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ABSTRACT

We sequenced the complete control region and adjacent tRNAs, partial 12S rRNA and Cytochrome b (over 3100 bp) from eight individuals of Madeiran Wall lizards *Lacerta dugesii* from four distinct island populations. The tRNAs exhibit a high degree of intra specific polymorphisms compared to other vertebrates. All control region sequences include a minisatellite that varies in length between populations but is apparently fixed within them. Variation in minisatellite length appears between populations separated by apparently very short evolutionary time spans. Many motifs identified in the CR of other vertebrates are not highly conserved, although conserved blocks are identifiable between the few published reptile CR sequences. Overall there are extensive differences in the internal organization of the reptile CR compared to the more widely studied mammals and birds. Variability in the CR is lower than in Cytochrome b, but higher than in 12S rRNA. Phylogenetic analysis of these sequences produces a well-resolved estimate of relationships between populations.

The complete eight sequences used in this study are available from GeneBank accession numbers AY147872-AY147879.
COMPLEMENTARY MATERIAL

The following material was included in early versions of the manuscript but was cut off in later revisions. Nevertheless, it is intended to help those wishing to better understand the structure and evolution of *Lacerta dugesii*’s mitochondrial DNA.

**Materials & Methodology**

The entire Control Region including 3 tRNAs and partial Cytb and 12SrRNA gene sequences were amplified in one single step using two primers:

(Forward) cBL (5’-CTGCATCTACCTCCACATCGGACG-3’) and

(Reverse) 12L (5’-AAGTTTTT CACTTGTAGTTCTCTG GCGG-3’)

We used *TaqPlus Long™* from Stratagene, following the manufacturers instructions. PCR cycle conditions were 30 sec at 94°C, 30 sec at 60°C and 2 min at 70°C, for 35 cycles. Following the sequencing of this fragment, nine primers were successively designed from conserved regions among the 8 sequences, to amplify consecutive segments, until sequences from both extremities overlapped (primer-walking strategy). Primer sequences and their relative locations are as follows:
Amplified fragments were always sequenced three times and in both directions, on a
377 Applied Biosystem DNA Sequencing Apparatus, with the same set of primers used for
amplification. One 10-mer primer (cBLint5-5’TTGCGCTAC3’) was specifically designed
to work with primer 12Lint5. These two primers were used to amplify the region containing
a repeat motif. We amplified this fragment in ten individuals from each site, to check for
variation of number of repeats within populations.
Results

The tRNAs

Here we present additional material regarding the tRNA sequences found in *L. dugesii* as well as in comparisons with other animals. Particularly in relation to tRNA$^{\text{Pro}}$ it is true that not all forms present the four stems usually present in tRNAs, some having shifted the TΨC stem for a bigger replacement loop. Most of these tRNA forms present one or two variable loops. Four different sequences of the tRNA$^{\text{Phe}}$ were found in the 8 individuals analysed, all of which are capable of folding into cloverleaf structures (see figure 3 of Brehm et al. 2003).

Below are aligned light-strand DNA sequences of a) tRNA$^{\text{Thr}}$, b) tRNA$^{\text{Pro}}$ and c) tRNA$^{\text{Phe}}$ genes of the different forms occurring in *L. dugesii* as well as other reptiles used for comparisons. Taxa shown are *Eumeces* (Kumazawa and Nishida 1999), *Iguana* (Janke et al. 2001), *Ovophis* and *Dinodon* (Kumazawa et al. 1998), *Cnemidophorus* (Stanton et al. 1994). The anticodons are underlined.

a) tRNA$^{\text{Thr}}$

| Taxon          | Sequence                                                                 |
|----------------|--------------------------------------------------------------------------|
| *Dugesii* 1   | GTCCCATGCTAGCTTAGACCACTAAAGCAGCGGTCCTGCTGAAACCGAGACCAGACCTCATC-CT---TCCTGAGACA |
| *Dugesii* 2   | ..........................................................G......................... |
| *Eumeces*     | ..........................................................A--T..AC......TT........ |
| *Iguana*      | ..C..T.........A--..T........TT.............A....T.-.GACTTAAA.CGCC...A..G.. |
| *Chelonia*    | A.T.T........A--..-C.......TT.............A....ATT..A.ACT..AA.C-T...AGA..T. |
| *Ovophis*     | .CT.T.A.......AA.T.TA........GTT.T........A....T.-.G-C-.C-.C--.--AGAG. |
| *Dinodon*     | .C.T........A--..G.......TT.T.............A....AT--.C..-A.C-------AGAG. |
b) tRNA\textsuperscript{Pro}

| Dugesii 1 | TCAAAAGAAGATCTACAGGCTCTGGCAACCCCCAATGCGCTTTTAAT----TTAAACTATCTTTTG |
| Dugesii 2 | ..............................................TG.............................. |
| Dugesii 3 | ..............................................T.A.............................. |
| Dugesii 4 | ..............................................A.............................. |
| Dugesii 5 | ..............................................C.A.............................. |
| Dugesii 6 | ..............................................C.A.............................. |
| Chelonia  | ..............................................GA.TA.A.......................... |
| Cnemidophorus | ..............................................GG.CC.C.......................... |
| Ovophis | ..............................................GAG.G.C.......................... |
| Eumeces | ..............................................-.GAT...T...TAGTCTCTCTAGAATGACAGCTGCTCTGCTTCCTTTGTTAAGGGGAGATGAGGAACAGAAAAACCTCCACAGACA |
| Iguana | ..............................................-.G.-AGAT...T...AATTTAAAAGCAGGGCTCTGGCAGAGATGAGGAACAGAAAAACCTCCACAGACA |

The Control Region (CR)

Lacerta dugesii CR is depicted schematically in Figure 4 of Brehm et al. (2003), but here we include the CR – Complete Sequence (D1 of one individual analysed (D1, Deserta Isl.) so it should make much more easier to follow the scheme:

c) tRNA\textsuperscript{Phe}

| Dugesii 1 | GTCATTGCTTTATTATTTTTTTAAAGCAGGGCTCTGGCAGAGATGAGGAACAGAAAAACCTCCACAGACA |
| Dugesii 2 | ..............................................-.G.............................. |
| Dugesii 3 | ..............................................-.G.............................. |
| Dugesii 4 | ..............................................-.G.............................. |
| Dinodon | ..............................................-.G.............................. |
| Eumeces | ..............................................-.G.............................. |
| Iguana | ..............................................-.G.............................. |
| Chelonia | ..............................................-.G.............................. |

The Control Region (CR) is depicted schematically in Figure 4 of Brehm et al. (2003), but here we include the CR – Complete Sequence (D1 of one individual analysed (D1, Deserta Isl.) so it should make much more easier to follow the scheme:
Reference sequence of the Control Region of *Lacerta dugesii* D1 individual (CR – Complete sequence). The 8 sequences studied vary in number of 37-38 repeats, as well as in polymorphic variable positions. The complete 8 sequences are available from GeneBank. The reference sequence shown presents all putative blocks mentioned in the text or shown in detailed analysis in the figures. Base nomenclature follows the international code in those cases more than one base was found in the same position (R=A or G; Y=C or T; W=A or T; S=C or G; K=G or T; M=A or C; B=C, G or T; D=A, G or T; H= A, C or T; and V= A, C or G).

Small letters indicate that some individuals present a gap in that position.
The Minisatellite

As reported in Brehm et al. (2003) the number of repeats from the minisatellite are constant from each collection site. Some repeats show length heteroplasmy and it is interesting to note that the first 5 bases of each repeat are identical to the last 5 bases of the tRNA-Pro (5′TTTTG3′). Moreover each repeat presents two perfect 7 bp mirror sequences separated by 2 cytosines (5′GCCGCTACCTAGCGGC3′) which are responsible for strait and consecutive stem and loop structure formed in this region (ΔG>-71Kcal.).

We have also identified three (CC)₃ repeats (in the L strand), one localized in the middle of the CR and the other two as part of the CSB-2 region, notorious because of a high content of A/Ts (Figure 4 from Brehm et al. 2003).

Conserved structures

Lacerta dugesii CR reveals interesting features when compared with sequences from other taxa retrieved from GenBank. The figure bellow summarizes these findings by comparing L. dugesii sequence with other similar sequences. Sequence a (see Figure CR-Complete Sequence) for example, a 43 bp long sequence following the tandem repeats of the 5′ left region of the CR, is strongly conserved across reptiles, amphibians, mammals and even plants. This sequence includes a TATA box followed by the already mentioned highly conserved sequence ACATTAA (a). Included in a rather variable region in L. dugesii CR are two 28 bp sequences. The first was found conserved in a fish (b in Figure CR-Complete Sequence) but the second is found extremely conserved across a broad array of organisms (c). In humans it is often found within repeat regions of the L1 family, either in the X chromosome or in tandem repeats in the autossomes. Although sometimes slightly modified, the same sequence is found in mammals (Mus), insects (Drosophila) and plants (Arabidopsis). It is interesting to note, however, that the known TATAA box precedes both
sequences b and c. The 43 bp fragment depicted in (d in CR-Complete Sequence) is another highly conserved region in the CR, with almost perfect homologies with the birds *Pionus chalcopterus* and *Amazona amazonica*. This region includes the conserved “F box” of Randi and Lucchini (1998) for *Alectoris*, but in a much bigger extension. However, no such structure was found in *Iguana*. Fragment e of *L. dugesii* (see Figure CR-Complete Sequence) includes the duplicated highly conserved regions R1. In *Iguana iguana* this fragment is also present (e) but with no repeats. Fragment f (also from Figure CR-Complete Sequence) includes the CSB-1. It is found in the turtle *Kinosternon hirtipes* CR in a much bigger extension. Finally, fragment g (figure CR-Complete Sequence), which includes CSB-2, is almost intact in *L. dugesii*, *I. iguana* and the fish *Sardinops melanostictus*.

The aligned sequences of several conserved regions in the Control Region of *Lacerta dugesii* and other organisms were retrieved from Genebank and have the following accession numbers: reptile *Iguana iguana* (AJ278511); amphibian *Rana porosa* (AB036404); fish *Sebastes paucispinis* (AF031499), *Sardinops melanostictus*...
(NC002616.1); mammals *Homo sapiens* (AC002485, AC006473), *Apodemus agrarius* (AAU21161), *Microtus mexicanus* (AF251260), *Acomys percivali* (APE012039), *Setonix brachyrus* (AF380320), *Neotoma lepida* (AF091260); birds *Pionus chalcopterus* (AF338318), *Amazona amazonica* (AF338280). The relative positions occupied by fragments a-e are pointed in the CR scheme of Figure 4 (Brehm et al. 2003) and in the above scheme of the control region.

The search for secondary structures also uncovered numerous stable inverted perfect or imperfect repeats that constantly turn into stable stem-loop structures when the molecule is single stranded. These repeats are mainly grouped in the CR extremities, following the minisatellite and near the L strand 3’ region. Inverted repeats may be tandemly or closely arranged forming hairpin structures by use of complementarity between the underlined parts, such as 5’ATGTAATAGTACAT3’, 5’ATAAAAAA TTGGTTAT3’, 5’GCTTTGTCAAAACAAACAAAGC3’, or separated by up to one hundred bases like 5’TAAAATTAATACATAAAA3’... 5’TGGTTATTAGTTA ATGAA3’ (see general CR scheme above). Other repeats, even imperfect ones, systematically form stem-loop structures using MFOLD. It is worthwhile to note that all conserved segments depicted in fragments a-c form hairpin-like structures meaning that these regions have mirror like sequences (e.g. inverted tandem repeats) but with the possible exception of CSB-1, none of the CSBs form stable secondary structures. Finally, two TCCC motifs exist in our reptile CR, which have been linked to termination of H strands in mammalian and bird D-Loops (Douzery and Randi 1997; Randi and Lucchini 1998) but none of them are linked to putative cloverleaf secondary structures.
CBS (Conserved Block Sequences)

Below we show the best alignment of sequences including the Conserved Sequence Blocks 1-3 (CSB1-3) in a lizard (L. dugesii, this work), a skink (Eumeces egregious, Kumazawa and Nishida 1999), an iguanidae (Iguana iguana, Janke et al. 2001), a turtle (Kinosternon hirtipes GenBank AF316136), a snake (Dinodon semicarinatus, Kumazawa et al. 1998), a gull and a chicken (Crochet and Desmarais 2000), two partridges (Alectoris barbara and A. graeca, Randi and Lucchini 1998) and six mammals (Rattus and Mus, Brown et al. 1986; Glis glis, Sbisà et al. 1997; Canis familiaris, Rothuizen, et al. direct submission to GenBank X97343; Canis lupus, Vila et al. 1997; Equus caballus, Ishida et al. 1994). In the alignment we did not always use the CSB boundaries proposed by Brown et al. (1986) and instead used the complete sequences that gave a better score on bp matching when compared with reptile sequences. An asterisk denotes that among several options for CSB-1 element, that was the best match. Bases common to all vertebrates are underlined. We present here an alternative sequence for Alectoris graeca from the one proposed by Randi and Lucchini (1998). We could not find any such pattern for Ursus arctus.

CBS-1

5’ TTGGGTGCTGATTCTTTGCTGAAGGCTTTTCATTTTGGA---TGCTATGACT-CAGCT 3’ E. caballus*
5’ TTGGGGGAACCTGGACTTATGATT---CAGCT 3’ U. arctos*
5’ TTGGGGAACCTGGACTTATGATT---CAGCT 3’ C. lupus
5’ TTTTTA---GGGGGGGGAATCTGCTACT-CATCT 3’ C. familiaris
5’ GTTAGACTATTTAAACCATGCTTGTTTGGACATAA 3’ G. glis
5’ GGGTGA---TTGCTGTACTTTTGCTGAAGGCTTTTCATTTTGGA---TGCTATGACT-CAGCT 3’ H. sapiens*
5’ CAAATACATTAAGATAA-TGCTTATTTTAGCTTTTAA-TGGTGG-CATGG 3’ R. norvegicus
5’ TATAT-AGTGAATGCTGATGCTGACATAT 3’ A. barbara
5’ TTTTTT-AGTGTAGTGCTTAAATGGACATG 3’ gulls
5’ TATTTT-AGTGAATGCTGATGCTGACATAA 3’ chicken
5’ ATGG-TATATTT-AGTGAATGCTGATGCTGACATAT 3’ C. careta
5’ GATTTCTTTTAA-TGCTGTTGGGGGCAATAA 3’ E. egregious

CTATACGGGATAC-ATT-C-TTTCA-TGCTGTATTTAGACATAC 3’ D. semicarinatus
5’ CTATACGGGATAC-ATT-C-TTTCA-TGCTGTATTTAGACATAC 3’ I. iguana
5’ CTATACGGGATAC-ATT-C-TTTCA-TGCTGTATTTAGACATAC 3’ K. hirtipes
5’ CTATACGGGATAC-ATT-C-TTTCA-TGCTGTATTTAGACATAC 3’ L. dugesii
CBS-2

5' CAAACCCCC-TACCCCCC 3' L.dugesii, E.egregius, I.iguana, Sardinops
5' TAAACCCCC--GCCCCGA 3' E.egregious
5' CAAACCCCCCTACCCCCC 3' E.caballus
5' CAAACCCCCC-ACCCCT 3' R.norvegicus
5' CAAACCCCCC--ACCCCT 3' M.domesticus
5' TNAAMCCCCC--ACCCCA 3' C.careta

CBS-3

5' TCGC-AAACCCCT----AAAACGA 3' L.dugesii
5' CCGCCAAACCC----AAAAACAA 3' E.egregious, C.familiaris
5' TTGTCAAACCC----AAAAACAA 3' I.iguana
5' TGCCAAACCC----AAAAACAA 3' R.norvegicus
5' TCCCAAACCC----AAAAACAA 3' M.domesticus
5' TTGCCAAACCC----AAAAACAA 3' E.caballus
5' CACAAAAAACC----AAAAAC 3' A.graeca
5' AAG--AAACCCCTAAAAACA 3' gulls
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