of *I. monticola* female (**D**). C-banding on *I. monticola* male (**E**) and *I. galani* female (**F**) metaphases. The W chromosome of *I. galani* is indicated by an arrow.

DISCUSSION

Satellite DNAs represent rapidly evolving genomic elements, and therefore, even among most closely related species, they usually differ in nucleotide sequence, copy number, and/or composition of satellite families (Csink and Henikoff, '98). However, some satDNA families evolve more slowly than others and occur in several closely related species with different degrees of sequence similarity (Bachmann and Sperlich, '93; Mantovani et al., '97; Watabe et al., '97). Some satDNAs seem to be rather ancient and are widely distributed among higher taxa (Modi et al., 2004; Robles et al., 2004). Consequently, some satDNAs may be valuable

taxonomic identification tools while others might be useful for phylogenetic analyses at higher taxonomic levels. In the present study, we compared sequences of two different satDNA families (*Hin*dIII and *Taq*I) in four closely related lacertid species, allopatrically distributed in mountain areas of the Iberian Peninsula. These satDNAs seem to evolve at different rates in the studied lizards, with *Hin*dIII showing a 10-fold faster evolutionary rate than *Taq*I. Indeed, Southern blot analysis using *Iberolacerta* satellite probes revealed a clear hybridization pattern also in other lizard genera (namely, *Lacerta*, *Podarcis*, and *Timon*) only for *Taq*I repeats, whereas *Hin*dIII seems to be restricted to the genus *Iberolacerta*. However, a significant level of genetic divergence was detected only in comparisons involving *I. cyreni* when *Hin*dIII satDNA was considered. For this satDNA, analysis of turnover dynamics indicate the effectiveness of the molecular drive process, after species split, in the spreading of new sequence variants leading to intraspecific homogeneity (0.56% of sequence variation within *I. cyreni*) and interspecific divergence (around 9% of sequence divergence between *I. cyreni* and the other species), an evolutionary pattern known as concerted evolution (Dover, '82). The fact that the other species are scarcely differentiated at *Hin*dIII repeats can be interpreted in two alternative ways: (i) it may represent the outcome of the relatively recent (approximately 2 mya, Arribas et al., 2006) and rapid succession of speciation events within this group. In fact, previous molecular analyses based on nuclear and mitochondrial markers also failed to resolve the phylogenetic relationships or even track lineage splitting at this taxonomic level (Mayer and Arribas, 2003; Carranza et al., 2004; Crochet et al., 2004; Arribas et al., 2006; Arnold et al., 2007); (ii) the specific status for these three taxa might not have been reached yet. Indeed, estimation of divergence times among these three *Iberolacerta* species are similar to those recorded for different populations of the lizard *Podarcis muralis* that diverged genetically in separate refuges during glaciations, currently not showing evidence for reproductive isolation (Giovannotti et al., 2010).

The deep divergence observed between *I. cyreni* and the other *Iberolacerta* species here investigated with *Hin*dIII satellite is in good accordance with the molecular phylogenies published so far (Mayer and Arribas, 2003; Carranza et al., 2004; Crochet et al., 2004; Arribas et al., 2006; Arnold et al., 2007). This analysis showed that this species was the most diverged clade of the tree, with an estimated splitting time of about 7.5 million years. The relatively scarce representation of transitional stages (only 5% of the nucleotide positions) might suggest that the concerted evolution mechanisms have led to sequence differentiation between *I. cyreni* and the other species, probably due to the efficiency of the molecular-exchange homogenizing mechanisms among chromosomes.

The occurrence of two different types of monomeric variants or subfamilies was described for *Hin*dIII satDNA sequences. These subfamilies were defined according to a set of particular nucleotide substitutions or indels, in two of the four species examined. However, given the almost simultaneous speciation processes between *I. monticola*, *I. galani*, and *I. martinezricai*, it seems unlikely that subfamily II constitutes a specific variant of *I. monticola* and *I. galani*. An interspecific analysis of the pattern of nucleotide change was not possible for subfamily II due to the lack of a representative number of sequences in *I. monticola* or *I. martinezricai*. Even so, our results show that both subfamilies are presumably evolving independently, as indicated by the substantially high percentage of transitions stages IV and V between the monomers of subfamily II (*I. galani*) and the sequences of subfamily I, either belonging to *I. galani*, *I. monticola*, or *I. martinezricai*. The coexistence and divergent evolution of satellite subfamilies in the genomes of these species could be in agreement with the Nijman and Lenstra model (2001), in which mutations inhibiting the interactions of repeat units in a satellite family would lead to sequence diversification and the independent amplification or contraction of concurrent sequence variants. Nevertheless, a more extensive survey of *Hin*dIII satDNA will be the subject of further studies, in order to assess the presence and abundance of both monomeric variants in other *Iberolacerta* species, as well as to elucidate the processes driving the evolution of this satellite family.

Conversely to *Hin*dIII sequences, the tandem arrays of *Taq*I show a low sequence change rate when comparing *I. cyreni* with the other *Iberolacerta*. In fact, we detected a low rate of sequence change (0.1% per Myr), a rate 10-fold lower than that estimated for *Hin*dIII sequences (about 1.2% per Myr) and only 1.1% of Strachan stages IV–VI compared to 18% of II–III stages. In addition, we also observed some shared polymorphic sites and a comparatively higher intraspecific heterogeneity, suggesting that most of the intraspecific variability in each species is ancestral, originated prior to the separation of these lineages; moreover, the high number of transitional stages of differentiation (Strachan stages II–III) suggest that after the allopatric isolation, processes of concerted evolution were less efficient than in the *Hin*dIII repeats. In addition, contrarily to *Hin*dIII, Southern hybridization with *Taq*I probe produced a clear signal also in other lacertid genera, like *Lacerta*, *Podarcis*, and *Timon*, also suggesting a strong conservation of this satellite DNA family.

Various factors were invoked to explain different evolutionary turnover rates between satDNA families, like interchromosomal and intrachromosomal recombination rates, copy number, array size and structure, chromosomal distribution, chromosomal structure, population size, divergence time and reproductive mode. Moreover, evolutionary conservation of satDNA repeats might be a likely indication of functional constraints and natural selection (see Plohl et al., 2008). Unfortunately, very few examples are found in the literature with both fast-evolving and slow-evolving satDNAs found within the same species. For instance, in the genus *Dolichopoda*, a comparison among three satDNA families showed a trend of sequence variability and copy number

being positively correlated, and a trend of sequence variability and length of repeats being negatively correlated (Martinsen et al., 2009). Like in *Dolichopoda*, it seems that also in the studied lizards an increase in copy number is linked to a trend of sequence homogenization. In fact, it was observed that *Hin*dIII repeats represent between 5% and 10% of the *Iberolacerta* genome, while *Taq*I satDNA between 2.5% and 5%. The different chromosome localization of the two satellites may also play a role in the different rate of sequence homogenization recorded for the two satDNA families. First of all, it should be noted that *Hin*dIII repeats are centromerically located on all the acrocentric chromosomes of *I. galani* and *I. monticola* karyotypes. In fact, it is reported that satellite DNAs at centromeres of acrocentric chromosomes show greater homology and a higher rate of homogenization than in noncentromeric locations or nonacrocentric chromosomes (Jantsch et al., '90; Bandyopadhyay et al., 2001). It has been hypothesized that homogenization occurs through physical association and crossing-over between nonhomologous chromosomes (Ohno et al., '61). Indeed, acrocentric chromosomes associate at the heterochromatic regions during meiotic prophase and somatic interphase (Schmid et al., '83; Tuck-Muller et al., '84; Kuznetsova et al., 2007) and we also observed typical "bouquet" figures, where chromosomes are linked together at the level of centromeres (Fig. 4B). This process may be the most important mechanisms for spontaneous chromosomal mutation, concerted evolution, and homogenization of satellite subfamilies of DNA among acrocentric chromosomes (Maeda and Smithies, '86).

Conversely, *Taq*I repeats are pericentromerically located on a lower number of chromosomes (10 pairs in *I. monticola* and 9 in *I. galani*). In this case, we could explain the low homogenization rate within single species in terms of primary rate of the homogenization process. That is, it is possible that the exchange between nonhomologous chromosomes having *Taq*I sequences is limited. The *Taq*I repeats are indeed restricted to a subset of chromosomes in these species and located in a pericentromeric position less prone to physical association: this could reduce interchromosomal exchange and homogenization, thus determining a lower rate of interspecific divergence and a higher degree of intraspecific repeat heterogeneity. Similar considerations were reported for satDNAs of *Rumex*, where repeats in nonrecombining Y chromosomes show low rates of concerted evolution and intraspecific variability increase with no interspecific divergence (Navajas-Pérez et al., 2009; see also Kuhn et al., 2008), and to explain the lower mutation rate of satDNAs in sturgeons as compared to sparids. In fact, the more symmetrical karyotypes of these latter fishes would represent no physical barrier to interchromosomal exchange (de la Herrán et al., 2001a,b). However, also these AT-rich pericentromeric repeats could represent chromosome sites favoring spontaneous rearrangements. Indeed, we observed that the majority of the *Taq*I repeats are flanked by interstitial telomeric sequences that would insert in these chromosome points during the repair of double strand breaks (see Bolzán and Bianchi, 2006). These unstable sequences might explain the high rate of Robertsonian translocation observed in Pyrenean *Iberolacerta* (Odierna et al., '96).

In conclusion, our study suggests the effect of differential location and repeat copy number in the evolution of satDNAs, revealing features that could also improve the use of this genomic component as a molecular marker in phylogenetic analyses. Moreover, these results indicate that some molecular markers should be used cautiously in species identification when divergence times are shallow among the taxa compared.

ACKNOWLEDGMENTS

Special thanks are given to Pedro Galán for providing the samples of *Iberolacerta monticola* and *I. galani* used in this study. All authors declare that no conflict of interests exists for this manuscript.

LITERATURE CITED

Arnold EN, Arribas O, Carranza S. 2007. Systematics of the palaearctic and oriental lizard tribe Lacertini (Squamata: Lacertidae: Lacertinae), with descriptions of eight new genera. Zootaxa 1430:1–86.

Arribas O. 1999. Taxonomic revision of the Iberian "Archaeolacertae" 2: diagnosis, morphology, and geographic variation of "*Lacerta*" *aurelioi* Arribas, 1994 (Squamata: Sauria: Lacertidae). Herpetozoa 11:155–180.

Arribas O, Carranza S, Odierna G. 2006. Description of a new endemic species of mountain lizard from Northwestern Spain: *Iberolacerta galani* sp. nov. (Squamata: Lacertidae). Zootaxa 1240:1–55.

Bachmann L, Sperlich D. 1993. Gradual evolution of a specific satellite DNA family in *Drosophila ambigua*, *D. tristis*, and *D. obscura*. Mol Biol Evol 10:647–659.

Bandyopadhyay R, McQuillan C, Page SL, Choo KHA, Shaffer LG. 2001. Identification and characterization of satellite III subfamilies to the acrocentric chromosomes. Chromosome Res 9:223–233.

Bolzán AD, Bianchi MS. 2006. Telomeres, interstitial telomeric repeat sequences, and chromosomal aberration. Mutat Res 612:189–214.

Cafasso D, Cozzolino S, Chinali G, De Luca P. 2003. An unusual satellite DNA from *Zamia paucijuga* (Cycadales) characterised by two different organisations of the repetitive unit in the plant genome. Gene 311:69–77.

Capriglione T. 2000. Repetitive DNA as a tool to study the phylogeny of cold-blooded vertebrates. Chromosomes Today 13:183–194.

Capriglione T, Olmo E, Odierna G, Smith DI, Miller OJ. 1989. Genome composition and tandemly repetitive sequence at some centromeres in the lizard *Podarcis s. sicula* Raf. Genetica 79:85–91.

Capriglione T, Cardone A, Odierna G, Olmo E. 1991. Evolution of a centromeric satellite DNA and phylogeny of lacertid lizards. Comp Biochem Physiol B 100:641–645.

Capriglione T, Cardone A, Odierna G, Olmo E. 1994. Further data on the occurrence and evolution of satellite DNA families in the lacertid genome. Chromosome Res 2:327–330.

Capriglione T, De Santo MG, Odierna G, Olmo E. 1998. An alphoid-like satellite DNA sequence is present in the genome of lacertid lizards. J Mol Evol 46:240–244.

Carranza S, Arnold EN, Amat F. 2004. DNA phylogeny of *Lacerta* (*Iberolacerta*) and other lacertine lizards (Reptilia: Lacertidae): did competition cause long-term mountain restriction? Syst Biodivers 2:57–77.

Charlesworth B, Sniegowski P, Stephan W. 1994. The evolutionary dynamics of repetitive DNA in eukaryotes. Nature 371:215–220.

Ciobanu D, Roudykh IA, Grechko VV, Kramerov DA. 2001. Molecular mechanisms of satellite DNA evolution in a group of closely related Caucasian rock lizards. In: Piruzian E, Volkova L, editors. International Symposium Molecular Mechanisms of Genetic Processes and Biotechnology, Moskow [Thesis]. p 26.

Ciobanu D, Grechko VV, Darevsky IS. 2003. Molecular evolution of satellite DNA CLsat in lizards from the genus *Darevskia* (Sauria: Lacertidae): correlation with species diversity. Russ J Genetics 39:1527–1541.

Ciobanu DG, Grechko VV, Darevsky IS, Kramerov DA. 2004. New satellite DNA in *Lacerta* s. str. lizards (Sauria: Lacertidae): evolutionary pathways and phylogenetic impact. J Exp Zool 302B:505–516.

Crochet PA, Chaline O, Surget-Groba Y, Debain C, Cheylan M. 2004. Speciation in mountains: phylogeography and phylogeny of the rock lizards genus *Iberolacerta* (Reptilia: Lacertidae). Mol Phylogenet Evol 30:860–866.

Csink AK, Henikoff S. 1998. Something from nothing: the evolution and utility of satellite repeats. Trends Genet 14:200–204.

Dawe RK, Henikoff S. 2006. Centromeres put epigenetics in the drivers's seat. Trends Biochem Sci 31:662–669.

de la Herrán R, Fontana F, Lanfredi M, et al. 2001a. Slow rates of evolution and sequence homogenization in an ancient satellite DNA family of sturgeons. Mol Biol Evol 18:432–436.

de la Herrán R, Ruiz Rejon, Ruiz C, Rejon M, Garrido-Ramos MA. 2001b. The molecular phylogeny of the Sparidae (Pisces, Perciformes) based on two satellite DNA families. Heredity 87:691–697.

Dover GA. 1982. Molecular drive: a cohesive mode of species evolution. Nature 299:111–117.

Excoffier L, Smouse P, Quattro J. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131:479–491.

Ezaz T, Quinn AE, Miura I, et al. 2005. The dragon lizard *Pogona vitticeps* has ZZ/ZW micro-sex chromosomes. Chromosome Res 13:763–776.

Feliciello I, Picariello O, Chinali G. 2006. Intra-specific variability and unusual organization of the repetitive units in a satellite DNA from *Rana dalmatina*: molecular evidence of a new mechanism of DNA repair acting on satellite DNA. Gene 383:81–92.

Giovannotti M, Nisi Cerioni P, Caputo V, Olmo E. 2009. Characterization of a GC-rich telomeric satellite DNA in *Eumeces schneideri* Daudin (Reptilia, Scincidae). Cytogenet Genome Res 125:272–278.

Giovannotti M, Nisi Cerioni P, Caputo V. 2010. Mitochondrial DNA sequence analysis reveals multiple Pleistocene glacial refugia for *Podarcis muralis* (Laurenti, 1768) in the Italian Peninsula. Ital J Zool 77:277–288.

Grechko VV, Ciobanu DG, Darevsky IS, Kramerov DA. 2005. Satellite DNA of lizards of the genus *Lacerta* s. str. (the group *L. agilis*), the family Lacertidae. Dokl Biochem Biophys 400:44–47.

Grechko VV, Ciobanu DG, Darevsky IS, Kosushkin SA, Kramerov DA. 2006. Molecular evolution of satellite DNA repeats and speciation of lizards of the genus *Darevskia* (Sauria: Lacertidae). Genome 49:1297–1307.

Helaers R, Milinkovitch MC. 2010. MetaPIGA v2.0: maximum likelihood large phylogeny estimation using the metapopulation genetic algorithm and other stochastic heuristics. BMC Bioinformatics 11:379.

Ijdo JW, Wells RA, Baldini A, Reeders ST. 1991. Improved telomere detection using a telomere repeat probe (TTAGGG)n generated by PCR. Nucleic Acids Res 19:4780.

Jantsch M, Hamilton B, Mayr B, Schweizer D. 1990. Meiotic chromosome behavior reflects levels of sequence divergence in *Sus scrofa domestica* satellite III DNA. Chromosoma 99:330–335.

King LM, Cummings MP. 1997. Satellite DNA repeat sequence variation is low in three species of burying beetles in the genus *Nicroporus* (Coleoptera: Silphidae). Mol Biol Evol 14:1088–1095.

Kuhn GCS, Franco FF, Manfrin MH, Moreira-Filho O, Sene FM. 2007. Low rates of homogenization of the DBC-150 satellite DNA family restricted to a single pair of microchromosomes in species from the *Drosophila buzzatii* cluster. Chromosome Res 15:457–469.

Kuhn GCS, Sene FM, Moreira-Filho O, Schwarzacher T, Heslop-Harrison JS. 2008. Sequence analysis, chromosomal distribution and long-range organization show that rapid turnover of new and old pBuM satellite DNA repeats leads to different patterns of variation in seven species of the *Drosophila buzzatii* cluster. Chromosome Res 16:307–324.

Kuznetsova IS, Enukashvily NI, Noniashvili EM, et al. 2007. Evidence for the existence of satellite DNA-containing connection between metaphase chromosomes. J Cell Biochem 101:1046–1061.

Larkin MA, Blackshields G, Brown NP, et al. 2007. ClustalW and ClustalX version 2.0. Bioinformatics 23:2947–2948.

Librado P, Rozas J. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25:1451–1452.

Luchetti A, Cesari M, Carrara G, et al. 2003. Unisexuality and molecular drive: Bag320 sequence diversity in *Bacillus* taxa (Insecta Phasmatodea). J Mol Evol 56:587–589.

Mace GM. 2004. The role of taxonomy in species conservation. Philos Trans R Soc Lond B 359:711–719.

Maeda N, Smithies O. 1986. The evolution of multigene families: human haptoglobin genes. Annu Rev Genet 20:81–108.

Mantovani B, Tinti F, Bachmann L, Scali V. 1997. The Bag320 satellite DNA family in *Bacillus* stick insects (Phasmatodea): different rates of molecular evolution of highly repetitive DNA in bisexual and parthenogenic taxa. Mol Biol Evol 14:1197–1205.

Martinsen L, Venanzetti F, Johnsen A, Sbordoni V, Bachman L. 2009. Molecular evolution of the *pDo500* satellite DNA family in *Dolichopoda* cave crickets (Rhaphidophoridae). BMC Evol Biol 9:1–14.

Mayer W, Arribas O. 2003. Phylogenetic relationships of the European lacertid genera *Archaeolacerta* and *Iberolacerta* and their relationships to some other 'Archaeolacertae' (*sensu lato*) from Near East, derived from mitochondrial DNA sequences. J Zool Syst Evol Res 41:157–161.

Miller WJ, Nagel A, Bachmann J, Bachmann L. 2000. Evolutionary dynamics of the SGM transposon family in the *Drosophila obscura* species group. Mol Biol Evol 17:1597–1609.

Modi WS, Ivanov S, Gallagher DS. 2004. Concerted evolution and higher-order repeat structure of the 1.709 (satellite IV) family in bovids. J Mol Evol 58:460–465.

Navajas-Pérez R, de la Herrán R, Jamilena M, et al. 2005. Reduced rates of sequence evolution of Y-linked satellite DNA in *Rumex* (Polygonaceae). J Mol Evol 60:391–399.

Navajas-Pérez R, Ruiz Rejón M, Garrido-Ramos MA, Aznarte JL, Rubio-Escudero C. 2007. SatDNA Analyzer: a computing tool for satellite-DNA evolutionary analysis. Bioinformatics 23:767–768.

Navajas-Pérez R, Quesada del Bosque ME, Garrido-Ramos MA. 2009. Effect of location, organization, and repeat-copy number in satellite-DNA evolution. Mol Genet Genomics 282:395–406.

Nijman IJ, Lenstra JA. 2001. Mutation and recombination in cattle satellite DNA: a feedback model for the evolution of satellite repeats. J Mol Evol 52:361–371.

Odierna G, Aprea G, Arribas O, et al. 1996. The karyology of the Iberian rock lizards. Herpetologica 52:542–550.

Ohno S, Trujillo JM, Kaplan WD, Kinosita R. 1961. Nucleolus-organizer in the causation of chromosomal abnormalities. Lancet 2:123–126.

Plohl M. 2010. Those mysterious sequences of satellite DNAs. Periodicum 112:403–410.

Plohl M, Luchetti A, Meštrovi N, Mantovani B. 2008. Satellite DNAs between selfishness and functionality: structure, genomics and evolution of tandem repeats in centromeric (hetero) chromatin. Gene 409:72–82.

Preacher KJ. 2001. Calculation for the chi-square test: an interactive calculation tool for chi-square tests of goodness of fit and independence [Computer software]. Available online at: http://quantpsy.org [Accessed February 13, 2013].

Rambaut A, Drummond AJ. 2009. Tracer version 1.5 [computer program]. Available online at: http://beast.bio.ed.ac.uk.

Robles F, de la Herrán R, Ludwig A, et al. 2004. Evolution of ancient satellite DNAs in sturgeon genomes. Gene 338:133–142.

Ronquist F, Huelsenbeck JP. 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574.

Rudd MK, Wray GA, Willard HF. 2006. The evolutionary dynamics of alpha-satellite. Genome Res 16:88–96.

Salvador A. 1985. Guia de campo de los anfibios y reptiles de la Peninsula Iberica, islas Baleares y Canarias. León, Spain: Santiago García Ed.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Schmid M, Grunert D, Haaf T, Engel W. 1983. A direct demonstration of somatically paired heterochromatin of human chromosomes. Cytogenet Cell Genet 36:554–561.

Schneider S, Roessli D, Excoffier L. 2000. Arlequin, version 2.000: a software for population genetic data analysis. Geneva, Switzerland: Genetics and Biometry Laboratory. University of Geneva.

Schwarzacher T, Heslop-Harrison P. 2000. Practical in situ hybridization. New York, NY: Springer-Verlag.

Schweizer D. 1976. Fluorescent chromosome banding in plants: applications, mechanisms and implications for chromosome structure. In: Davies DR, Hopwood AP, editors. The plant genome. Proc 4th John Innes Symposium. Norwich, UK: John Innes Charity. p 61–72.

Sindaco R, Jeremcenko VK. 2008. The reptiles of the Western Paleartic. Latina, Italy: Edizioni Belvedere.

Smith GP. 1976. Evolution of repeated DNA sequences by unequal crossover. Science 191:528–535.

Stephan W, Cho S. 1994. Possible role of natural selection in the formation of tandem repetitive noncoding DNA. Genetics 136:333–341.

Strachan T, Webb D, Dover G. 1985. Transition stages of molecular drive in multiple-copy DNA families in *Drosophila*. EMBO J 4:1701–1708.

Sumner AT. 1972. A simple technique for demonstrating centromeric heterochromatin. Exp Cell Res 75:304–306.

Swofford DL. 2002. PAUP* Phylogenetic Analysis Using Parsimony (*and other methods), Version4.0b10a. Sunderland, MA: Sinauer Associates.

Tamura K, Peterson D, Peterson N, et al. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739.

Tuck-Muller CM, Bordson BL, Varela M, Bennett JW. 1984. NOR associations with heterochromatin. Cytogenet Cell Genet 38:165–170.

Walsh JB. 1987. Persistence of tandem arrays: implications for satellite and simple-sequence DNAs. Genetics 115:553–567.

Watabe H, Bachmann L, Haring E, Sperlich D. 1997. Taxonomic and molecular studies on *Drosophila sinobscura* and *D. hubeiensis*, two sibling species of the *D. obscura* group. J Zool Syst Evol Res 35:81–94.