



**HAL**  
open science

## Development of thirty-four new microsatellite loci and multiplexing of seven existing loci for *Zootoca vivipara* (Squamata: Lacertidae)

Jose Luis Horreo, Maria Luisa Peláez, Teresa Suárez, Benoit Heulin, Patrick Stefan Fitze

### ► To cite this version:

Jose Luis Horreo, Maria Luisa Peláez, Teresa Suárez, Benoit Heulin, Patrick Stefan Fitze. Development of thirty-four new microsatellite loci and multiplexing of seven existing loci for *Zootoca vivipara* (Squamata: Lacertidae). *Phyllomedusa: Journal of Herpetology*, 2017, 16 (1), pp.89-96. 10.11606/issn.2316-9079.v16i1p89-96 . hal-01580360

**HAL Id: hal-01580360**

**<https://univ-rennes.hal.science/hal-01580360>**

Submitted on 20 Jun 2018

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# Development of thirty-four new microsatellite loci and multiplexing of seven existing loci for *Zootoca vivipara* (Squamata: Lacertidae)

Jose Luis Horreo,<sup>1,2</sup> Maria Luisa Peláez,<sup>3,4</sup> Teresa Suárez,<sup>4</sup> Benoit Heulin,<sup>5</sup> and Patrick Stefan Fitze<sup>1,3</sup>

<sup>1</sup> Department of Ecology and Evolution (DEE), University of Lausanne, 1015 Lausanne, Switzerland. E-mail: horreojose@gmail.com, psfitze@gmail.com.

<sup>2</sup> Department of Biodiversity and Ecologic Restoration, Instituto Pirenaico de Ecología (IPE-CSIC), Avenida Nuestra Señora de la Victoria 16, 22700 Jaca, Spain.

<sup>3</sup> Department of Biodiversity and Evolutionary Biology, Museo Nacional de Ciencias Naturales (MNCN-CSIC), José Gutiérrez Abascal 2, 28006 Madrid, Spain.

<sup>4</sup> Department of Cellular and Molecular Physiopathology, Centro de Investigaciones Biológicas (CSIC), Calle Ramiro de Maetzu 9, E-28040 Madrid, Spain. E-mails: maaller@yahoo.es, teresa@cib.csic.es.

<sup>5</sup> Station Biologique, CNRS UMR 6553, Paimpont 35380, France. E-mail: benoit.heulin@univ-rennes1.fr.

## Abstract

**Development of thirty-four new microsatellite loci and multiplexing of seven existing loci for *Zootoca vivipara* (Squamata: Lacertidae).** Few microsatellite loci exist for the European common lizard, *Zootoca vivipara*, a common model species in studies of population dynamics, sexual selection, population genetics, parity evolution, and physiology. The existing primers did not amplify in all lineages, and multiplexes were not optimized. A total of 34 new polymorphic microsatellite markers have been developed for this species and tested in 64 specimens belonging to oviparous and viviparous clades (B and D). The microsatellites were combined into seven different multiplexes. Results showed that all but one loci successfully amplified in all samples and both clades. The number of alleles detected per locus ranged 7–22 alleles and the effective number 1.58–7.82. The observed heterozygosity ranged 0.312–0.930, showing that all loci were highly variable. Oviparous and viviparous clades exhibited significant genetic differences (in  $F_{ST}$ ). In addition to these new markers, the seven previously published and widely used microsatellite loci have been multiplexed and tested in oviparous clades. These innovations will allow for timesaving and robust analyses in *Zootoca vivipara*, boosting evolutionary and population studies and easing paternity analyses.

**Keywords:** European Common Lizard, *Lacerta vivipara*, multiplex, NGS, nuclear DNA, viviparity, oviparity.

---

Received 1 August 2016  
Accepted 7 November 2016  
Distributed June 2017

## Resumo

**Desenvolvimento de trinta e quatro novos locos de microssatélites e otimização de PCR multiplex de sete locos existentes para *Zootoca vivipara* (Squamata: Lacertidae).** Existem poucos locos de microssatélites para o lagarto-comum-europeu, *Zootoca vivipara*, uma espécie-modelo comum em estudos de dinâmica de populações, seleção sexual, genética de populações, evolução do modo reprodutivo e fisiologia. Os oligonucleotídeos existentes não amplificaram em todas as linhagens, e a reação em cadeia da polimerase (PCR) multiplex não foi otimizada. Um total de 34 novos marcadores polimórficos de microssatélite foi desenvolvido para essa espécie e testado em 64 espécimes dos clados ovíparo e vivíparo (B e D). Os microssatélites foram combinados em sete diferentes agrupamentos. Os resultados mostraram que todos os locos, com uma única exceção, amplificaram com sucesso em todas as amostras e em ambos os clados. O número de alelos detectado por loco variou entre 7 e 22 e o número efetivo, entre 1,58 e 7,82. A heterozigosidade observada variou de 0,312 a 0,930, mostrando que todos os locos foram altamente variáveis. Os clados ovíparo e vivíparo exibiram diferenças genéticas significativas (em  $F_{ST}$ ). Além desses novos marcadores, os sete locos de microssatélite previamente publicados e largamente utilizados foram otimizados em PCR multiplex e testados em clados ovíparos. Essas inovações permitirão análises rápidas e robustas em *Zootoca vivipara*, impulsionando estudos evolutivos e populacionais e facilitando análises de paternidade.

**Palavras-chave:** DNA nuclear, *Lacerta vivipara*, lagarto-comum-europeu, PCR multiplex, NGS, oviparidade, viviparidade.

## Introduction

The European Common Lizard, *Zootoca vivipara* (Lichtenstein, 1823), is the most widely distributed reptile species of the world (Guillaume *et al.* 2006). Its distribution ranges from Ireland and western Spain in the west to Japan (Hokaido) in the east, and from the Balkans and Pyrenees in the south to the polar circle in the north; there are several clearly distinct lineages across Eurasia (Clades A–F; Surget-Groba *et al.* 2006). One of these clades has been proposed to be a new species (Clade A; Cornetti *et al.* 2015a). The species is reproductively bimodal; two lineages are oviparous (Clades A, B) and all the other clades are viviparous (Clades C–F). Owing to the range and reproductive bimodality, *Z. vivipara* is a highly interesting species in terms of its evolution, geography, and population dynamics. Evolutionary studies (e.g., Surget-Groba *et al.* 2006, Cornetti *et al.* 2015b) and studies analyzing individual paternities (e.g., Laloï *et al.* 2004, Fitze *et al.* 2005, 2008, Richard *et al.* 2005, 2009, Eizaguirre *et al.* 2007, Fitze and Le

Galliard 2008, Le Galliard *et al.* 2008, San-Jose *et al.* 2014, Breedveld and Fitze 2016) have been conducted on this species, whereas population genetic studies are rather rare. Such studies need reliable, time- and cost-efficient genetic tools; however, few microsatellite loci had been developed, protocols did not work in all lineages (Boudjemadi *et al.* 1999, Remon *et al.* 2008, Molecular Ecology Resources Primer Development Consortium 2011), and multiplexes were not optimized (Laloï *et al.* 2004). Efficient multiplexing (co-amplification of several loci in a single PCR) can significantly decrease the cost of genotyping and increase throughput of microsatellite loci (Guichoux *et al.* 2011).

We have used next generation sequencing methods to develop a large new panel of microsatellite loci and generated cost-effective multiplexes for new and existing microsatellite loci.

## Materials and Methods

For the development of new microsatellite loci, a total of 64 *Zootoca vivipara* individuals (Table 1) were sampled. Thirty individuals

belonged to Clade D (the Eastern Viviparous Clade) and another 34 belonged to Clade B (the Western Oviparous Clade; Surget-Groba *et al.* 2001). Their genomic DNA was extracted from ethanol-preserved lizard tissue using DNeasy Blood & Tissue Kit (Qiagen, Verlo, Netherlands), which produces DNA of better quality than other methods (Horreo *et al.* 2015).

The genomic DNA of one specimen of the Western Oviparous Clade was enriched following the protocol of Santana *et al.* (2009). A 454 library was obtained from a partial run using the 454 Life Sciences/Roche GS-FLX genome sequence system (Roche Applied Science) (Margulies *et al.* 2005). A total of 709,643 sequence reads (153,531,887 base pairs) was generated, of which 38,000 contained a minimum of five tandem repeats: 17,297 dinucleotides; 16,432 trinucleotides; 3,750 tetranucleotides; 337 pentanucleotides; and 184 hexanucleotides. Ninety-eight of these sequences (24, 58, and 16 tri-, tetra-, and penta-nucleotides, respectively) were selected to design primers using PRIMER3 (Rozen and Skaletsky 2000). Selected repeats were amplified in eight specimens belonging to all clades described (one individual per lineage: A from Italy, B1 from Spain, B2 from France, C and E from Austria, D from Romania, F from Hungary, and G from Galicia Spain) (Surget-Groba *et al.* 2001, Milá *et al.* 2013), using 10–100 ng of DNA in a total reaction volume of 10 µL and the Taq DNA Polymerase (5PRIME GmbH, Germany). The proportions of the primers, Taq, 5Prime mastermix, magnesium, and molecular-biology grade-water followed the manufacturer's protocol. The PCR started with an initial denaturation step at 95°C for 5 min,

followed by 35 cycles of denaturation at 95°C for 20 s, annealing at 57°C for 20 s, extension at 70°C for 30 s, and a final extension of 72°C for 10 min. PCR products were visualized in 2% agarose gels. If the amplification was successful in all eight specimens, the Forward primer was labelled with a fluorescent-dye (VIC, NED, PET and FAM). Thereafter, successful loci were amplified in a subset consisting of 16 samples of the 64 studied individuals (B and D; Table 1). PCR conditions corresponded to those described above and the total reaction volume was 25 µL. PCR products were visualized using an automatic sequencer (an ABI 3100, Applied Biosystem) and the software GENEMAPPER 4.0 (Applied Biosystems). Thirty-four loci (6, 19 and 9 tri-, tetra-, and penta-nucleotides, respectively; Table 2) were polymorphic and exhibited consistently good electropherograms.

The 34 microsatellite loci were combined in seven multiplexes (Table 2), and their amplification followed the “QUIAGEN Type-it Microsatellite PCR Kit” protocol and an annealing temperature of 57°C. In these multiplexes, 31 primers were employed using the manufacturer's protocol and the proportions of ZV12 (Multiplex 3), ZV29 (Multiplex 6), and ZV32 (Multiplex 7) were 1.5 times higher than those of the other primers. Multiplexes were then amplified in the 64 samples and Tandem v.1.09 (Matschiner and Salzburger 2009) was used for allele binning. Thirty-three loci successfully amplified in all 64 samples. Locus ZV14 amplified in all oviparous samples (Clade B), but it did not amplify in most of the 30 viviparous specimens (Clade D; Table 1) despite its amplification during the development of the method.

**Table 1.** Sampling details for *Zootoca vivipara*. The clades have been named according to Surget-Groba *et al.* (2001). *N* = sample size. Acronyms: ND = newly developed microsatellites; EM = existing microsatellites.

Microsatellites	<i>N</i>	Clade	Country	Reproductive mode
ND	30	D	Hungary/Romania	Viviparous
ND	34	B	France/Spain	Oviparous
EM	12	B	Spain	Oviparous

**Table 2.** Genetic variability, primers, and repeat motif of the 34 newly developed and the seven previously known (Boudjemadi et al. 1999) microsatellite loci of *Zootoca vivipara* (ZV1–ZV34 and LV 1–4, respectively). The genetic variability of the newly developed loci was calculated based on 64 specimens and the 7 previously existing (named “Lv”) on another 12 specimens. Acronyms: Na = number of alleles per locus; Eff\_Na = effective number of alleles per locus; H<sub>E</sub> = expected heterozygosity; H<sub>O</sub> = observed heterozygosity; Motif = repetition motif; Color = used fluorescent dye; Mn = multiplex number.

Locus	Size (bp)	Eff_Na	Na	H <sub>O</sub>	H <sub>E</sub>	Forward Primer (5'-3')	Reverse Primer (5'-3')	Motif	Color	Mn
ZV1	141–225	7.82	20	0.732	0.904	TGACTCCACTTTTGCTGGCAT	CACATGTGAAGCTGCATCTAAG	(ATCT) <sub>12</sub>	NED	1
ZV2	191–227	5.22	10	0.501	0.840	GAGCACCTCCAAATTTTACTGTT	GTTTGTTCACCCACACTG	(GATA) <sub>11</sub>	FAM	1
ZV3	100–176	5.61	19	0.765	0.846	TGCCTTGAATCCACATCTC	TGAGAAGATACCCGGTCAGG	(GATA) <sub>10</sub>	FAM	1
ZV4	117–157	5.19	11	0.788	0.830	CACCTTGACGGGTTTGGACT	CCATCCCTGTAGAAAAGGCA	(AACA) <sub>11</sub>	VIC	1
ZV5	185–248	5.07	12	0.559	0.833	AAGTTGCAGGGAAGTAGGCA	TGTTGCCATCTATTGTCTCA	(TTA) <sub>16</sub>	VIC	1
ZV6	179–239	5.27	13	0.724	0.835	ACCAACAACACCTCAACCT	TCTGGACATATAGCCTGGTCC	(TATTC) <sub>11</sub>	FAM	2
ZV7	240–348	5.30	18	0.694	0.837	CAGAGCTGAGCCTGGAAGTT	AGCACCCGAGAGTGAAAAC	(AGAT) <sub>10</sub>	FAM	2
ZV8	215–287	5.49	14	0.811	0.841	CTCCAATTTGGCAGGTGAAT	TCCTCCTTGCTGCCAAGACT	(GTTT) <sub>11</sub>	NED	2
ZV9	203–242	6.01	12	0.747	0.859	AGGTGTAGAGAATGGGCACG	ATGGCAGGTATCTGGAGCAT	(TAG) <sub>13</sub>	PET	2
ZV10	119–224	6.21	17	0.705	0.869	AACCATCTTGGGTGCTTTGG	TCAGGAATCTTTGGATGGAGA	(ATAAC) <sub>19</sub>	VIC	2
ZV11	220–300	4.90	16	0.774	0.819	CAAGAGTCTCTCCACAGCAC	TGTGTCATGCATGAAATG	(GATA) <sub>11</sub>	PET	3
ZV12	121–205	2.64	13	0.609	0.639	TGTTTGTTAATCCCCCGTC	GGAGAAGCAATGGAAACTGG	(TTGT) <sub>9</sub>	PET	3
ZV13	203–245	3.80	14	0.577	0.762	TGAGCCACAGTCATCAAAGG	TCCAACCTAGAGGCTCTCTG	(TAT) <sub>16</sub>	FAM	3
ZV14	140–164	3.00	7	0.710	0.796	CCCACCTTCACCAATATGT	CCAGATGAGCGGGGTATAAA	(AAC) <sub>11</sub>	FAM	3
ZV15	110–210	6.67	20	0.695	0.880	TGTAAGTATGATGCAAAACACC	CATTAGCGGCAGATTCAT	(AGTAT) <sub>16</sub>	PET	4
ZV16	177–241	3.12	11	0.893	0.692	TGTAATCTGATCCCGTGTCTG	CTGAAGCAGCCCTGTTTAG	(ACAT) <sub>9</sub>	FAM	4
ZV17	171–236	3.85	14	0.649	0.766	TGAAATGCTTCTCCCTCTG	TTCGAAAAGCAGGAATTGAA	(ATGTA) <sub>10</sub>	NED	4
ZV18	141–206	5.67	14	0.816	0.846	CAGTATAGCCTTGGGGTCCA	TCTCAGGCCCTCGTTTCACT	(TTTCT) <sub>9</sub>	VIC	4
ZV19	153–201	6.75	11	0.836	0.876	TCCAGGTGTACACTGGGGCTA	TGGGCTAAAAGCCCACTAGAGC	(TGTA) <sub>9</sub>	NED	5

Table 2. Continued.

Locus	Size (bp)	Eff_Na	Na	Ho	He	Forward Primer (5'-3')	Reverse Primer (5'-3')	Motif	Color	Mn
ZV20	126–174	5.64	12	0.579	0.852	AGGGTGTTTCTGTGAGTCGG	CCTTCCCTCTTTTCCAG	(AAG) <sub>9</sub>	PET	5
ZV21	095–139	5.10	10	0.649	0.832	CTTGCAACACCAGGAACCTCA	TGGTTAGACCACAGCACCA	(GATA) <sub>9</sub>	FAM	5
ZV22	152–204	1.58	9	0.312	0.383	GGCAAGGTAACAATTTGGCAT	TGCTTACAGCAATTCGATG	(GATA) <sub>12</sub>	FAM	5
ZV23	212–256	3.00	12	0.619	0.686	GGAGGCTACTATTGGGGGCTC	AGCAATCAATGTGAGTTATGGC	(GATA) <sub>12</sub>	FAM	5
ZV24	164–224	3.92	10	0.727	0.766	GCCATGAAAAGTCGTGTGTG	TGTAACCGTCCACAGAACT	(GTTT) <sub>10</sub>	VIC	5
ZV25	081–126	4.14	9	0.777	0.779	AGAGCATGAGGTGAGAGGGA	CCCCACCCACATATTACAG	(TTCTA) <sub>10</sub>	FAM	6
ZV26	213–283	4.99	14	0.683	0.825	GCCAAAGCAATTTCAAGTATGT	GAGTCACTCCATTGTGCAG	(TTCTA) <sub>10</sub>	FAM	6
ZV27	104–149	3.49	10	0.689	0.733	GATGGCAAGTGTGGCAGTAA	TGCTTGAATGAGGTGTGGA	(TATAG) <sub>10</sub>	VIC	6
ZV28	154–198	5.18	13	0.746	0.831	CATGGTTCCAACAATCCCTT	CTGCTGCTTGTGGAACGTAA	(TTA) <sub>16</sub>	FAM	6
ZV29	085–165	3.10	11	0.418	0.704	TCACATGAGTCAACGGCCT	GTGAGTCATCTCGGACTGGA	(TGT) <sub>9</sub>	PET	6
ZV30	113–189	5.81	13	0.832	0.854	CCCGGAGTAAAGAGAGGTA	GTGCAAGTGGTGTGATGTG	(TCTT) <sub>10</sub>	NED	7
ZV31	111–151	6.12	11	0.506	0.870	CAGAAGAATGCCACTCTGGA	AGGTCTCTTGGCCACTTTGA	(TTTC) <sub>10</sub>	PET	7
ZV32	202–298	6.45	20	0.930	0.869	CAGGTTAAGAACCGATCTCCA	AGCCTGCACATCCCAGTATC	(GATA) <sub>10</sub>	FAM	7
ZV33	105–137	4.01	8	0.708	0.773	GACACCCCTTGTGCCTCATT	TCCCTCCCTGTCTGAAAGAA	(TGT) <sub>9</sub>	FAM	7
ZV34	127–208	6.04	22	0.906	0.856	GGAGATTGTTAGCCGCCTTTG	CAGCAATCTAGTCTGCTTCCA	(TCT) <sub>22</sub>	VIC	7
Lv-4-72	122–146	7.78	7	0.917	0.909	TGCCGTCAAAAGCCAAACAAG	CCGCCCTCCACAATACACCT	(AC) <sub>18</sub>	NED	8
Lv-2-145	296–310	3.39	4	0.667	0.739	CCATTGTAGGCTCAGGTTG	GGTGCCAACTATGCAGG	(TG) <sub>20</sub>	NED	8
Lv-4-x	167–190	6.86	9	0.917	0.890	TGGATTAGAGGCTGAAAGAG	TGAGAAGGCTGTGAATGTG	(GT) <sub>22</sub>	NED	8
Lv-4-∞	157–181	5.33	7	1.000	0.841	ATTACTGCAGGGAAACAGA	CCAGAAAGCAITTTCCACAC	(AC) <sub>14</sub> (GA) <sub>11</sub> (CAGAGA) <sub>9</sub> CAGAT(AG) <sub>3</sub>	FAM	8
Lv-1-139	134–140	1.97	3	0.333	0.527	GGGAGATGTTGCCTTATGG	CTGCATTTAAAACCTGAAGTGGC	(CA) <sub>26</sub>	FAM	8
Lv-4-115	125–158	5.14	8	0.917	0.837	CCCAACCCACAAGACTGA	CCGGTGTACTCAATGATGCT	(CA) <sub>17</sub>	PET	8
Lv-3-19	134–162	9.29	12	1.000	0.928	GCTGTGCTATTTTGTATGCTTA	CCCTGTGACTGTCTCCAGAG	(AC) <sub>22</sub>	VIC	8

In addition to all this, seven previously published (Lv-3-19, Lv-4-72, Lv-4-alpha, Lv-2-145, Lv-4-X, Lv-4-115, Lv-1-139; Table 2) (Boudjemadi *et al.* 1999) and commonly used microsatellite loci (e.g., Breedveld and Fitze 2016) were multiplexed to save money and time. PCR conditions were the same as those described above, except that annealing was conducted at 58°C. In the newly developed multiplex, the primers Lv-4-X, Lv-4-alpha, and Lv-3-19 were employed in a proportion three times higher than the rest. A set of 12 oviparous samples was amplified using the multiplex protocol and the previous protocols (following Laloi *et al.* 2004).

The genetic variability of all the loci (number of alleles per locus, effective number of alleles per locus, and observed and expected heterozygosity), as well as the genetic differentiation among sample groups ( $F_{ST}$ ) were calculated with GenoDive 2.0b25 (Meirmans and Van Tienderen 2004). The linkage disequilibrium among pairs of loci was calculated with GENEPOP v.4 (Rousset 2008).

## Results

The 34 newly developed loci exhibited high genetic variability (Table 2). The mean number of alleles per locus ( $\pm$  standard deviation, obtained through jackknifing) was  $13.29 \pm 0.63$ ; the effective mean number of alleles per locus was  $4.89 \pm 0.24$ . The mean expected heterozygosity was  $0.79 \pm 0.02$  and the mean observed heterozygosity was  $0.69 \pm 0.02$ . No linkage disequilibrium was detected among pairs of loci. The fixation index ( $F_{ST}$ ) among clades (B and D) using 33 loci was significant ( $p = 0.01$ ,  $F_{ST} = 0.082$ ), indicating that genetic differences among clades can be detected with the newly developed loci.


In the case of the seven previously published loci, both the multiplex protocol developed here and the previous protocols rendered the exactly same genotypes. The mean number of alleles per

locus ( $\pm$  standard deviation) was  $7.57 \pm 1.21$ ; the effective mean number of alleles per locus was  $5.68 \pm 0.95$ . The mean expected heterozygosity was  $0.81 \pm 0.05$  and the mean observed heterozygosity was  $0.82 \pm 0.05$ . Thus, the range of variability of both the newly developed and the old loci was similar ( $A_e$  new: 1.58–7.82;  $A_e$  old: 1.97–9.29). ZV22 was the least variable of the new loci and, of the previously published loci, LV-1-139 was the least variable in the oviparous clade, but not in the viviparous Clade E (Boudjemadi *et al.* 1999).

## Discussion

Thirty-four newly developed, highly polymorphic microsatellite loci (combined in seven different multiplexes) and new multiplexing techniques for seven existing loci (Boudjemadi *et al.* 1999) described here were tested in viviparous and oviparous clades of the European Common Lizard, *Zootoca vivipara*. Thirty-three of the new and all seven old markers amplified in all samples. ZV14 only amplified in the oviparous individuals belonging to Clade B, but not in the viviparous Clade D. Because Clade A is strongly divergent (Cornetti *et al.* 2015a) in addition to the fact that we tested the amplification of the newly developed microsatellite markers in only one individual belonging to Clade A, it may be interesting to test the effectiveness of the new microsatellite markers in this clade further. Previous to this study, only seven microsatellite loci were available for this species (Boudjemadi *et al.* 1999, Remon *et al.* 2008, Molecular Ecology Resources Primer Development Consortium 2011) and their amplification protocol was not optimized. The loci and protocols we developed provide strong, economical tools for evolutionary and population genetic studies, including the identification of population structure and management/conservation units, the estimation of gene flow, effective population sizes, and for other applications including cost-efficient paternity analyses.

## Acknowledgments

Jose L. Horreo was supported by a FICYT Clarín-EU Marie Curie Co-Found (ACA14-26) and a Spanish MINECO postdoc grant FPD1-2013-16116. Project funds were provided by the Swiss National Science Foundation (PPOOP3\_128375, PP00P3\_152929/1 to P. S. Fitze). Benoit Heulin was funded by the French National Research Center (CNRS). The study conducted complies with the current Spanish laws and with ASAB/ABS Guidelines for the Treatment of Animals in Behavioural Research. The authors declare that they have no conflict of interest. 

## References

- Boudjemadi, K., O. Martin, J. C. Simon, and A. Estoup A. 1999. Development and cross-species comparison of microsatellite markers in two lizard species, *Lacerta vivipara* and *Podarcis muralis*. *Molecular Ecology* 8: 518–520.
- Breedveld, M. C. and P. S. Fitze. 2016. The timing and interval of mate encounter affects investment during mating. *Biological Journal of the Linnean Society* 118: 613–617.
- Cornetti, L., F. Belluardo, S. Ghielmi, G. Giovine, G. F. Ficotola, G. Bertorelle, C. Vernesi, and H. C. Hauffe. 2015a. Reproductive isolation between oviparous and viviparous lineages of the Eurasian common lizard *Zootoca vivipara* in a contact zone. *Biological Journal of the Linnean Society* 114: 566–573.
- Cornetti, L., G. F. Ficotola, S. Hoban, and C. Vernesi. 2015b. Genetic and ecological data reveal species boundaries between viviparous and oviparous lizard lineages. *Heredity* 115: 517–526.
- Eizaguirre, C., D. Laloi, M. Massot, M. Richard, P. Federicim, and J. Clobert. 2007. Condition dependence of reproductive strategy and the benefits of polyandry in a viviparous lizard. *Proceedings of the Royal Society B, Biological Sciences* 274: 425–430.
- Fitze, P. S., J. F. Le Galliard, P. Federici, M. Richard, and J. Clobert. 2005. Conflict over multiple-partner mating between males and females of the polygynandrous common lizards. *Evolution* 59: 2451–2459.
- Fitze, P. S., J. Cote, J. P. Martínez-Rica, and J. Clobert. 2008. Determinants of male fitness: disentangling intra- and inter-sexual selection. *Journal of Evolutionary Biology* 21: 246–255.
- Fitze, P. S. and J. F. Le Galliard. 2008. Operational sex ratio, sexual conflict and the intensity of sexual selection. *Ecology Letters* 11: 432–439.
- Guichoux, E., L. Lagache, and S. Wagner, P. Chaumeil, P. Léger, O. Lepais, C. Lepoittevin, T. Malausa, E. Revardel, F. Salin, and R. J. Petit. 2011. Current trends in microsatellite genotyping. *Molecular Ecology Resources* 11: 591–611.
- Guillaume, C. P., B. Heulin, I. Y. Pavlinov, D. V. Semenov, A. Bea, N. Vogrin, and Y. Surget-Groba. 2006. Morphological variations in the common lizard, *Lacerta (Zootoca) vivipara*. *Russian Journal of Herpetology* 13: 1–10.
- Horreo, J. L., M. L. Peláez, and P. S. Fitze. 2015. Skin sheds as a useful DNA source for lizard conservation. *Phyllomedusa* 14: 73–77.
- Laloi, D., M. Richard, J. Lecomte, M. Massot, and J. Clobert. 2004. Multiple paternity in clutches of common lizard *Lacerta vivipara*: data from microsatellite markers. *Molecular Ecology* 13: 719–723.
- Le Galliard, J. F., J. Cote, and P. S. Fitze. 2008. Lifetime and intergenerational fitness consequences of harmful male interactions for female lizards. *Ecology* 89: 56–64.
- Margulies, M., M. Egholm, W. E. Altman, S. Attiya, J. S. Bader, L. A. Bemben, J. Berka, M. S. Braverman, Y. J. Chen, Z. Chen, S. B. Dewell, L. Du, J. M. Fierro, X. V. Gomes, B. C. Godwin, W. He, S. Helgesen, C. H. Ho, G. P. Irzyk, S. C. Jando, M. L. I. Alenquer, T. P. Jarvie, K. B. Jirage, J. B. Kim, J. R. Knight, J. R. Lanza, J. H. Leamon, S. M. Lefkowitz, M. Lei, J. Li, K. L. Lohman, H. Lu, V. B. Makhijani, K. E. McDade, M. P. McKenna, E. W. Myers, E. Nickerson, J. R. Nobile, R. Plant, B. P. Puc, M. T. Ronan, G. T. Roth, G. J. Sarkis, J. F. Simons, J. W. Simpson, M. Srinivasan, K. R. Tartaro, A. Tomasz, K. A. Vogt, G. A. Volkmer, S. H. Wang, Y. Wang, M. P. Weiner, P. Yu, R. F. Begley, and J. M. Rothberg. 2005. Genome sequencing in microfabricated high-density picolitre reactors. *Nature* 437: 376–380.
- Matschiner, M. and W. Salzburger. 2009. TANDEM: integrating automated allele binning into genetics and genomics workflows. *Bioinformatics* 25: 1982–1983.
- Meirmans, P. G. and P. H. Van Tienderen. 2004. GENOTYPE and GENODIVE: two programs for the analysis of genetic diversity of asexual organisms. *Molecular Ecology Resources* 4: 792–794.
- Milá, B., Y. Surget-Groba, B. Heulin, A. Gosá, and P. S. Fitze. 2013. Multilocus phylogeography of the common



- lizard *Zootoca vivipara* at the Ibero-Pyrenean suture zone reveals lowland barriers and high-elevation introgression. *BMC Evolutionary Biology* 13: 192.
- Molecular Ecology Resources Primer Development Consortium. 2011. Permanent genetic resources added to Molecular Ecology, Resources Database – 1 December 2010–31 January 2011. *Molecular Ecology Resources* 11: 586–589.
- Remon, N., M. Vila, P. Galán, and H. Naveira. 2008. Isolation and characterization of polymorphic microsatellite markers in *Iberolacerta monticola*, and cross-species amplification in *Iberolacerta galani* and *Zootoca vivipara*. *Molecular Ecology Resources* 8: 1351–1353.
- Richard, M., M. Lecomte, M. Fraipont, and J. Clobert. 2005. Age-specific mating strategies and reproductive senescence. *Molecular Ecology* 14: 3147–3155.
- Richard, M., S. Losdat, J. Lecomte, M. Fraipont, and J. Clobert. 2009. Optimal level of inbreeding in the common lizard. *Proceedings of the Royal Society B, Biological Sciences* 276: 2779–2786.
- Rousset, F. 2008. Genepop'007: a complete re-implementation of the Genepop software for Windows and Linux. *Molecular Ecology Resources* 8: 103–106.
- Rozen, S. and H. J. Skaletsky. 2000. Primer3 on the WWW for general users and for biologist programmers. Pp 365–386 in S. Misener and S. A. Krawetz (eds.), *Methods in Molecular Biology, Series Bioinformatics Methods and Protocols, Volume 132*. Totowa. Humana Press Inc.
- San-Jose, L. M., M. Peñalver-Alcázar, B. Milá, V. Gonzalez-Jimena, and P.S. Fitze. 2014. Cumulative frequency-dependent selective episodes allow for rapid morph cycles and rock-paper-scissors dynamics in species with overlapping generations. *Proceedings of the Royal Society of London, Series B, Biological Sciences* 281: 20140976.
- Santana, Q. C., M. P. A. Coetzee, E. T. Steenkamp, O. X. Mlonyeni, G. N. A. Hammond, M. J. Wingfield, and B. D. Wingfield. 2009. Microsatellite discovery by deep sequencing of enriched genomic libraries. *BioTechniques* 46: 217–223.
- Surget-Groba, Y., B. Heulin, C. P. Guillaume, R. S. Thorpe, L. Kupriyanova, N. Vogrin, R. Maslak, S. Mazzotti, M. Venczel, I. Ghira, G. Odierna, O. Leontyeva, J. C. Monney, and N. Smith. 2001. Intraspecific phylogeography of *Lacerta vivipara* and the evolution of viviparity. *Molecular Phylogenetics and Evolution* 18: 449–459.
- Surget-Groba, Y., B. Heulin, C. P. Guillaume, M. Puky, D. Semenov, V. Orlova, L. Kupriyanova, I. Ghira, and B. Smajda. 2006. Multiple origins of viviparity, or reversal from viviparity to oviparity? The European Common Lizard (*Zootoca vivipara*, Lacertidae) and the evolution of parity. *Biological Journal of the Linnean Society* 87: 1–11.

Editor: Jaime Bertoluci