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(Article begins on next page)

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Keywords:	satDNA, W-specific repeats, FISH, sex chromosome differentiation

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Characterization of a satellite DNA in the genera *Lacerta* and *Timon* (Reptilia, Lacertidae) and its role in the differentiation of the W chromosome.

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Seven figures and four tables.

Abbreviated title: Satellite DNA in *Lacerta* and *Timon*.

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Abstract

In this study, IMO-TaqI satDNA, previously isolated in several species of Lacertidae, was isolated and characterized from four species of the genus *Lacerta* and three of the genus *Timon* with the aim of gaining further insights into its evolutionary dynamics, its occurrence among lacertids and to understand if it plays any role in sex chromosome evolution in these seven species. The results here obtained highlighted the presence of this genomic element in the genome of all the species investigated, thus indicating that IMO-TaqI satDNA is evolutionary conserved among a wide variety of lacertids. In addition, this element was found as very abundant in the constitutive heterochromatin of the W-sex chromosome of the four *Lacerta* species investigated. The occurrence of IMO-TaqI satDNA on *Lacerta* heterochromosome suggests that it is involved in the differentiation of the W chromosome by heterochromatinisation, and the fact that it is absent in the W of other lacertids investigated seems to confirm that repetitive DNA sequences would remain randomly trapped into the sex chromosomes, undergoing amplification as a consequence of the suppression of recombination.

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Key words: satDNA, W-specific repeats, FISH; sex chromosome differentiation.

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Introduction

Satellite DNAs (satDNAs) are tandem head-to-tail arranged highly repetitive DNA sequences located in the constitutive heterochromatin (Charlesworth et al., 1994) at centromeric, pericentromeric and/or subtelomeric chromosomal positions, and in few cases they have been detected interstitially on chromosomes (e.g., Plohl et al., 2012). Many thousands of copies of satDNA monomers occur in a genome and the repeats belonging to a satellite-DNA family do not evolve independently of one another but evolve as a unit, following a process named concerted evolution (e.g., Plohl et al., 2008). This process is influenced by rates of transfer between homologous and non-homologous chromosomes, arrangement of repeats, array sizes, and population structure. Alteration of any of these factors can interfere with rates of concerted evolution. Therefore, reduced rates of concerted evolution would be expected in satDNA sequences in the non-recombining W or Y chromosomes (e.g. Navajas-Pérez et al., 2009) or depending on the location of satDNA clusters on the chromosomes (e.g. terminal position *vs* interstitial position; see Giovannotti et al., 2014).

Although satDNAs are abundant genomic elements in eukaryotes, they have been extensively studied in only few taxa such as insects (Palomeque and Lorite, 2008), fish (e.g., Vicari et al., 2010) and mammals (Enukashvily and Ponomartsev, 2013), whereas scarce information is available for other organisms. Several studies on satDNA have been carried out also in squamate reptiles. In this taxon, satDNAs have been isolated and characterized in skinks (Giovannotti et al., 2009, 2013), snakes (Singh et al., '76; Matsubara et al., 2015a), monitor lizards (Chaiprasertsri et al., 2013; Prakhongcheep et al., 2017) and lacertids (Capriglione et al., '89, '91, '94, '98, 2000; Ciobanu et al., 2001, 2003, 2004; Grechko et al., 2005, 2006; Giovannotti et al., 2014; Rojo et al., 2015), with this latter taxon being the most studied squamates with reference to satDNAs so far.

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3 The familiy Lacertidae consists of about 42 genera (Vitt and Caldwell, 2014)
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5 including 323 species (Uetz et al., 2017) distributed in most of Africa, Europe, and Asia
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7 southward into the northern East Indies (Vitt and Caldwell, 2014). According to Hipsley et al.
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9 (2009) modern lacertids arose around 60 million years ago and underwent quite rapid
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11 evolutionary diversification splitting into two subfamilies, namely Gallotiinae and Lacertinae
12
13 (this latter including Lacertini and Eremiadiini tribes). Green lizards belonging to *Lacerta*
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15 *sensu stricto* (tribe Lacertini) form a monophyletic clade (Harris, '99; Godinho et al., 2005;
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17 Arnold et al., 2007; Marzahan et al., 2016) and have a distribution range that extends from
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19 western Europe to central Asia and include several species whose taxonomic relationships are
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21 still not completely resolved (see Godinho et al., 2005). These lizards are characterized by the
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23 typical lacertid karyotype (36 uniarmed macrochromosomes plus 2 microchromosomes) with
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25 sex chromosomes of the ZZ/ZW type (e.g., Olmo and Signorino, 2005). *Timon* is a small
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27 genus comprising six currently recognized species that it is distributed across the eastern and
28
29 western Mediterranean (see Ahmadzadeh et al., 2016). The karyotype of *Timon* is composed
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31 of 36 chromosomes (34 macro- plus two microchromosomes) with a metacentric pair 1 and a
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33 chromosomal mechanism of sex determination of the ZZ/ZW type. (e.g., Olmo and Signorino,
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35 2005). In both *Lacerta* and *Timon* the W chromosome is almost completely heterochromatic
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37 (see Olmo et al., '93).
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41 Despite a substantial uniformity in the karyotypes of lacertids and a widespread
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43 diffusion of a Genetic Sex Determination (GSD) system with ZW heterogamety (e.g., Olmo
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45 and Signorino, 2005), the W chromosome exhibits different morphologies, interpreted as
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47 different stages of evolution in an almost linear trend (e.g., Olmo et al., '87). According to
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49 Olmo et al. (e.g., '87, '90) heterochromatin could have played a paramount role in the
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51 differentiation of sex chromosomes, starting with the storage of a specific highly repetitive
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53 DNA on either homologues accompanied by heterochromatinization of a homomorphic and
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heterochromatic W chromosome, with a mechanism similar to that of snakes. In fact, Capriglione et al. ('94) speculated that this early stage of W differentiation would have replication and spiralization cycles different from that of Z chromosome, thus hampering the recombination. The next step in this "linear model" would be a progressive degeneration of the W, transforming it in a microchromosome (Olmo et al., '87; Odierna et al., '93). A deviation from that linear trend would be represented by some populations of *Zootoca vivipara* and the Pyrenean *Iberolacerta*, where original W would be fused with an autosome, giving rise to a Z_1Z_2W system (Odierna et al., '96; Odierna et al., 2004). Despite this wide theoretical background, only for three lacertid species data on the composition of heterochromatin in the W chromosome is reported. Two cases refer to the eremiadine *Eremias velox* and *Acanthodactylus lineomaculatus*. In the former species Pokorná et al. (2011) observed an enrichment in some microsatellite sequences on either the whole W chromosome (repeated motif: A, C, TA, CAA, CAG, CAT, GAC, GAG, TAA, TAC) or its centromeric region (repeated motif: CA, GC, GA), whereas in the latter species Giovannotti et al. (in press) observed and enrichment in telomeric-like sequences of the W-chromosome heterochromatin. Also in the lacertine *Lacerta agilis* the (micro-)W chromosome was found to be enriched in telomeric repeats (Srikulnath et al., 2014; Matsubara et al. 2015b).

IMO-TaqI satDNA is widespread in the lacertid genome (Capriglione, '91; Giovannotti et al., 2014, Rojo et al. 2015) and it was detected in the genome of *Lacerta bilineata* and *Timon lepidus* by Southern blot analyses by Giovannotti et al. (2014) but it was neither isolated nor characterized. In the present study, we isolated this genomic element from the genome of species representative of the genera *Lacerta* and *Timon* in order to characterize it and to assess its possible involvement in the differentiation of W sex chromosome and if W-specific repeats undergo a reduced rate of homogenisation compared to those of the autosomes.

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Materials and methods

Animals, DNA extraction, isolation and analyses of IMO-TaqI satDNA repeats

One female and one male of *Lacerta agilis, L. bilineata, L. strigata, Timon lepidus, T. pater*, *T. tangitanus* and a female of *L. trilineata* were used in this study (Table 1). Genomic DNA was extracted from whole blood, using standard protocols with proteinase K digestion followed by phenol/chloroform extraction (see Sambrook et al., '89). Individuals of *Lacerta, T. lepidus* and *T. pater* were made available by private breeders. Individuals of *T. tangitanus* were collected at Oukaimden in the Central High Atlas (31°12'N- 7°52'W). Permissions for fieldwork and ethics approval of experimental procedures were issued by the High Commissariat for Water and Forest (Decision Number 05/2013 HCEFLCD/DLCDPN/DPRN/CFF) - Morocco.

A pair of degenerate primers were designed to PCR-amplify IMO-TaqI satDNA repeats in all the above species: TaqI-F: 5'-AAATTCTGACCSYGSGGGTTAG-3'; TaqI-R: 5'-AAAATVGTGCCAAACTGTTG-3'. PCR products were run in 2% agarose gels, and the band corresponding to the amplified monomers was 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) following manufacturer's recommendations. Clones of IMO-TaqI were sequenced on an ABI PRISM 3730XL (Applied Biosystems) automatic sequencer. These sequences were then aligned using the MAFFT v7.0 online server with the L-INS-i option (Katoh and Standley, 2013). . In order to compare IMO-TaqI satDNA with other satDNAs, a BLAST (Altschul et al., '90) search was performed against the "nucleotide collection (nr/nt)" database at NCBI (http://www.ncbi.nlm.nih.gov/).

Digoxigenin labelled probes were produced by PCR amplification of single clones and used in Southern hybridisation experiments to verify that the elements isolated were tandemly

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arranged, as expected for satDNAs. In these experiments, TaqI digested genomic DNAs from *Lacerta* and *Timon* were used. The hybridization with the digoxigenin-labelled satDNA probes was performed at 50°C overnight with the Sure Blot CHEMI Hybridisation and Detection Kit (Intergen) following the manufacturer's recommendations. The hybridisation was detected with the same kit.

Analyses of IMO-TaqI genetic variability

Maximum Likelihood (ML) and Bayesian (BA) analyses were applied to infer the phylogenetic relationships among the sequences of IMO-TaqI from the species analysed. The best fit model of nucleotide substitution for IMO-TaqI repeats was selected among the 88 models available in jModeltest 2.1.3 (Darriba et al., 2012) using the Akaike Information Criterion (AIC). The most appropriate model was GTR + G. ML analysis was performed in MetaPIGA v3.1 (Helaers and Milinkovitch, 2010) using the metapopulation genetic algorithm (metaGA) with probability consensus pruning among four populations of four individuals each (Lemmon and Milinkovitch, 2002). Prior to running the analysis, the alignment was checked for sequence redundancy (only one sequence kept for each group of identical sequences) and automated trimming of poorly aligned regions using the trimAl algorithm (Capella-Gutierrez et al., 2009) applied as implemented in MetaPIGA. 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%. MetaGA values ≥70% were considered as statistically significant. BA analysis was carried out with MrBayes v3.2 (Ronquist et al., 2012) using the appropriate model of nucleotide substitution (GTR + G) selected with jModeltest 2.1.3. The alignment checked for redundancy and subject to automated trimming in MetaPIGA was used also for this analysis. The BA analysis was run with four incrementally heated Markov chains for 10×10^6

generations in two independent runs with samplings at intervals of 500 generations that produced 20000 trees. Once the stationarity had been reached, both in terms of likelihood scores and parameter estimation, 5×10^3 trees (25% 'burn-in') were discarded in both runs and a majority-rule consensus tree was generated from the 15 x 10^3 remaining (post burn-in) trees. The posterior probability (pp) was calculated as the percentage of samples recovering any particular clade (Huelsenbeck and Ronquist, 2001), with pp ≥ 95% indicating a statistically significant support (Wilcox et al., 2002). Both ML and BA trees were edited using FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

Average AT content of the monomeric unit of IMO-TaqI was determined with MEGA version 5 (Tamura et al., 2011). Intraspecific nucleotide diversity (π) [Jukes and Cantor (JC) method], haplotype number and haplotype diversity (h) were estimated for each species using DnaSP v. 5 (Librado and Rozas 2009).

The overall consensus sequence, consensus sequence of *Timon* and *Lacerta* autosomal repeats and *Lacerta* W-specific repeats were determined with the program EMBOSS that is available on-line at http://emboss.bioinformatics.nl/.

Net average genetic distances between groups were calculated under the appropriate substitution model (see above) with MEGA v. 5. Rate of IMO-TaqI evolution was determined for the species here studied according to the divergence times estimated for *Lacerta* and *Timon* by Ahmadzadeh et al. (2016).

The occurrence of genetic differentiation between monomeric repeats in the seven species analysed was also assessed by the analysis of molecular variance (AMOVA) (Excoffier et al. '92) calculating Φ-statistics. The test was carried out on the sequences from *Timon* (*T. lepidus, T. pater, T. tangitanus*) and *Lacerta* (*L. agilis, L. bilineata, L. strigata*, *L. trilineata*). In *Timon*, TaqI satDNA monomeric repeats were divided into two groups

according to the sex of the individual from which they had been isolated. In *Lacerta*, four different groupings were made: i) one on the basis of the sex of the individual from which monomeric repeats had been isolated; ii) one according to the clades recovered by the phylogenetic analyses [putative W-specific repeats (W) and putative autosome-specific repeats (A)]; iii) a third grouping by considering only the monomeric repeat sequences from females separating the W-specific repeats from the autosome-specific repeats (based on data from the phylogenetic analyses); iv) the fourth grouping considered a W-specific group of sequences and a group containing only sequences isolated from males. This test was performed at two hierarchical levels to test how satDNA sequence variability was distributed within a group of monomeric repeats and among groups of such repeats. The test was based on pair wise genetic distances between clones and performed as implemented in ARLEQUIN 2.000 (Schneider et al., 2000), using 1000 permutations.

The repeats were compared using satDNA Analyzer version 1.2 (Navajas-Pérez et al. 2007). This program allows the discrimination between shared and non-shared 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 intra-specific sequence homogenisation and inter-specific 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 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 given pair of species, whereas Classes II, III, and IV represent

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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 nucleotide in most members of the repetitive family in the other species. Class V represents diagnostic sites in which a new variant is fully homogenised 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 analyses

Metaphase chromosomes were prepared from females and males of *Lacerta bilineata* (LBI), *L. agilis* (LAG), *L. strigata* (LST), *Timon lepidus* (TLE), and a female of *L. trilineata* (LTR). Fibroblast cell cultures were established from autotomized tail tips and cultured as reported by Rojo et al. (2014). Metaphase spreads were prepared according to Rens et al. (2006).

Fluorescence *in situ* hybridisation (FISH) experiments were performed on metaphase preparations using i) a telomeric probe (TTAGGG)n produced by PCR according to Ijdo et al. ('91) to check if W chromosome constitutive heterochromatin was enriched in telomeric-like sequences, and ii) the probes obtained by PCR amplification of IMO-TaqI satDNA clones from each of the studied species to determine the chromosomal location of this satDNA. The probes were labelled by PCR with biotin-16-dUTP (Roche). Slide pretreatment, denaturation, hybridisation, post-hybridisation washes and detection were performed according to

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Schwarzacher and Heslop-Harrison (2000). Telomeric probes were revealed with Tetramethyl Rhodamine Iso-Thyocianate (TRITC) whereas IMO-TaqI probes with Fluorescein Iso-Thyocianate (FITC). Chromosomes were observed with a Leica Leitz DMRBE epifluorescence microscope and the images were captured and processed with a Leica CytoVision version 7.2 system.

In order to identify the relationships between satDNAs and the constitutive heterochromatin, C-banding was performed on metaphase chromosomes following Sumner ('72). C-banded metaphases were mounted and stained with Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories).

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Results

Isolation and analysis of satDNA sequences.

Southern blot hybridisation of genomic DNAs digested with TaqI restriction enzyme revealed a typical ladder-like pattern consisting of multimeric units, with the size of monomeric units ranging from 173 to 190 bp in both *Lacerta* and *Timon*. These results suggest that the satDNA studied is tandemly arrayed in the two genera, with a hybridization signal stronger in *Lacerta* than in *Timon* (data not shown).

A total of 162 clones containing sequences of IMO-TaqI monomeric unit were sequenced for the three species of *Timon* and the four species of *Lacerta*. These sequences were deposited in GenBank (accession numbers: MF069256-MF069417). One hundred and twelve haplotypes were detected (Table 1). This satDNA showed an average AT content of 59.2%, indicating an enrichment in AT, as typical for these genomic elements, with short A and T stretches ranging from 3 to 7 base pairs (Fig. 1). The BLAST search (using either megablast or blastn algorithm) found significant similarity with *Iberolacerta* sequences by

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3 Giovannotti et al. (2014) and Rojo et al. (2015) for both *Lacerta* and *Timon* satDNA repeats.
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5 pLCS consensus sequences (Capriglione et al., '91) copied by hand from the paper and run in
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7 BLAST (using "somewhat similar sequences" search option) also showed a significant
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9 similarity with *Iberolacerta* sequences with a maximum query cover of 89% and identity
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11 ≤83%.
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14 Intraspecific π values (JC method), ranged from 0.1526±0.0075 to 0.0810±0.0123. In
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16 *Lacerta*, when π values were calculated separately for sequences of the two sub-clades (W-
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18 specific and autosome-specific, see below), the W-specific repeats resulted more
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21 heterogeneous than the autosomal ones (Table 1). Nucleotide diversity values in *Timon* were
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23 similar to those of *Lacerta* W chromosome (Table 1).
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25 The phylogenetic tree obtained from the ML analysis of IMO-TaqI satDNA is shown
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27 in Figure 2. The ML and Bayesian analyses yielded very similar topologies, with some minor
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29 incongruences. Three major clades were recovered with maximum support, one harbouring
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31 *Timon* (ML= 100%; pp= 100%) clones and the other two harbouring the sequences of the four
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33 *Lacerta* species (*Lacerta* Autosomes: ML= 100%; pp= 100%; *Lacerta* W: ML= 100%; pp=
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35 100%). For this latter genus, one clade contained only sequences from females that were
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37 considered as W-specific IMO-TaqI satDNA repeats, and the other clade contained repeats
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40 from both males and females and that were therefore considered as repeats located in the
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42 autosomal arrays of IMO-TaqI satDNA. Finally, relationships between these three main clades
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44 were poorly resolved. (Fig. 2).
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47 The evolutionary rate for IMO-TaqI satDNA among *Lacerta* species ranged from 0.04
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49 to 0.85%/Million years (Myr, henceforth) when only autosomal sequences were considered,
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51 from 0.22 to 0.53%/Myr for W-specific sequences and from 0.11 to 0.34%/Myr when IMO-
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54 TaqI repeats were not separated into autosomal and W-specific sequences. The evolutionary
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rate was 0.53%/Myr when *Lacerta* (autosomal + W-specific repeats) and *Timon* were compared. The rate ranged from 0.27 to 0.50%/Myr among the three *Timon* species (Table 2).

AMOVA analysis was performed on IMO-TaqI sequences of *Lacerta* and *Timon*. The analysis produced results quite different in the two genera. In *Timon* most of the molecular variation was distributed within groups of sequences (88.24%; Φ_{ST} 0.11756, P < 0.0001) whereas the percentage of variation among groups of sequences (males *vs* females) was much lower, representing only 11,76% of the total variation (Φ_{ST} 0.11756, P < 0.0001). The variance among groups of sequences became higher when the AMOVA was carried on *Lacerta* considering two groups of sequences. In three tests, one group was always represented by the putative W-specific IMO-TaqI satDNA repeats, the other group always contained autosome-specific repeats (see Materials & Methods section for details). In these three comparisons, the percentage of molecular variation among groups of sequences ranged from 44.74 to 49.54%, with Φ_{ST} values always significant (P < 0.0001), thus confirming the differentiation between autosomal and W-specific repeats (Table 3). In one test, one group was represented by sequence repeats isolated from females and the other from males. In this case, the percentage of variation among groups was much lower (25.72% with P< 0.0001) than in the comparisons involving the separation between W-specific and autosomal sequences and the percentage of variation within groups of sequences was higher (74.28% with P< 0.0001). This is explained by the fact that in this case the groups are more heterogeneous because highly divergent sequences from W chromosome and autosomes are mixed together.

This differentiation between W-specific and autosomal repeats is also clearly shown by the net nucleotide distance between autosomal and W-specific IMO-TaqI sequence repeats within each species (values ranging from 12.44 to 16.69%, average 14%). These values are higher than net distances obtained from the comparison of autosomal (values ranging from 0.5

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3 to 11.88%, average 6.17%) or W-specific repeats (values ranging from 3.05 to 6.21%, average
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5 4.90%) between the four *Lacerta* species (Table 2).
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8 Strachan analysis of variable sites confirms what has already been highlighted by
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10 phylogenetic and AMOVA analyses. Indeed, the presence of sites occurring in a group of
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12 sequences but not in the other with various degrees of homogenisation (stages IV-VI
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14 according to Strachan et al. '85) are detected in the comparisons involving both genera
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16 (*Timon vs Lacerta*) and sequences from the same genus (*Lacerta* Autosomes vs *Lacerta* W-
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18 specific sequences) when one of the two groups of sequences compared is represented by W-
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20 specific repeats (Table 4).
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24 *Chromosome analyses*
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27 The analysis of chromosomal location of IMO-TaqI satDNA repeats by FISH with species-
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29 specific probes confirmed the results of Southern hybridisation that produced a weaker signal
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31 on *Timon* as compared to *Lacerta*, indicating a smaller amount of IMO-TaqI repeats in the
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33 former genus. Indeed, in *Timon* the species-specific probe produced a fluorescent signal in
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35 pericentromeric regions on nine autosomes in both males and females. In *Lacerta* the signal
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37 was detected in pericentromeric position (except for one pair of small acrocentrics bearing a
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39 weak signal in distal position in *L. strigata*) on a higher number of autosomes than in *Timon*
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41 (16 in *L. bilineata*, 20 in *L. agilis,* 24 in *L. strigata* and 16 in *L. trilineata*). In addition, the
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43 probe produced a bright signal also on the W sex-chromosomes of all the *Lacerta* species,
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45 whereas no signal was produced by IMO-TaqI satDNA probe onto *Timon* W chromosome
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47 (Figs. 3-4). In addition, FISH experiments confirmed the occurrence in *Lacerta* species of two
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49 clearly distinguished groups of repeats, already highlighted by phylogenetic, AMOVA and
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51 Strachan sites analyses. In fact, when W-specific probe was hybridised onto female
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53 metaphases a very bright signal on the heterochromosome and a dimmer signal on autosomes
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were produced (Fig. 4A). FISH with autosome-specific probe onto female metaphases produced a dim signal on the W and a bright one on the autosomes (Fig. 4B). Finally, FISH with a telomeric probe marked the telomeres of all the chromosomes of the complement with a certain degree of enrichment in telomeric repeats of the W chromosome in both *Timon* and *Lacerta* and the microchromosomes in *T. lepidus* (pair 17) and *L. bilineata* (pair 18) (Fig. 5).

C-banded metaphases showed that constitutive heterochromatin occurs in both pericentromeric and telomeric position. The pericentromeric heterochromatin is on a number of autosomes similar to that of autosomes labelled by IMO-TaqI satDNA probe. In all species the W chromosome is almost completely heterochromatic (Fig. 6).

Discussion

Among the interesting findings of this study is the fact that IMO-TaqI satDNA is widely represented in lacertid lizards genomes. This satDNA was isolated and characterized in eight *Iberolacerta* species (Giovannotti et al. 2014; Rojo et al. 2015), in *Podarcis muralis*, *P. sicula*, *P. tiliguerta*, *P. taurica*, *Algyroides fitzingeri*, *A. moreoticus* and *Teira dugesii* (in these species was named pLCS element by Capriglione et al., '89, '91). However, these latter authors did not detect this element in the genome of *Timon lepidus* and *Lacerta viridis*, whereas it was detected in the genome of *Lacerta* and *Timon* by Southern blot analysis made by Giovannotti et al. (2014), but it was neither isolated nor characterized. In the the present study, IMO-TaqI satDNA was successfully isolated and characterized in four species of the genus *Lacerta* and three of the genus *Timon*. These results point to a high level of conservation for this genomic element that could be attributed to its slow evolutionary rate as found in the present study with rates between 0.11 and 0.33%/Myr among *Lacerta* species, 0.27 to 0.50% Myr among *Timon* species and 0.53%/Myr between *Lacerta* and *Timon*.. These results are in accordance with the slow evolutionary rate already observed for this element in

the four Iberian rock lizards by Giovannotti et al. (2014) and similar to evolutionary rates of sturgeons (0.11 and 0.07%) (Robles et al., 2004), skinks (0.13%) (Giovannotti et al., 2013) and cetaceans (0.2%) (Arnason et al., '92) that are considered extremely low (e.g., Robles et al., 2004; Giovannotti et al., 2013). The slow evolutionary rate of these repeats could be related to their genomic distribution and chromosomal location. In fact, IMO-TaqI repeats are located in interstitial/pericentromeric position (less susceptible to physical association) on a subset of chromosomes (from 9 in *Timon* to 25 in *Lacerta strigata*), and it is possible that the exchange between non-homologous chromosomes bearing IMO-TaqI sequences is limited in these circumstances. This could reduce non-sister chromatid exchange and homogenisation, thus determining a lower rate of interspecific divergence and a higher degree of intraspecific repeat heterogeneity (as was shown from the comparison between HindIII and IMO-TaqI satDNAs in four *Iberolacerta* species by Giovannotti et al., 2014). These data on the phylogenetic distribution among lacertids indicate that the evolutionary history of this satellite DNA is ~35 Million years old, as inferred from the dating of Lacertinae radiation (Hipsley et al., 2009). This slow evolutionary rate is also responsible for the low phylogenetic resolution power shown by this satellite that can resolve genera but not species (see Giovannotti et al., 2014 and the present study). However, the slow evolutionary rate of IMO-TaqI satDNA could have an alternative explanation. In fact, the alignment of the consensus sequences of this satellite in the species here investigated show that most of the monomer sequence is conserved and that variable sites are confined to three regions of the monomeric unit. This observation could imply that the evolutionary conservation of this satDNA is due to selective pressures. Similar considerations were made for a 70 Million years old centromeric satellite DNA, described in two anurans families, by Vittorazzi et al. (2014). This hypothesis opens the way to a new scenario in which the resilience of this satDNA to mutational events would be the results of the constraints imposed by a putative function of this genomic element rather

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than the position along the chromosome. A function in the centromeric heterochromatin compaction was already hypothesized by Capriglione et al. ('91) for this element. In particular, the presence of A-T stretches would favour stable DNA curvature that would play a relevant role in heterochromatin condensation (Ng et al., '86; Radic et al., '87; Capriglione et al., '91; Caputo et al., 2009). Indeed, A-T stretches occur in the IMO-TaqI element and, as also observed by Capriglione et al. ('91), the fact that they are conserved across species investigated could indicate that they possess a functional role. However, given that more and more scientific evidence has been indicating that various important biological roles can be fulfilled by satDNA transcripts (e.g., Grewal and Elgin, 2007; Feliciello et al., 2015; Kuhn, 2015), despite satDNAs have been considered as useless genomic elements for a long time (Ohno, '72; Orgel and Crick, '80), it is clear that further investigations will be needed to assess the presence of transcripts of this elements that could help explain this remarkable conservation across the phylogeny of lacertid lizards.

Another interesting result of this investigation is represented by the finding that in the four representative of the genus *Lacerta* here investigated (*L. agilis*, *L. bilineata*, *L. strigata*, *L. trilineata*) the fluorescent probe of IMO-TaqI produced a very bright signal also on the heterochromatic W sex chromosome. Indeed, the fluorescent signal is absent on the W of the other species of lacertids so far tested by FISH with IMO-TaqI probe. In particular, this satellite occurs only on autosomes in the three species of *Timon* studied here (*T. lepidus*, *T. pater*, *T. tangitanus*), in *Iberolacerta* (Giovannotti et al., 2014; Rojo et al., 2015), and for the species investigated by Capriglione et al. ('89, '91) the occurrence on sex chromosomes was not reported. Therefore, it seems that in these *Lacerta* species, IMO-TaqI is involved in the differentiation of the W sex chromosome by heterochromatinization. Despite heterochromatinization is often found in reptilian sex-heterochromosomes, DNA repeats in the heterochromatin of Y and W chromosomes have been partially characterized in just few

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species of snakes (Singh et al., ‘80; Jones & Singh, ‘85; Lee et al., 2007; O’Meally et al., 2010), iguanians (Gamble et al., 2014; Altmanová et al., 2016), monitor lizards (Matsubara et al., 2014; Prakhongcheep et al., 2017) and lacertid lizards (Pokorná et al., 2011; Srikulnath et al., 2014). In most cases these repeats are represented by microsatellites (O’Meally et al., 2010; Pokorná et al., 2011; Gamble et al., 2014; Altmanová et al., 2016; Matsubara et al., 2014). Our study also showed the enrichment in telomeric-like sequences of the W chromosome of the four *Lacerta* species investigated, thus confirming what already reported by Srikulnath et al. (2014) and Matsubara et al. (2015b) for *L. agilis*, and in the W of the *Timon* species investigated. The enrichment in telomeric-like repeats of the sex heterochromosome has been recorded also in the lacertid *Acanthodactylus lineomaculatus* (Giovannotti et al., in press), in the agamid lizard *Pogona vitticeps* (Young et al., 2013) and in the gecko *Underwoodisaurus milii* (Pokorná et al., 2014).

The considerable heterogeneity in constitutive heterochromatin composition of the heterochromosomes in different reptilian lineages suggests, in line with the considerations by Pokorná et al. (2011), that chance, not sequence specificity, determines which repetitive elements will accumulate on the sex chromosome of a certain lineage. The fact that IMO-TaqI satDNA occurs only on autosomes and not sex chromosomes in most of the species analysed (Capriglione et al., ’89, ’91; Giovannotti et al., 2014; Rojo et al., 2015) seems to support this conclusion. In addition, the variability of the repeats among relatively closely related species confirms that sex heterochromosomes are the most variable part of the genome (e.g. Graves, 2008). Interestingly, *Lacerta* species are the only species among those so far investigated in which the sequences IMO-TaqI are clearly separated into two statistically supported clades in the phylogenetic analysis. This condition reflects the isolation of the repeats accumulated on the non-recombining W that would not undergo concerted evolution, consistently with the higher intraspecific heterogeneity recorded for W-specific repeats (π= 0,1195) when

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compared to autosomal repeats (π= 0,0834) (see Navajas-Pérez et al., 2009 for similar considerations). An intraspecific heterogeneity similar to that of *Lacerta* W-specific repeats is found in *Timon* (π= 0.12), in which the presence of IMO-TaqI on only 9 chromosomes could explain the low homogenisation rate among repeats. The absence of the homogenising effect in *Lacerta* autosomal and W-specific repeats is confirmed by AMOVA analysis that gives a clear indication of the differentiation that is accumulating between these two groups of IMO-TaqI sequences in *Lacerta*. In the three *Timon* species, where the sequences of this satDNA occur only on autosomes, the IMO-TaqI repeats form a single statistically supported cluster. The same indication comes from the analysis of Strachan transitional stages that show in *Lacerta* species a pattern similar to that found between genera due to the separation of IMO-TaqI repeats into two distinct groups.

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Recently, Rovatsos et al. (2016) demonstrated that sex chromosomes are homologous across lacertids. In this context, the enrichment in repeats represented by IMO-TaqI satDNA in the W of the four *Lacerta* species here investigated and the lack of these repeats on the W of other lacertids in which IMO-TaqI was isolated (e.g., Giovannotti et al., 2014; Rojo et al., 2015) would indicate a certain turnover of heterochromatin involved in the differentiation of the heterochromosome. Therefore, it is likely that these repeats replaced pre-exisitng repetitive DNA on the heterochromosome of the common ancestor of modern *Lacerta* species after the split of this lineage. In addition, the sharing of repetitive elements on the W among *Lacerta* species also support the monophyletism of these lizards, that appear as a distinct group from *Timon* because of the lack of IMO-TaqI on the W of this latter genus.

In conclusion, tandemly repetitive DNA sequences would remain randomly trapped into the sex chromosomes, undergoing amplification as a consequence of the suppression of recombination. In addition, comparing W-specific repeats with autosomal repeats, we found that the former escaped the concerted evolution as shown by the relatively deep genetic

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divergence between them and the autosomal repeats. These data imply that dynamics of satDNA evolution depend on the chromosomal location of the repeats, such as the absence of recombination between sex chromosomes, that seriously influences satDNA sequence change. On the other hand, the strong conservation of IMO-TaqI in the genome of Lacertidae also suggests a possible functional role to be investigated in a future study.

Conflict of interest

The authors declare that they have no conflict of interest.

For Peer Review

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Figure legends

Figure 1. Consensus sequences of IMO-TaqI satDNA repeats (all) obtained by aligning the consensus sequences of *Timon* (TIM), W-specific (W) and autosome-specific (A) repeats of *Lacerta*. Highighted in grey: stretches of A/T characterising the repeats.

Figure 2. ML tree depicting the phylogenetic relationships among IMO-TaqI satDNA repeats isolated from three species of Timon and four species of Lacerta. At nodes only MetaGA values >70% (ML) and posterior probability values >95% (BA) are reported. A hyphen was inserted whenever BA method did not support a given node in the ML topology. Pink background: *Lacerta* autosomal-specific repeats; pale blue background: *Lacerta* W chromosome-specific repeats; purple background: *Timon* repeats.

Figure 3. FISH with IMO-TaqI probe onto metaphases from females of: *Timon lepidus* (A), *Lacerta agilis* (B), *L. trilineata* (C), *L. strigata* (D). A species-specific probe was used for each species. The arrow indicates the W chromosome. Scale bar =10 µm.

Figure 4. FISH with IMO-TaqI probe onto metaphases from *L. bilineata* female. A) the probe was produced from W-specific repeats; B) the probe was produced using autosome-specific repeats. The arrow indicates the W chromosome. Scale bar =10 µm.

Figure 5. FISH with telomeric (TTAGGG)n probe onto metaphases from females of: (A) *Timon lepidus*, (B) *Lacerta agilis*, (C) *L. trilineata*, (D) *L.* strigata, (E) *L. bilineata*. Arrows indicate: W chromosome, pair 17 of *T. lepidus* and pair 18 of *L. bilineata*. Scale bar =10 µm.

Figure 6. C-banded methaphase plates of females of *Timon lepidus* (A), *Lacerta agilis* (B), *L. trilineata* (T), *L. strigata* (D) and *L. bilineata* (E). Scale bar = 10 µm.

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all    NCGAGGCCTGATTTCCTTTCCTNTGATNAAAAACCCTTCTGTTTTCACCGCCAAATCTTC 60
TIM    -C.............T......A..-TT................C.C....C........ 58
W      NT.............C......G..-AT................T.A....G........ 59
A      -C.............C......T..ATG................T.A....C........ 59

all    CAGGGGAGTTNTGGCAACAGTTTGGCACCATTTTTGANGNAAATTGGAGAACGTCAGATT 120
TIM    .AGGG..G.TC.TG...C.G................AG-TG................... 117
W      .AGGG..A.TG.GC...C.G................NAGAA................... 119
A      .TTCA..G.CT.GG...A.T................AG-GA................... 118

all    TTTGGTGAAATTCTGACCGCGNGGGGTTAGGGATTTTTTCAAAAAAANNNTTTTTCNGCA 180
TIM    ....A.............C..G...-..............A.....ANNN....TCCG.. 176
W      ....G.............G..C...-..............N.....-TNN....TCNG.. 177
A      ....G.............G..C...N..............A.....AGTT....CNNN.. 178

all    GGTNAAGTTGTNGN 194
TIM    G..T..G....C.- 189
W      A..A..G....N.- 190
A      G..C..C....N.N 192
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154x109mm (300 x 300 DPI)

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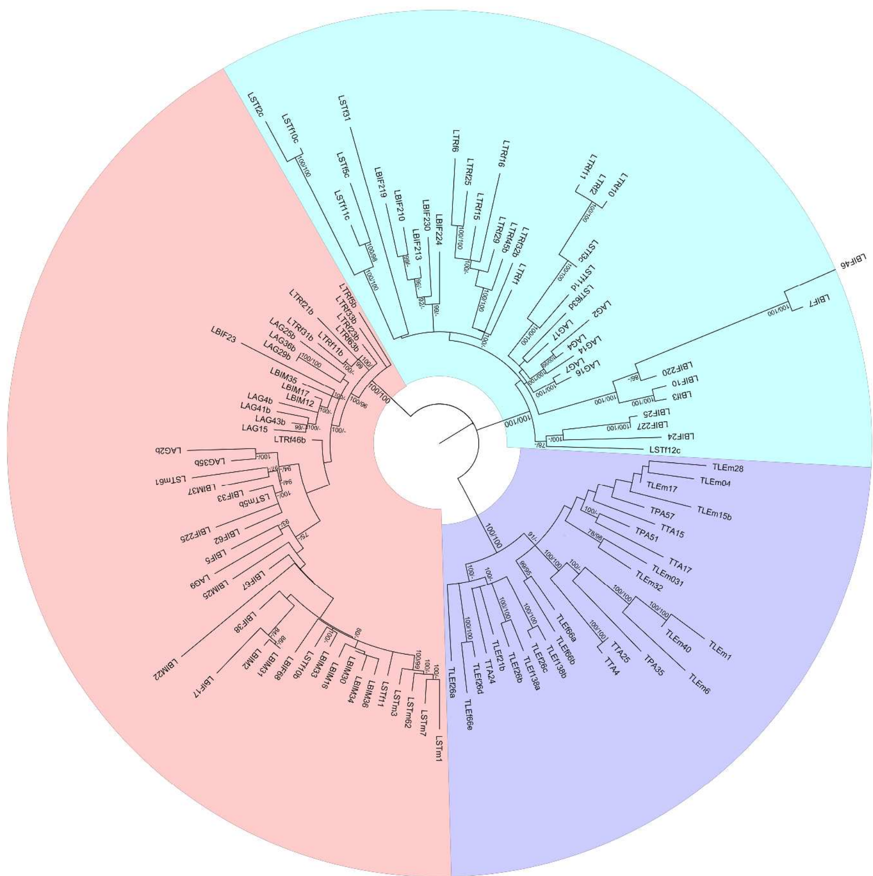

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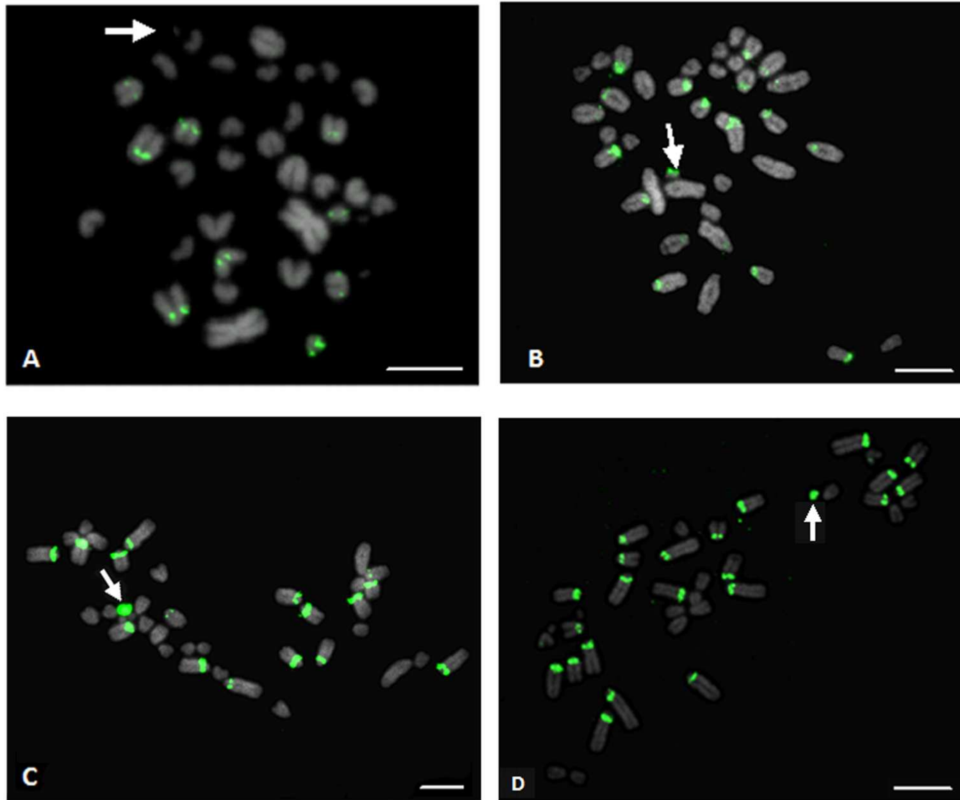

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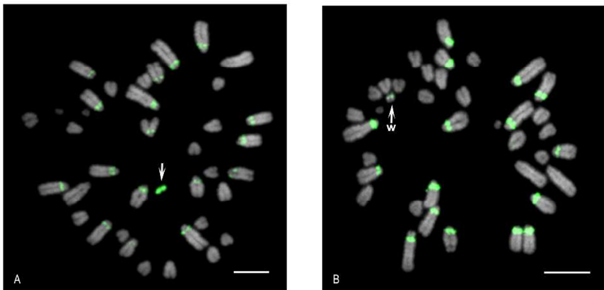

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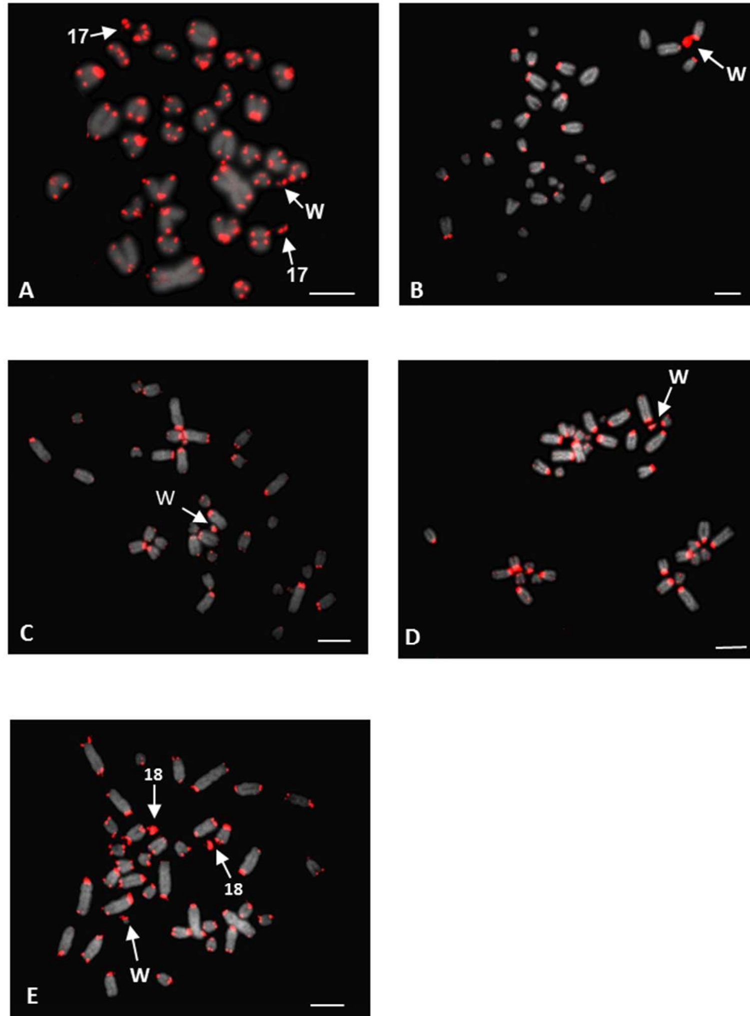

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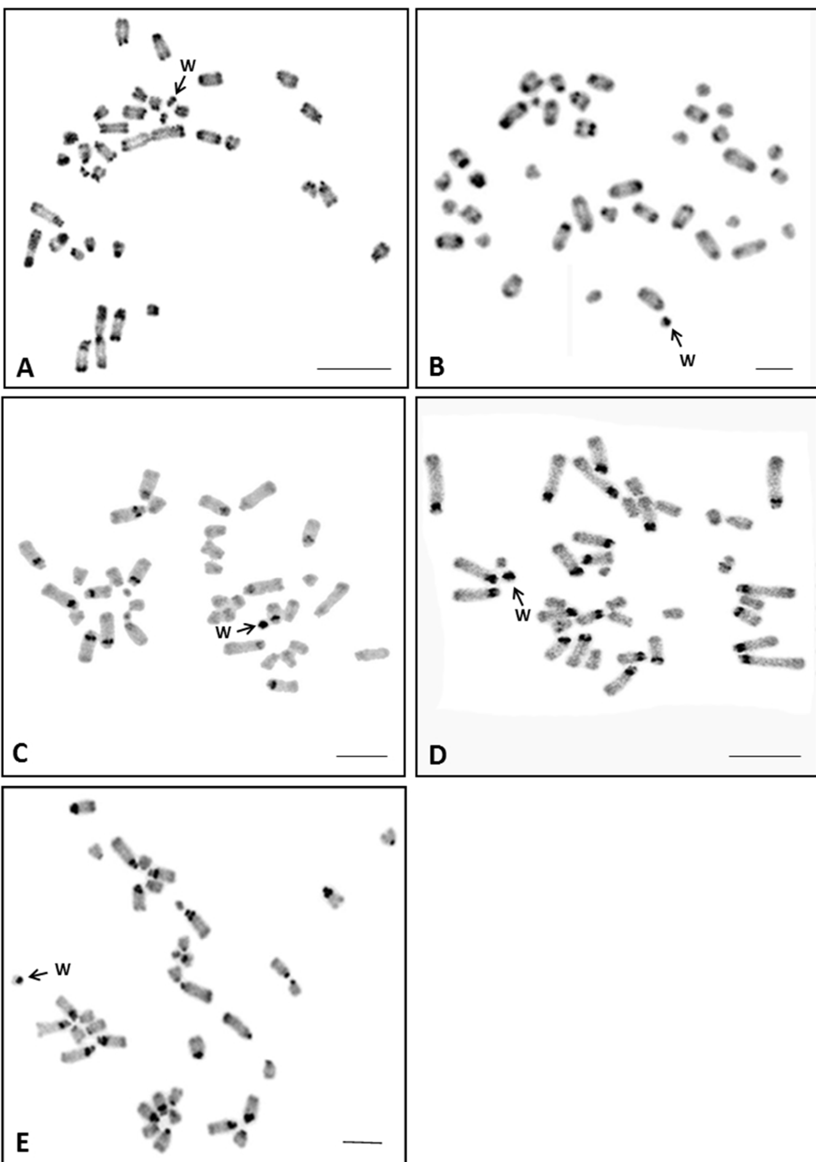

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Table 1. Summary of repeat genetic diversity estimates for the species studied.

Species	Number of clones			% AT	Repeat Length (base pairs)	Nucleotide diversity (π)	Number of Haplotypes (H)	Haplotype diversity (Hd)
	Female	Male	Total					
L. agilis	12	10	22	58.7	186-189	0.1182±0.0056	15	0.944
L. bilineata	28	15	43	59.0	187-190	0.1432±0.0075	35	0.990
L. trilineata	26	-	26	59.2	186-190	0.1096±0.0048	18	0.951
L. strigata	19	11	30	60.2	166-187	0.1526±0.0075	18	0.956
Lacerta A	36	26	62	58.8	166-190	0.0834±0.0031	47	0.989
Lacerta W	59	-	59	59.8	173-189	0.1195±0.0047	38	0.964
T. lepidus	10	10	20	59.3	182-189	0.1124±0.0047	19	0.995
T. pater	2	8	10	59.3	182-188	0.0810±0.0123	3	0.733
T. tangitanus	4	7	11	58.8	187-189	0.1086±0.0124	5	0.818
Timon	16	25	41	59.1	182-189	0.1125±0.0059	27	0.951

The columns female and male indicate how many clones were sequenced from either sex. A= autosome-specific repeats of IMO-TaqI satDNA; W= W chromosome-specific repeats of IMO-TaqI satDNA.

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Table 2. Evolutionary rates of IMO-TaqI satDNA in the species investigated.

Pair-wise comparison	Net genetic distances among groups average	Divergence in million years	IMO-TaqI evolutionary rate
LAG_A vs LBI_A	0.0214 ± 0.0057	13.91*	0.15%
LAG_A vs LST_A	0.0373 ± 0.0103	13.91*	0.27%
LAG_A vs LTR_A	0.0835 ± 0.0197	11.65*	0.72%
LBI_A vs LST_A	0.0050 ± 0.0020	12.24*	0.04%
LBI_A vs LTR_A	0.1045 ± 0.0217	13.91*	0.75%
LST_A vs LTR_A	0.1188 ± 0.0261	13.91*	0.85%
LAG_W vs LBI_W	0.0462 ± 0.0106	13.91*	0.33%
LAG_W vs LST_W	0.0526 ± 0.0112	13.91*	0.38%
LAG_W vs LTR_W	0.0621 ± 0.0126	11.65*	0.53%
LBI_W vs LST_W	0.0461 ± 0.0096	12.24*	0.38%
LBI_W vs LTR_W	0.0565 ± 0.0111	13.91*	0.41%
LST_W vs LTR_W	0.0305 ± 0.0081	13.91*	0.22%
LAG vs LBI	0.0149 ± 0.0040	13.91*	0.11%
LAG vs LST	0.0180 ± 0.0044	13.91*	0.13%
LAG vs LTR	0.0365 ± 0.0063	11.65*	0.31%
LBI vs LST	0.0111 ± 0.0029	12.24*	0.09%
LBI vs LTR	0.0467 ± 0.0078	13.91*	0.34%
LST vs LTR	0.0363 ± 0.0072	13.91*	0.26%
TLE vs TPA	0.0202 ± 0.0045	7.42*	0.27%
TLE vs TTA	0.0258 ± 0.0057	7.42*	0.35%
TPA vs TTA	0.0296 ± 0.0051	5.98*	0.50%
LAC vs TIM	0.0925 ± 0.0198	17.55*	0.53%
LBI_A vs LBI_W	0,1244 ± 0,0250	-	-
LAG_A vs LAG_W	0.1458 ± 0.0295	-	-
LST_A vs LST_W	0.1669 ± 0,0338	-	-
LTR_A vs LTR_W	0.1257 ± 0.0258	-	-

Net genetic distances in pair-wise comparisons between IMO-TaqI satDNA repeats ± standard deviation; divergence in million years among the taxa compared and estimated evolutionary rate (% of sequence change/ million years) for IMO-TaqI satDNA. The distance method (Maximum Composite Likelihood with Gamma distribution) was inferred from the best fit model of nucleotide evolution estimated by MEGA v. 6. Distances calculations were carried out with the same program. Gamma shape parameter = 1.8. * data from Ahmadzadeh et al., 2016.

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Table 3. AMOVA analysis.

Source of variation	Groups	Variance components	Percentage of variation	F_{ST}
Among groups of sequences	TIM f vs m	2.93421	11.76	0.11756***
	LAC f vs m	5.44448	25.72	0.25724***
	LAC W vs A	10.76439	47.00	0.47005***
	LAC W vs Af	10.18352	44.74	0.44739***
	LAC W vs m	12.10009	49.54	0.49541***
Within group of sequences	TIM f vs m	22.02493	88.24	0.11756***
	LAC f vs m	15.72024	74.28	0.25724***
	LAC W vs A	12.13616	53.00	0.47005***
	LAC W vs Af	12.57831	55.26	0.44739***
	LAC W vs m	12.32418	50.46	0.49541***

The test Was carried on TaqI satDNA sequences from *Timon* and *Lacerta*. Grouping details are given in the text.
*** = P<0.001. f: monomeric repeats isolated from females; m: monomeric repeats isolated from males; W: W-specific repeats; A: autosome-specific repeats isolated from both males and females; Af: autosome-specific repeats isolated from females. LAC: *Lacerta* (*L. agilis*, *L. bilineata*, *L. strigata*, *L. trilineata*); TIM: *Timon* (*T. lepidus*, *T. pater*, *T. tangitanus*).

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Table 4. Variable sites classified according to Strachan et al. (1985).

PAIR-WISE COMPARISONS	II-III	IV-VI
LACw *vs* LACa	26	5
LACw *vs* TIM	58	3
LACa *vs* TIM	50	4

LAC_W: W-specific repeats from *Lacerta*; LAC_A: autosome-specific repeats from *Lacerta*; TIM: repeats from *Timon*.

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