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Comparisons of mitochondrial DNA (mtDNA) sequences (16S rRNA gene) between oviparous and viviparous strains of *Lacerta vivipara*: a preliminary study

B. HEULIN,* Y. SURGET-GROBA,* A. GUILLER,† C. P. GUILLAUME‡ and J. DEUNFF†
**CNRS UMR 6553, Station Biologique de Paimpont, 35380 Paimpont, France, †CNRS UMR 6553, Faculté des Sciences Pharmaceutiques et Biologiques, 35043 Rennes, France, ‡EPHE, Laboratoire de Biogéographie et Ecologie des Vertébrés, Université de Montpellier II, 34095 Montpellier, France*

Abstract

The lizard *Lacerta vivipara* has allopatric oviparous and viviparous populations. The mitochondrial DNA (mtDNA) gene coding for the 16S rRNA was sequenced for several viviparous lizard populations from France, Switzerland, Bulgaria, Czech Republic, The Netherlands, Sweden, and for oviparous lizard populations from the Pyrenean and Cantabric Mountains. Seven distinct groups (three oviparous and four viviparous) were identified. The net nucleotide divergence between oviparous and viviparous haplotypes was 1.3% ± 0.5 (mean ± standard deviation). These results on mtDNA, together with other data obtained previously, led us to formulate a biogeographical scenario that could be tested by further research.

Keywords: *Lacerta vivipara*, mitochondrial DNA, quaternary, viviparity evolution

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Introduction

Lacerta vivipara is a bimodal reproductive lizard species, with allopatric oviparous (egg-laying + incubation *in natura*) and viviparous (gestation + parturition) populations. Viviparous populations of this species are widely distributed and range from central France (Massif Central) and the British Isles up to Scandinavia and Russia. Oviparous populations are isolated in the extreme southwestern part of the distribution area: in the Pyrenean mountains and in Aquitaine in southwest France, and in northwest Spain (Heulin *et al*. 1991, 1993, 1997). No contact zone has been found between oviparous and viviparous populations. The characteristics of viviparity (with persistence of nonfunctional oviparous structures), morphological and ecological resemblances, small genetic distances calculated from allozyme studies, and successful experimental (laboratory) hybridizations all indicate that oviparous and viviparous strains are very closely related (Heulin *et al*. 1993; Arrayago *et al*. 1996; Guillaume *et al*. 1997). This also suggests that the separation between the two forms is probably very recent.

Specifically, studies of allozymes revealed that, despite the existence of diagnostic alleles separating oviparous (aspartate transaminase enzyme, ATA-150 or 200) from viviparous (ATA-100) lizards for all the populations of *L. vivipara* studied so far (in France, Spain and Bulgaria), the overall genetic differentiation (Nei's genetic distance $D = 0.102$, for 13 polymorphic loci) between oviparous and viviparous animals remained small (Guillaume *et al*. 1997). According to this value ($D = 0.102$) and to the available calibration clocks of Nei's genetic distance (Sarich 1977; Wilson *et al*. 1977; Nei 1987), the divergence between oviparous and viviparous lineages of *L. vivipara* could have occurred between 0.5 and 2 million years ago. That is during the Pleistocene.

Hence, it seemed worthwhile to examine whether another kind of genetic marker could improve our understanding of the genetic differentiation between the oviparous and viviparous strains of *L. vivipara*. The aim of the present study was to provide a preliminary insight into the divergence of some mitochondrial DNA (mtDNA) sequences between the two reproductive forms.

Materials and methods

Tissue samples were obtained from six oviparous and 16 viviparous specimens of *Lacerta vivipara* preserved in 95%

Correspondence: B. Heulin. Fax: +33-02-99-61-81-87; E-mail: benoit.heulin@univ-rennes1.fr

Table 1 Origin, mitochondrial DNA haplotypes and GenBank Accession nos of the analysed specimens

Specimen code	Locality, country	Haplotype code	GenBank Accession no.
Oviparous			
OF1	Louvie, North of Vallée d'Ossau, Pyrénées, France	OH1	AF086955
OF23	Louvie, North of Vallée d'Ossau, Pyrénées, France	OH1	AF086958
OF2	Brousset, South of Vallée d'Ossau, Pyrénées, France	OH2	AF086957
OF17	Pourtalet, South of Vallée d'Ossau, Pyrénées, France–Spain	OH3	AF086956
OE1	Puerto de Letariegos, Cantabric mountains, Spain	OH2	AF086953
OE2	Puerto de Tarna, Cantabric mountains, Spain	OH2	AF086954
Viviparous			
VB1	Rila mountains, Bulgaria	VH1	AF086959
VB8	Vitocha mountains, Bulgaria	VH1	AF086962
VB10	Balkan mountains, Bulgaria	VH2	AF086960
VB14	Pirin mountains, Bulgaria	VH1	AF086961
VF1	Paimpont, Bretagne, France	VH1	AF086963
VF10	Paimpont, Bretagne, France	VH1	AF086964
VF11	Paimpont, Bretagne, France	VH3	AF086965
VF12	Paimpont, Bretagne, France	VH1	AF086966
VF14	Frasnes, Jura, France	VH1	AF086967
VF15	Haute Savoie, Alps, France	VH1	AF086968
VF16	Mont Lozère, Cévennes, France	VH1	AF086969
VN1	Overasseltse-Haterste Vennen, The Netherlands	VH4	AF086970
VS1	Vallorbe, Canton de Vaud, Switzerland	VH1	AF086971
VS2	Chatel Saint Denis, Canton de Fribourg, Switzerland	VH4	AF086972
VSU1	Umea, Sweden	VH1	AF086973
VT1	Trebon, south Bohemia, Czech Republic	VH1	AF086974

ethanol (list of origins in Table 1). Two species of green lizards, *L. viridis* (GenBank Accession no. AF086952) and *L. lepida* (GenBank Accession no. AF042561), were used as outgroups.

DNA was extracted from small amounts of the tails (procedures in Hedges *et al.* (1991)). A fragment of the mitochondrial 16s rRNA gene was amplified using primers 984 and 986 (Clary & Wolstenholme 1985). The sequencing of double-stranded DNA was performed in both directions on an Applied Biosystems (Perkin-Elmer) 377 automated DNA sequencer (Genome Express). Sequence alignments for 336 bp were made using CLUSTAL W, version 1.7. Phylogenetic analyses (excluding positions with insertion/deletion) were performed with PAUP* version 4.0d64. A neighbour-joining analysis was performed and a bootstrap consensus tree (2000 pseudoreplicates) was generated, using Jukes–Cantor distances. The average sequence divergence and the net nucleotide divergence (*Da*), calculated from pairwise distances (Jukes–Cantor), were obtained from DNASP software. The net nucleotide divergence (*Da*) between oviparous and viviparous forms (i.e. between-form variation corrected for within-form variation) can be used in calculating the group splitting time (Nei 1987).

Results

From a total of 336 bases aligned unambiguously (Fig. 1), 55 sites were found to be variable in the whole (ingroup + outgroup) data set, and 18 in the ingroup data set. Twelve of the ingroup polymorphic sites were polymorphic in the oviparous group but monomorphic in the viviparous group, two were polymorphic in the viviparous group but monomorphic in the oviparous group, and four corresponded to fixed differences between the oviparous and viviparous groups.

Three distinct haplotypes (OH1, OH2 and OH3 representing 33.3%, 50% and 16.7%, respectively) were observed in the six oviparous lizards studied. Four distinct haplotypes (VH1, VH2, VH3 and VH4 representing 75%, 6.3%, 6.3% and 12.5%, respectively) were observed in the 16 viviparous lizards studied (Table 1). The bootstrap consensus tree of haplotypes (Fig. 2) robustly supported the monophyly of *Lacerta vivipara* (100% bootstrap value). The oviparous and the viviparous haplotypes branched off separately in the tree (Fig. 2), although the monophyletic nature of the oviparous group was less well supported (65% bootstrap value) than that of the viviparous group (95% bootstrap value). Nevertheless, there were four diagnostic mutations, in positions 110, 183, 248 and 305, unambiguously separating the three oviparous (OH) haplotypes from the four viviparous (VH) haplotypes (Fig. 1). The average sequence divergence, calculated from the pairwise distances (Jukes–Cantor), was 1.35 ± 0.65% (mean ± standard deviation (SD)) within the oviparous group, 0.73 ± 0.04%

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          1                                                          60
Lvir      ATGAGGACTA AACTGTCTCT TGCGAACTGG CCAATGAAAT TGATTTTCCA GTACAAAAGC
Llep      .......... .......... ...AGGTA-A .......... ....A.C... ..........
OH1       .......... .T........ .A..G..-AA .......... .A..C..T.. ..........
OH2       .......... .T........ .A..G..CAA .......... .A..C..T.. ..........
OH3       .......... .TA....... .A..G..CAA .......... .A..C..T.. ..........
VH1       .......... .T........ .A..G..CAA .......... .A..C..T.. ..........
VH2       .......... .T........ .A..GG.CAA .......... .A..C..T.. ..........
VH3       .......... .T........ .A..G..CAA .......... .A..C..T.. ..........
VH4       .......... .T........ .A..G..CAA .......... .A..C..T.. ..........

                                                          *          120
Lvir      TGGAATATAC CCATAAGACG AGAAGACCCT GTGGAGCTTC AAA-ACCAAA TCACCCAGCA
Llep      ...G...A.T .......... .......... .........T ...AC.A.GC C....A.AT.
OH1       ..A....A.A A......... .......... .......... ....C.A..C A.....T...
OH2       ..A....A.A A......... .......... .......... ....C.A..C A.....T...
OH3       ..A....A.A A......... .......... .......... ....C.A..C A.....T.A.
VH1       ..A....A.A A......... .......... .......... ....C.A... A.....T...
VH2       ..A....A.A A......... .......... .......... ....C.A... A.....T...
VH3       ..A....A.A A......... .......... .......... ....C.A... A.....T...
VH4       ..A....A.A A......... ........-. .......... ....C.A... A.....T...
                                                                    *
                                                                    180
Llep      TGCTCATTGG ATTTTTAGTT GGGGCGACTT CGGAATATAG TAAAACTTCC AAAATAAATT
OH1       ..GC.C.... .....C.... .........C T...G..A.A C.....C... ..G.A...C.
OH2       .....C.CA. G......... .......... ....GC.C.A ......C... .....----.
OH3       .....C.CA. G......... .......... ....GC.C.A ......C... .....---..
VH1       .....C.AA. G....C..G. .......... ....GC.C.A ......C... .....---..
VH2       .....C.CA. G......... .......... ....GC.C.A ......C... .....---C.
VH3       .....C.CA. G......... .......... ....GC.C.A ......C... .....---C.
VH4       .....C.CA. G......... .......... ....GC.C.A ......C... .....---C.
          .....C.CA. G......... .......... ....GC.C.A ......C... .....---C.

                                                                    240
Lvir      TATTTCATCA AGATTAACAC ACCAAAGTTT CTTTATAGAC CCAGCAACTA ATTTT--ATT
Llep      .T----..T. ....C..... ......A.A. .AAC.C.-.. .......TAC CCACCCA...
OH1       .T.A..T... .........A ......AAC. T.A.-..... .......TGT ..GAC.....
OH2       .T.A..T... .........A ......AAC. T.A.-..... .......TGT ..AAC.....
OH3       .T.A..T... .........A ......AAC. T.A.-..... .......TGT ..AAC.....
VH1       .T-A..T... .........A ......AAC. T.A.-..... .......TGT ..AAC.....
VH2       .T-A..T... .........A ......AAC. T.A.-..... .......TGT ..AAC.....
VH3       .T-A..T... .........A ......AAC. T.A.-..... .......TGT ..AAC.....
VH4       .T-A..T... .........A ......AAC. T.A.-..... .......TGT ..AAC.....

                *                                                   300
Lvir      TGTCTGACCA ATGAACCAAG TTACCCCAGG GATAACAGCG CAATCTCCTT TTAGAGTCCA
Llep      ..C....TT. .C........ .......... .......... .C...C.... .........C
OH1       -......T.. C......... .......... .......... .C...C...C C......T..
OH2       -......T.. C......... .......... .......... .C...C...C C.........
OH3       -......T.. C......... .......... .......... .C...C...C C.........
VH1       -......... C......... .......... .......... .C...C...C C.........
VH2       -......... C......... .......... .......... .C...C...C C.........
VH3       -......... C......... .......... .......... .C...C...C C.........
VH4       -......... C......... .......... .......... .C...C...C C.........

              *                                 336
Lvir      TATCGACAAG GGGGTTTACG ACCTCGATGT TGGATC
Llep      .......... .......... .......... ......
OH1       .......... .......... .......... ......
OH2       .......... .......... .......... ......
OH3       ...A...... .......... .....C.GCA ......
VH1       ....A..... .......... .......... ......
VH2       ....A..... .......... .......... ......
VH3       ....A..... .........C .......... ......
VH4       ....A..... .......... .......... ......
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Fig. 1 DNA sequence alignments of portion of the 16S rRNA gene, for the two outgroups (Lvir = *Lacerta viridis*, Llep = *L. lepida*), and for the four viviparous (VH) and three oviparous (OH) haplotypes identified. A dot (.) denotes identity with the first sequence; a dash (–) denotes a gap; N denotes an ambiguity; * indicates the four diagnostic mutations between oviparous and viviparous haplotypes.

within the viviparous group and 2.03 ± 0.56% between the oviparous and the viviparous group. The net nucleotide divergence (*Da*) between the oviparous and viviparous groups was 1.3 ± 0.5%.

Discussion

Our analysis of mtDNA (16S rRNA gene) variation clearly separated two groups of haplotypes, OH and VH, which were, respectively, characteristic of the oviparous and viviparous forms of *Lacerta vivipara*. Similarly, the allozymes previously studied showed that it was possible to separate oviparous and viviparous lizards according to their ATA alleles (Guillaume *et al*. 1997). All our data showed that the variation of reproductive mode, allozyme frequency and mtDNA haplotype were not related in a simple manner to geographical distances between populations, nor to latitude. The genetic distance calculated from allozymes data was $D = 0.12$ between French oviparous and viviparous lizards, whereas it was approximately half that value ($D = 0.056$) between French and Bulgarian viviparous lizards (Guillaume *et al*. 1997). It is also worth noting that, contrary to what is to be expected from geographical distance, the base sequences of the French viviparous lizards were closer to those of other viviparous populations, however distant (e.g. Bulgarian or Swedish populations),

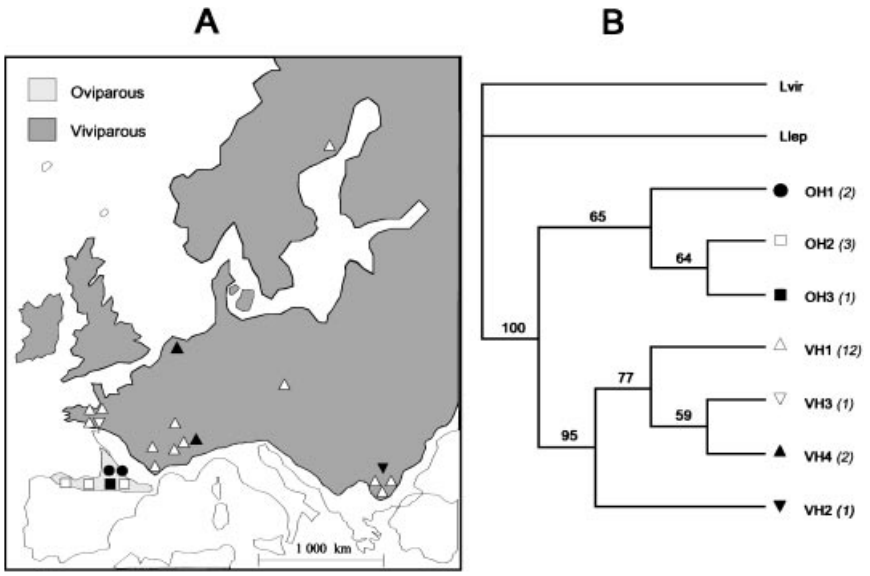

Fig. 2 Geographical distribution (A) and bootstrap consensus tree (B) derived from neighbour-joining analysis of haplotypes. Oviparous haplotypes (OH) and viviparous haplotypes (VH) as in Table 1 and Fig. 1. In the consensus tree: the number of individuals showing each haplotype is given in brackets; numbers above branches indicate the percentage of bootstrap replicates out of 2000 that support each branch node.

than to those of neighbouring oviparous populations. These studies underline a major phylogeographical break in southern France and an asymmetrical (west/east) pattern of distribution in southern Europe: lizards from the southwest limit of the distribution range are oviparous, exhibit the mtDNA haplotypes OH and the ATA-150 or 200 alleles, whereas, at a comparable latitude on the southern limit of the range, Bulgarian lizards are viviparous, exhibit the mtDNA haplotypes VH and the ATA-100 allele characteristic of other distant (French) viviparous populations. These asymmetric characteristics support, or at least do not contradict, an asymmetric biogeographical scenario predicting that viviparity could have evolved and been rapidly propagated in eastern populations of *L. vivipara* during the glacial phases of the Pleistocene, while oviparity remained unchanged in an isolated southwestern refuge (Heulin *et al*. 1993; Guillaume *et al*. 1997).

Assuming an overall rate of change of mtDNA of 2% per million years (Wilson *et al*. 1985), the net nucleotide divergence (*Da*) $1.3 \pm 0.5\%$ between oviparous and viviparous strains of *L. vivipara* could correspond to a divergence time of 0.65 ± 0.26 million years. This estimate should be considered with caution: further research on various populations with known separation time (for example: Britain/continent) would be necessary to calibrate directly mtDNA divergence/unit time in *L. vivipara*, and to verify whether this calibration is consistent with the above estimate (2% per million years). However, it is worth noting that our estimates of divergence time based on mtDNA and on allozyme data (see Introduction) seem convergent: they both suggest that the differentiation between the oviparous and viviparous lineages of *L. vivipara* could have occurred during the Pleistocene. The colder climates associated with the glacial phases of this period might have played a role in this evolution (Heulin *et al*. 1993; Guillaume *et al*. 1997).

Further research is needed to test our hypothetical scenario of the evolution of viviparity in *L. vivipara* more thoroughly. Its verification would provide interesting empirical support of: (1) the model of allopatric evolution during the quaternary glaciations in Europe (for a review see Hewitt (1996) and Taberlet *et al*. (1998)); and (2) the 'cold climate model' positing that cold climatic conditions are one of the most important selective forces acting in favour of the evolution of viviparity in reptiles (for a review see Shine (1985) and Heulin *et al*. (1991)). Future research should attempt to collect data allowing direct calibration of the divergence rate (see above), to improve the resolution of the phylogeny by analysing other mtDNA segments (for example, NADH gene, D-loop segment), and to investigate more thoroughly the phylogeographical history of the viviparous lineages (centre of origin and dispersion) by analysing larger samples.

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This work is part of a continuing study aimed at understanding the adaptive significance and the phylogeographical history of the evolution of viviparity in the lizard, *Lacerta vivipara*. Previous research focused on aspects of enzymatic differentiation, interbreeding possibilities, and demographic differences between the oviparous and viviparous strains of the species.
