Population structure of the oviparous South-West European Common lizard

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Abstract

Gene flow is an important factor determining the evolution of a species, since it directly affects population structure and species' adaptation. Here we investigated population structure, population history, and migration among populations covering the entire distribution of the geographically isolated South-West European common lizard (*Zootoca vivipara louslantzi*) using 34 newly developed polymorphic microsatellite markers. The analyses unravelled the presence of isolation by distance, inbreeding, recent bottlenecks, genetic differentiation, and low levels of migration among most populations, suggesting that *Zootoca vivipara louslantzi* is threatened. The results point to discontinuous populations and are in line with physical barriers hindering longitudinal migration South to the Central Pyrenean cordillera and latitudinal

migration in the central Pyrenees. In contrast, evidence for longitudinal migration exists from the lowlands north to the central Pyrenean cordillera and the Cantabrian mountains. The location of the populations South to the Central Pyrenean cordillera were identified as the first to be affected by global warming, thus management actions aimed at avoiding population declines should start in this area.

Keywords climate change; conservation; first-generation migrant, gene flow; IBD; *Zootoca vivipara*.

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Introduction

Gene flow is among the most important determinants of population structure, population persistence, and local adaptation (Garant et al. 2007). Gene flow reflects the transfer of genetic information from one population to another one, and is related to the number of migrants among populations. Several factors have been proposed to affect migration rate. Among the most prominent ones are physical barriers (e.g. rivers, mountains, deserts), climatic barriers (i.e., unsuitable climatic conditions hindering migration), and behavioural barriers (e.g. sexual selection; Geffen et al. 2004; Tobler et al. 2009). Moreover, the presence and permeability of such barriers can vary during the evolutionary history of a species (Coulon et al. 2004; Sharma et al. 2013). Connectivity among populations is suggested to be affected by climate change (Mieszkowska *et al.*, 2007; Horreo *et al.*, 2011; Anderson *et al.*, 2009; Munday et al. 2009 Coral Reefs) and recent results suggest that on the northern hemisphere climatic change may first affect populations at the southern range limit (Horreo *et al.*, 2014). Consequently, information about population structure, population history, and gene flow among populations is essential in order to understand past, current, and future population dynamics, as well as whether populations might be affected by climatic change.

Here we investigate migration, inbreeding, and bottlenecks in the European common lizard *Zootoca vivipara* to infer past and recent dynamics and to provide a base for future studies. More specifically, we estimated migration in *Z. vivipara louislantzi*, a subspecies inhabiting the southern limit of its wide geographic distribution (Horreo et al. 2018; Surget-Groba et al. 2006), and thus the geographic area where climate change is suggested to first affect populations (Horreo *et al.*, 2014).

Zootoca vivipara is a model species for the study of evolution, populations dynamics, behaviour, coloration, and ecology of dispersal (Aragon and Fitze 2014; Bestion et al.

2015; Cote et al. 2007; Cornetti et al. 2015; Cotto et al. 2015; Fitze et al. 2010; Fitze and Le Galliard 2008; Horváthová et al. 2013; Le Galliard et al. 2005; Peñalver-Alcázar et al. 2016; Recknagel et al. 2018; San-Jose et al. 2014; Sinervo et al. 2010; Surget-Groba et al. 2006). It exhibits two oviparous (clade A and B) and four viviparous clades (clades: C, D, E, and F; Horreo et al. 2018). The oviparous South-West European common lizard, *Z. vivipara lousilantzi* (clade B; Surget-Groba et al. 2006), inhabits south-western Europe (northern Spain and south-western France), and its geographic distribution does not overlap with the geographic distribution of the other clades (Horreo et al. 2018; Surget-Groba et al. 2006). Fine-scale analyses revealed that *Z. v. lousilantzi* consists of four major subclades: B1, the NW Spain (Galicia and Cantabrian Mountains); B2, the southern France (northern slopes of the Pyrenees; Mila et al. 2013); B3, the North-Central (NC) Spain (Basque country); and B4, the NE Spain (southern slopes of the Pyrenees) subclade (Horreo et al. 2018; Mila et al. 2013).

Despite the large research interest (see above), only two studies investigated gene flow and migration in *Z. vivipara* (Mila et al. 2013; Cornetti et al 2015). Milá et al. (2013) concluded that mountain ranges act as drivers of isolation and differentiation in *Z. v. lousilantzi*, and Cornetti et al. (2015) found no introgression between clade A and E in the Alps (*Z. v. vivipara* and *Z. v. carniolica*) despite the absence of physical barriers. Here we sampled a larger number of individuals and used specific newly developed nuclear markers (Horreo et al. 2018) to provide the first direct estimates of gene flow in *Z. v. lousilantzi*.

Material and Methods

Study Area

Samples from ten different lizard populations (20 samples/population) covering the distribution of *Z. v. lousilantzi* were collected (Fig. 1). These populations belong to four different oviparous subclades (Mila et al. 2013). All sampled populations were located in northern Spain and southern France (Fig. 1): Xistral and Andara (subclade B1; Western Cantabrian Mountains); Pinet, Louvie, and Aubisque (subclade B2; northern slopes of the Pyrenees); Estacas and Barazar (subclade B3: Eastern Cantabrian Mountains and Basque country); and Culivillas, Zuriza and Beret (subclade B4; southern slopes of the Pyrenees), following AFLP-inference by Mila et al. (2013). Non-invasive tissue sampling was conducted in 2014 and 2015 under licence of different institutions (Instituto Aragonés de Gestión Ambiental LCE/Mp 24/2012/426 and 500201/24/2013/12572; Service Nature Languedoc-Roussillon arrêté N° 2014304-004; DREAL Aquitaine; Parc National des Pyrénées 2012-55 & 2016_8; Xunta de Galicia 071/2014; Generalitat de Catalunya; Asturias; Parque Nacional Picos de Europa; Bizkaiko Foru Aldundia Zk/N° 1079). All sampling was performed in accordance with relevant guidelines and regulations.

The airline distances among sampled populations were highly variable, from less than 20 km between Louvie and Aubisque, to more than 800 km from Xistral to Pinet. Specimens were mainly adults (only 6 juveniles were included), and the sex ratio was balanced (97 males and 103 females in total).

DNA analyses

After DNA extraction from skin and tail tips (for methodological details see Horreo and Fitze 2015), 34 newly developed microsatellite loci were amplified in all specimens following the original protocol (seven multiplexes; Horreo et al. 2017). MICROCHECKER (Van Oosterhout et al. 2004) was employed to check for the presence of null alleles and large allelic dropout. All loci were checked for linkage

disequilibrium (LD) using GENEPOP online (<http://genepop.curtin.edu.au>). The average number of alleles (N_a) as well as the observed (H_o) and expected heterozygosity (H_e), were estimated with GENETIX (Belkhir et al. 2004). Departures from Hardy Weinberg Equilibrium (HWE), heterozygote deficiency (F_{is}), and the fixation index (F_{st}), were calculated using GENODIVE (Merimans and Van Tienderen 2004). Test significance was determined using sequential Bonferroni adjustments (Rice 1989), which accounts for multiple testing. Differences among subclades in the number of alleles per locus as well as in observed and expected heterozygosity were tested by analysis of variance (ANOVA) employing JMP 11.2.0 software.

To analyse the population structure, the program STRUCTURE v.2.3.4 (Falush et al. 2007) was employed using the Bayesian assignment probability test. A model of no admixture with the *Locprior* option and correlated allele frequencies was used to assign individuals to a given number of genetic clusters (K). Ten different repetitions of one million steps were run for each number of K (from 2 to 10; note that genetic information from 10 populations was analysed) after a 10% burn-in. The optimal K was assessed with Structure selector (<http://lmme.qdio.ac.cn/StructureSelector>), a web based software to select and visualize the optimal number of genetic clusters (Li & Liu 2018), using the methods by Puechmaille (2016) and Evanno et al. (2005).

The number of migrants per generation (N_m) among populations (using the private alleles method), and the presence of isolation by distance (IBD) were calculated with GENEPOP online (link above). To test for recent gene flow, first-generation migrants were determined with GeneClass v.2.0 (Piry et al. 2004) using the following settings: L_max/L_home likelihood computation, a Bayesian criteria of computation (Rannala & Mountain 1997), and Monte Carlo resampling (Paetkau et al. 2004) with 10,000 simulated individuals and 0.05 of type I error (α).

The presence of recent genetic bottlenecks (reduced genetic diversity due to drastic reductions in population sizes) was tested with Bottleneck v.1.2.02 using settings for microsatellite data (Piry et al. 1999): the two phase model (TPM settings: variance for TPM = 30, proportion of SMM in TPM = 70%) and the 2-tailed Wilcoxon sign-rank test with 10,000 interactions. In these analyses, significant *P*-values point to the presence of recent bottlenecks. To detect older bottlenecks, the Garza-Williamson index (2001) was estimated using Arlequin v.3.11 (Excoffier and Lischer 2010). Garza and Williamson's (2001) method estimates *M* ($M = k/r$ being *k* the number of alleles and *r* the allelic range) and $M < 0.68$ corresponds to past bottlenecks and $M > 0.80$ allows to conclude that no past bottlenecks existed.

Results

The 34 microsatellite loci successfully amplified 92.9% of the genotypes and the genetic diversity within populations was high (Table 1). MICROCHECKER detected the presence of null alleles in three of the 34 microsatellite loci (ZV17, ZV25 and ZV33) and another three loci were not in HWE in the majority of the populations (ZV11, ZV12, ZV13 and ZV17; Table 1). These loci were thus discarded from subsequent analyses, and the final dataset included 28 microsatellite loci. The average *N_a* measured over all populations was 20.86 alleles per locus with the least variable locus (ZV33) comprising 12 different alleles and the most variable one (ZV34) comprising 37 alleles. The average *N_a* per population ranged between 7.36 and 12.07, *H_o* ranged between 0.60 and 0.70, and *H_e* between 0.60 and 0.82.

No significant differences among subclades (B1, B2, B3, B4) existed in the number of alleles per locus (*N_a*; ANOVA: $F_{3,6} = 3.855$, $P = 0.075$) and the observed heterozygosity (*H_o*; ANOVA: $F_{3,6} = 4.529$, $P = 0.055$). Significant differences existed in

the expected heterozygosity (H_e ; ANOVA: $F_{3,6} = 6.445$, $P = 0.026$). The Tukey's HSD test showed that H_e was significantly lower in subclade B1 compared to subclades B2 and B3.

F_{is} ranged from -0.004 to 0.146, being positive and significant in all populations except in Xistral, where it was negative and not significant (Table 2). In seven populations, Wilcoxon tests unravelled a recent reduction of effective population size (Wilcoxon test: $P < 0.05$; Table 2) and Garza-Williamson indices unravelled no evidence for past reductions (G-W: $M > 0.80$; Table 2), while in three populations: Culivillas, Barazar, and Beret, no evidence for recent (Wilcoxon test: $P > 0.05$) and past reductions (G-W: $M > 0.80$; Table 2) existed.

F_{st} analyses showed genetic differentiation (P -value < 0.01) among all population pairs with the exception of Louvie and Aubisque (Table 3), both belonging to subclade B2 from southern France. Both, Puechmaille's (2016) and Evanno *et al.*'s (2005) methods showed that $K = 7$ was the optimal number of clusters for the STRUCTURE analyses (Fig. 2). The structure analyses attributed most of the populations to different clusters (Xistral, Andará, Culivillas, Pinet, Beret). Estacas and Barazar were assigned to same genetic unit and also Louvie, Aubisque, and partly Zuriza.

The number of migrants (Nm) between populations was low, in general less than one migrant per generation (Table 3, Fig. 1). Nm values above 1 existed among the following population pairs: Louvie and Barazar, Pinet, Beret, or Zuriza, and Aubisque and Zuriza. Nm values were highest ($Nm > 2$) among Barazar and Estacas (both belonging to subclade B3) and Louvie and Aubisque (both belonging to subclade B2). Low migration existed between populations whose airline distance crosses mountains (e.g. from Culivillas to Beret $Nm = 0.3$, Barazar $Nm = 0.4$, Estacas: $Nm = 0.4$, or Zuriza

$N_m = 0.7$; or from Andara to Xistral $N_m = 0.4$, Estacas $N_m = 0.6$, or Barazar $N_m = 0.5$). Isolation By Distance (IBD) analyses over the entire distribution (maximal distance 800 km) were statistically significant ($F_{1,43} = 20.961$, $P < 0.01$, $R^2 = 0.328$), and IBD was also present within populations located close to the Pyrenees (maximal distance 460 km: subclades B2, B3, B4; $F_{1,26} = 5.026$, $P = 0.034$, $R^2 = 0.162$). A total of fifteen first-generation migrants (7.5% of the total individuals) were detected within the sampled individuals: one in Zuriza (coming from Louvie), six in Louvie (all of them coming from Aubisque), another six in Aubisque (all of them from Louvie), and two in Estacas (one coming from Barazar and the other one from Andara).

Discussion

Knowledge about gene flow and migration provides valuable insight into the process of lineage divergence and the past history; and it is essential for conservation. Here we investigated migration among populations covering the geographic distribution of the South-West European common lizard *Zootoca vivipara lousiantzi* (clade B of *Z. vivipara*, Horreo et al. 2018). The results provide evidence for strong genetic differentiation (Table 3), with most populations belonging to a different genetic unit (Figs. 1, 2). Two genetic units included several populations, and among those the number of migrants per generation was estimated to be higher than two (Figs. 1, 2). Significant heterozygote deficiency, which points to inbreeding, was present in 90% of the studied populations and evidence for recent bottlenecks existed in the 70% of the populations (significant Wilcoxon tests, Table 2), while no evidence existed for past bottlenecks in any of the populations (all Garza-Williamson indices: $M > 0.84$; Table 2). The presence of significant Wilcoxon tests and the absence of past bottlenecks suggest that effects on effective population sizes are caused by recent factors (e.g. the current

climatic change) that negatively affect *Z. v. louislantzi*'s populations. Moreover, the strong genetic differentiation and population structure, the omnipresence of inbreeding, and the generally low migration rates detected in *Z. v. louislantzi*, point to the existence of isolated populations. In line with that was the genetic distance, which increased with geographic distance over the entire distribution of *Z. v. louislantzi* (Fig. 3), and also over shorter distances, i.e. within populations located close to the Pyrenees.

The number of migrants per generation (N_m) between Louvie and Aubisque, both populations belonging to subclade B2 (S France), was highest (> 2 migrants per generation). Given that N_m reflects the proportion of genetic exchange existing among two populations, but not necessarily the number of individuals that migrate from one population to the other population (Slatkin 1985), we also quantified the number of first-generation migrants. Twelve first-generation migrants were detected between Louvie and Aubisque, which points to high recent gene flow between Louvie and Aubisque, which is in line with the absence of significant genetic differentiation among these populations and attribution to the same genetic cluster (Table 3; Fig. 2). The air-line distance among these populations is less than 20 km and both populations are located in the same valley (Fig. 1). Given that 20 km is the shortest distance among sampled population pairs, the lack of differentiation is fully congruent with the detected IBD (Fig. 3).

The number of migrants per generation in population pairs including Louvie was generally high (average = 1.106 ± 0.143 SE) and the majority of these population pairs exhibited more than one migrant per generation ($N_m > 1$; Table 3). Moreover, only seven of the 45 comparisons (16%) exhibited $N_m > 1$ and five of them were among population pairs including Louvie (Louvie-Aubisque, Aubisque-Zuriza, Louvie-Zuriza, Louvie-Pinet, Louvie-Beret, Louvie-Barazar, Estacas-Barazar; Table 3). In addition, 13

of the 15 first-generation migrants detected in the dataset included Louvie. Seven individuals migrated from Louvie to Aubisque or Zuriza and six individuals migrated from Aubisque to Louvie. Louvie is located at the northern foothills of the Pyrenees (372 m asl; Fig. 1) and the population pairs including Louvie and exhibiting $N_m > 1$ were located in the North of the Pyrenees (Pinet) and in the North of the Cantabrian mountains (Barazar), in valleys with a northward-drainage system (Aubisque, Beret), or close to the central cordillera and a low altitude pass connecting the southern with the northern slope (Zuriza). This points to migration through the northern lowlands (e.g. from Louvie to Pinet, from Louvie along the coast to Barazar, or vice versa) and from the northern lowlands southwards through the valleys, or vice versa (e.g. from Louvie, to Beret, or to Zuriza, or vice versa). The high differentiation and low migration detected between populations whose airline distance crosses mountains suggests that longitudinal migration through the northern lowlands and latitudinal migration through the valleys are much easier than longitudinal migration through the mountains. This suggests that topography (i.e. mountain ranges) forms a physical barrier to migration, which agrees with conclusions by Milá et al. (2013). The results on *Z. v. louisiantzi* contrast to those of Cornetti et al. (2015), which in the absence of physical barriers did not detect genetic exchange between clades A and E of *Z. vivipara*. Given that the common ancestor of clades A and E is much older than the common ancestor of subclades B2 and B4 (4.4 Mya versus 1.6 Mya; Horreo et al. 2018), the detected difference in reproductive isolation between the studies on *Z. v. louisiantzi* and Cornetti et al.'s study (2015) suggests that the age of genetic differentiation among involved lineages might explain the observed differences.

As commented above, migration was detected also among populations belonging to different subclades of *Z. v. louisiantzi* (Fig. 1) and more specifically, among

populations belonging to subclade B2 and B3, and populations belonging to subclade B2 and B4 (Table 3). Migration among populations belonging to B2 and B4 is congruent with the findings of Milá et al. (2013), who detected genetic introgression in several populations (Fig. 6 in Milá et al. 2013), as for example between Gabas and Turbera populations (Fig. 1B). This suggests that admixture among these subclades is possible (see populations SOP and SOU; Fig. 6 in Milá et al. 2013). The split of subclade B2 was estimated at 1.6 Mya (95% CI: 1.2 – 2.0 Mya; Horreo et al. 2018) and the split between subclade B1 and the other two subclades was estimated at 0.8 Mya (0.5 – 1.2 Mya; Horreo et al. 2018), suggesting the existence of recent mixing among relatively old subclades. The here detected strong genetic differentiation (Table 3; Fig. 2) also suggests that the observed gene flow was not sufficiently strong to balance the genetic differences caused by past divergence.

It has been suggested that on the northern hemisphere climatic warming may first affect populations at the southern range limit (Horreo et al. 2014) and at low altitudes (Sinervo et al. 2010). The here detected low migration rates among populations located south to the central Pyrenean cordillera (all belonging to subclade B4, the southern limit of the natural distribution of *Z. vivipara*) might be in line with climate warming that first disrupted connections through the lowlands South of the Pyrenees and that may have led to the detected recent bottlenecks. Low migration rates among populations belonging to the same subclade, together with strong genetic structuring, significant heterozygote deficiency and the presence of recent bottlenecks point to currently isolated populations (Allendorf et al. 2010; Bretman et al. 2011). B1 and B4 exhibited low migration rates among populations belonging to the same subclade (Table 3), significant heterozygote deficiency existed in all but one population (Table 2), and evidence for recent bottlenecks existed in all populations of B1 and B2 and in some but

not all populations of B3 and B4 (Table2), suggesting that most subclades might be threatened and thus the oviparous subspecies *Z. v. louislantzi*. On the other hand, the results also indicate that climatic warming may homogenise existing allele frequency differences thus removing genetic structure. Two first-generation migrants were detected in Zuriza, one coming from Louvie and the other one from Aubisque and there existed gene flow between Zuriza and the other two populations ($N_m > 1$, Table 3). The detected migration together with the genetic introgression from Aubisque/Louvie detected in Zuriza (Fig. 2) indicates that recent gene flow may homogenise allele frequency differences thus removing genetic structures. This suggests that the fate of populations located in the southern area of the Pyrenees (subclade B4) may depend on whether or not gene flow with northern populations may become possible.

Management implications

Our results on the population structure of *Z. v. louislantzi* unravelled latitudinal (north-southwards or vice versa) migration in the Pyrenees and longitudinal migration through the lowlands north of the Pyrenees and the Cantabrian mountains, while in the South of the central Pyrenean cordillera longitudinal (east-west) migration was very weak and significant heterozygote deficiency existed. This suggests that topography (mountains) forms a barrier to migration, that climatic conditions limit longitudinal migration at the southern distribution limit, and that subclade B4 will be the first to be affected by global warming. Management actions aiming at avoiding population declines in *Z. v. louislantzi* (recent reductions in population sizes have been detected in 70% populations) might be required in all subclades, and start in populations belonging to subclade B4. The strong population structure and the reduced migration among different subclades also indicate that reintroductions and population reinforcements

should be conducted with individuals belonging to the same subclade, in order to avoid the loss of the existing allele frequency differences and potential negative effects on population dynamics due to outbreeding depression.

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442 **Figures**

443

444 **Figure 1.** Schematic maps showing the location of the sampled *Zootoca vivipara lousilantzi* populations. A) locations of the ten sampled *Z. v.*
445 *louizslantzi* populations in northern Spain/southern France (dots). B) close-up of the Ossau and upper Tena Valley with drainage system. Dot
446 colour indicates subclade affiliation (yellow: B1, NW Spain; green: B2, S France; red: B3, NC Spain; blue: B4, NE Spain subclade). Thin arcs
447 delimitate population pairs with significant gene flow ($Nm > 1$), thick arcs with high gene flow ($Nm > 2$), and the orange arc (panel B) reflects
448 published evidence for gene flow (populations GAB: Gabas, TUR: Turbera; Mila et al. 2013). Map shading from white to dark brown indicates
449 elevation above sea level, with dark brown representing highest elevation. The black line in the Pyrenees indicates the French-Spanish border,
450 which generally follows the central cordillera and separates northwards (French side) and southwards (Spain side) draining systems. Photo:
451 female of *Z. v. louislanzi* at Turbera, Tena Valley, Spain (author: P. S. Fitze).

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453

455 **Fig. 2.-** Structure output based on 28 microsatellite loci of the studied *Z. v. louislantzi* populations and an optimal number of genetic units of $K =$
 456 7. Each colour corresponds to a different cluster. Each vertical bar corresponds to the assignment probability to the genetic clusters of a single
 457 individual.
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463 **Fig. 3.-** Isolation By Distance (IBD) analysis. Genetic distance ($F_{ST}/(1-F_{ST})$) versus geographic distance ($\log_{10}$ distance (km)) of the sampled *Z. v.*
464 *louisianzi* populations and the predicted regression line are plotted. The *P*-value of the regression line was $P \ll 0.01$.

465

466 **Tables**

467 **Table 1.-** Genetic variability of the 34 microsatellites amplified in *Z. v. louislantzi*. Shown are the number of alleles per locus and population,
 468 and the total number of alleles detected per locus (referred to as ‘all’). ‘-’ in Beret indicates that this locus did not amplify, and this value was not
 469 considered for the further analyses. Significant deviations from Hardy-Weinberg Equilibrium (HWE) are indicated with asterisk: **P*-value <
 470 0.05; ***P*-value < 0.01. Three loci exhibited null alleles (ZV17, ZV25, and ZV33) and in another three loci (ZV11, ZV12, ZV13) the majority of
 471 the populations (≥ 6 populations) were not in HWE. Average number of alleles (*N_a*) per locus or for all loci, and the observed (*H_o*) and expected
 472 heterozygosities (*H_e*), were calculated for the remaining 28 bold-faced loci.

473

Subclade	B1		B2			B3		B4			All
Locus/Pop	Andara	Xistral	Aubisque	Louvie	Pinet	Barazar	Estacas	Beret	Culivillas	Zuriza	
ZV1	13	6	15	10	9	11	14	8	18	14	23
ZV2	2	13	12*	7	7**	12**	12**	11	14**	9	23
ZV3	9	9	15	12	6**	10	10	6**	9	9	23
ZV4	5	4	9	10	8	8	6	5	7	8	14
ZV5	8	7	12	13**	7	8	12**	9	10	10*	19
ZV6	8	8**	15	13	10	7	8**	9	7**	12	20
ZV7	6	11	11	12	9	8	10	7	7	9	19
ZV8	8	5	10	14	10	12**	11	9*	7	14**	20
ZV9	7	5	12	11	8	10**	8	7	7	13	20
ZV10	14	11	4	2	2	16	14	-	10	2	20
ZV11	13**	10**	12*	11*	13	12**	13**	10	10**	11**	21
ZV12	2	4	10*	15**	7**	9**	8**	5**	4**	10**	18

ZV13	5	5	7**	17**	12**	8**	7**	5	6	9**	23
ZV14	8	7*	12**	12	9*	12	12**	3	2	7	24
ZV15	6	12	15*	14	12**	13	12	13	14	15	26
ZV16	9	8	9	8**	6	9	10	8	11	10	17
ZV17	9	7	15*	8**	14**	12	9*	13**	9*	10	21
ZV18	2	3	11**	6	11	10**	11	13*	8	10*	19
ZV19	7	10	13	11	4	9*	11	8	8	9	20
ZV20	10	10	9	12	12	13	15	5	10*	10	22
ZV21	11	10	12	9	11	14	12	7	9	5*	18
ZV22	5**	7	13	14	14	10	10	8	5**	7	21
ZV23	8	3	15	15	12	10	8*	9	9	9*	20
ZV24	5**	3	12	11	7	9	8	6	4	10*	16
ZV25	10	8**	9	12	7	11**	10**	8	7	7	15
ZV26	5	9	15	16	9	12	18	6	9	11*	24
ZV27	10	2	8	8	14	8**	13	8	6	9*	24
ZV28	10	10	8**	10**	8	12	12*	5	8	9**	20
ZV29	3	2	7**	9	2	6	6**	4	7	9	15
ZV30	7*	17	14	14	10	11	12	9	9	13*	24
ZV31	6	12	17*	14	9**	10	9**	11	15**	12**	21
ZV32	11	10	15**	16	11	7	10	10	11	12	25
ZV33	4	7	11	9	7	6	6	6	6	10	12
ZV34	3	3	18	17*	9	9	7**	14	9**	9	27
Average Na	7.36	7.75	12.07	11.43	8.78	10.21	10.75	8.07	8.93	9.86	20.86
Ho	0.60	0.60	0.70	0.70	0.62	0.68	0.70	0.60	0.62	0.64	-
He	0.60	0.60	0.82	0.81	0.71	0.76	0.78	0.63	0.65	0.75	-

475 **Table 2.-** Heterozygote deficiency (F_{is} ; * indicates $P < 0.05$ after Bonferroni correction for multiple tests), P -values of Wilcoxon tests, and
476 Garza-Williamson (G-W) index for the studied *Z. v. louisianzi* populations. Significant Wilcoxon tests indicate recent bottlenecks, while G-W <
477 0.68 points to presence, and G-W > 0.8 to absence of past bottlenecks. Subclade corresponds to the subclade affiliation and the distribution range
478 following Horreo et al. (2018) and Milá et al (2013). GPS coordinates are shown in Latitude (Lat) and Longitude (Long).

Population	Subclade/distribution	Lat	Long	F_{is}	Wilcoxon	G-W
Andara	B1: NW Spain	43°13'2.62"N	4°42'57.37"W	0.002*	0.009	0.841
Xistral	B1: NW Spain	43°28'0.41"N	7°32'14.14"W	-0.004	0.000	0.954
Aubisque	B2: S France	42°58'17.87"N	0°20'43.19"W	0.146*	0.015	0.952
Louvie	B2: S France	43°5'40.19"N	0°22'52.52"W	0.135*	0.022	0.969
Pinet	B2: S France	42°51'38.22"N	1°58'44.62"E	0.128*	0.005	0.955
Barazar	B3: NC Spain	43°3'32.47"N	2°43'7.65"W	0.111*	0.063	0.929
Estacas	B3: NC Spain	43°7'2.02"N	3°42'3.56"W	0.103*	0.028	0.913
Beret	B4: NE Spain	42°42'57.58"N	0°57'16.97"E	0.053*	0.141	0.931
Culivillas	B4: NE Spain	42°47'8.61"N	0°23'51.50"W	0.046*	0.104	0.872
Zuriza	B4: NE Spain	42°51'31.59N	0°47'55.87"W	0.144*	0.011	0.934

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Table 3.- Genetic differentiation (F_{ST} ; below diagonal) and estimates of migration (Nm ; above diagonal) among the studied *Z. v. louislantzi* populations belonging to the four subclades. * P -value < 0.01 for F_{ST} comparisons after Bonferroni correction for multiple tests.

Subclade		B1		B2			B3		B4		
	Population	Andara	Xistral	Aubisque	Louvie	Pinet	Barazar	Estacas	Beret	Culivillas	Zuriza
B1	Andara	-	0.403	0.607	0.641	0.409	0.456	0.575	0.349	0.323	0.422
	Xistral	0.256*	-	0.789	0.948	0.485	0.359	0.527	0.361	0.528	0.579
B2	Aubisque	0.209*	0.167*	-	2.149	0.993	0.952	0.867	0.918	0.781	1.059
	Louvie	0.220*	0.167*	0.006	-	1.025	1.038	0.971	1.028	0.851	1.303
	Pinet	0.243*	0.272*	0.097*	0.109*	-	0.593	0.598	0.901	0.447	0.722
B3	Barazar	0.228*	0.245*	0.089*	0.100*	0.146*	-	2.039	0.479	0.382	0.721
	Estacas	0.205*	0.222*	0.098*	0.099*	0.147*	0.033*	-	0.488	0.409	0.652
B4	Beret	0.302*	0.264*	0.143*	0.149*	0.161*	0.239*	0.229*	-	0.325	0.627
	Culivillas	0.296*	0.223*	0.147*	0.142*	0.234*	0.222*	0.217*	0.263*	-	0.708
	Zuriza	0.255*	0.200*	0.071*	0.056*	0.139*	0.137*	0.138*	0.194*	0.146*	-