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Can neutral genetic differentiation explain geographical variation in body size of the natterjack toad, *Epidalea calamita*?

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Abstract. Population genetic studies are crucial for evolutionary biologists because the population is the basic substrate on which evolution is forged. However little empirical evidence has been able to demonstrate the role that isolation and gene flow play in maintaining differentiation in populations at short geographic scales. *Epidalea calamita* exhibits a steep variation in body size and reproductive traits in southwestern Spain, associated with changes in the geological substrate. This implies a decrease of 70.9% of body mass and 28.5% in snout-vent length, on a microgeographic scale of only 60 km. Previous results from both metamorphic and juvenile common garden experiments showed that genetic differentiation may be a causal determinant of geographic variation in adult. This study tested whether neutral genetic differentiation can explain the geographical variation in the body size observed in *E. calamita*. It was addressed analyzing the level of genetic structuring and gene flow among populations along the cline, comparing the genetic diversity between and within populations, as well as between ecological environments. The study showed that the geographic variation in body size observed in *E. calamita* has evolved in absence of geographic isolation, with moderate gene flow connecting the populations. Thus, neutral genetic differentiation cannot explain the geographical variation observed. Future studies are needed on the interaction between the genetic component with the environmental factors and will be necessary to analyze the contribution of the maternal effects in the origin and evolution of the geographical variation in the body size observed in *E. calamita* from southern Spain.

Keywords. *Epidalea*, F_{ST}, microsatellite loci, population differentiation, body size.

INTRODUCTION

Geographic variation in phenotypic and genetic characteristics among species' populations is a phenomenon that has been very well documented since the 1950s (Stebbins, 1950; Mayr, 1963; Harper, 1977). Adaptive explanations for the evolution and maintenance of geographic variation in body size have been put forward, particularly considering macrogeographical patterns as a response to environmental gradients (Bergmann, 1847; Ray, 1960; Lindsey, 1966; Adams and Church, 2008; Ashton, 2002; Cvetkovic et al., 2009; Sinsch et al., 2010). Nevertheless, few studies have evaluated it at smaller geographical scales with, in many cases, lack of genetic isolation between populations (e.g., Skelly, 2004; Gomez-Mestre and Tejedo, 2004; Lee et al., 2020; Albert and García-Navas, 2022), and thus, what causes and maintains those patterns is still not well understood. Therefore, to infer on the processes causing these patterns, we need to know the mechanisms underlying the observed phenotypic variation, how they are connected to genetic variation, and how they interact with other traits and the environ-

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ment (Stearns, 1989). This will also help us to understand the evolutionary significance of the geographic variation, which ultimately can lead to the formation of new species (Endler, 1977; Foster and Endler, 1999).

The agents that change the gene frequencies of populations, that is, the factors of evolution, are mutation, genetic drift, gene flow, and natural selection (Slatkin, 1987). While drift and selection tend to increase population differentiation, gene flow promotes homogenization among connected populations, either increasing or decreasing the genetic diversity of the system (Lenormand, 2002). Thus, gene flow is a major component of population structure because it determines the extent to which each local population of a species is an independent evolutionary unit. If there is a high gene flow between local populations, then all the populations evolve together; whereas in the presence of low gene flow, each population evolves almost independently (Slatkin, 1985).

The natterjack toad (*Epidalea calamita*) populations from southwestern Spain exhibit a steep variation in body size and reproductive traits associated with changes in the geological substrate (Marangoni et al., 2008). This implies a decrease of 70.9% of body mass and 28.5% in snout-vent length, on a micro-geographic scale of only 60 km (Fig. 1). Previous studies suggested that considerable genetic differentiation may be the mechanism underlying the observed geographic variation in metamorphic traits in *E. calamita* (Marangoni, 2006) and *Pelobates cultripes* (Marangoni and Tejedo, 2008), which exhibit the same geographic pattern of adult body size variation (Marangoni et al., 2008; Lee et al., 2020). Moreover, the study of age structure and growth pattern across populations of *E. calamita* suggests that both environmental variations in resources availability associated with the sandy substrate (Marangoni, 2023), but also different growth and maturity pathways, may happen in response to contrasting selective pressures (Marangoni et al., 2021). Two hypotheses could be suggested to explain the evolution and maintenance of the observed cline in body size and reproductive parameters in *E. calamita* (Marangoni et al., 2008). In the first place, it could be expected that in the presence of gene flow between populations, the alleles favored by a selection pressure of intensity s do not decrease their high frequency, because the rate of immigration m of other alleles is lower than the intensity of selection s, that is, $m < s$ (Slatkin, 1985). The second hypothesis would be that the differentiation between the populations along the cline had occurred in a context of relative population isolation or in the presence of scarce gene flow. Little empirical evidence has been able to demonstrate the role that gene flow plays in maintaining differentiation in populations at short geographical scales. It has been suggested that when gene flow is not homogeneous, evolutionary differentiation can be rapid and can occur on small spatial scales (Kennington et al., 2003; Garant et al., 2005; Postma and van Noordwijk, 2005).

In previous studies on *E. calamita* that exhibited local adaptation to osmotically stressful environments, microsatellite markers revealed little population differentiation, lack of an isolation-by-distance pattern, and moderate gene flow connecting the populations (Gomez-Mestre 2001; Gomez-Mestre and Tejedo, 2004). Present study assess whether neutral genetic differentiation can explain the geographical variation in the body size, age and reproductive parameters observed in natterjack toad, from southern Spain (Marangoni et al., 2008; 2021). This comparison between patterns of population genetic differentiation in neutral markers and quantitative traits can provide valuable insights into the mechanisms driving variation within populations (Leinonen et al., 2007). Thus, this comparison can help us understand evolutionary forces by discerning the relative influences of genetic drift and natural selection on the evolution of quantitative traits (Gomez-Mestre and Tejedo, 2004; Knopp et al., 2007; Páez-Vacas et al., 2001). Secondly, it aids in identifying adaptive traits, for example those traits which might are related to adaptation to different environmental conditions (Gomez-Mestre and Tejedo, 2003; Luque et al., 20015). In addition, it also allows us to understand the distribution of genetic effects within the architecture of quantitative traits (see Leinonen et al. 2007 and references therein).

The main goals in the present study were: i) analyze the level of genetic structuring and gene flow in *E. calamita* populations along the geographic variation in body size observed (Marangoni et al., 2008), ii) compare genetic diversity between and within populations, as well as between ecological environments and iii) test for the existence of an isolation-by-distance pattern of populations differentiation. We expect that the observed geographical variation in the body size of natterjack toad has been evolved in absence of isolation-by-distance, and with a gene flow connecting the populations.

MATERIAL AND METHODS

Populations

Seven populations of *Epidalea calamita* were selected, representing the cline in body size observed in a previous study (Marangoni et al., 2008), which encompass three areas with different geological substrates. These populations included: two Large-bodied populations, Pedroso (L1) and Navas (L2), from Sierra Morena (old hercinic granites schist soils): four Small-bodied popula-

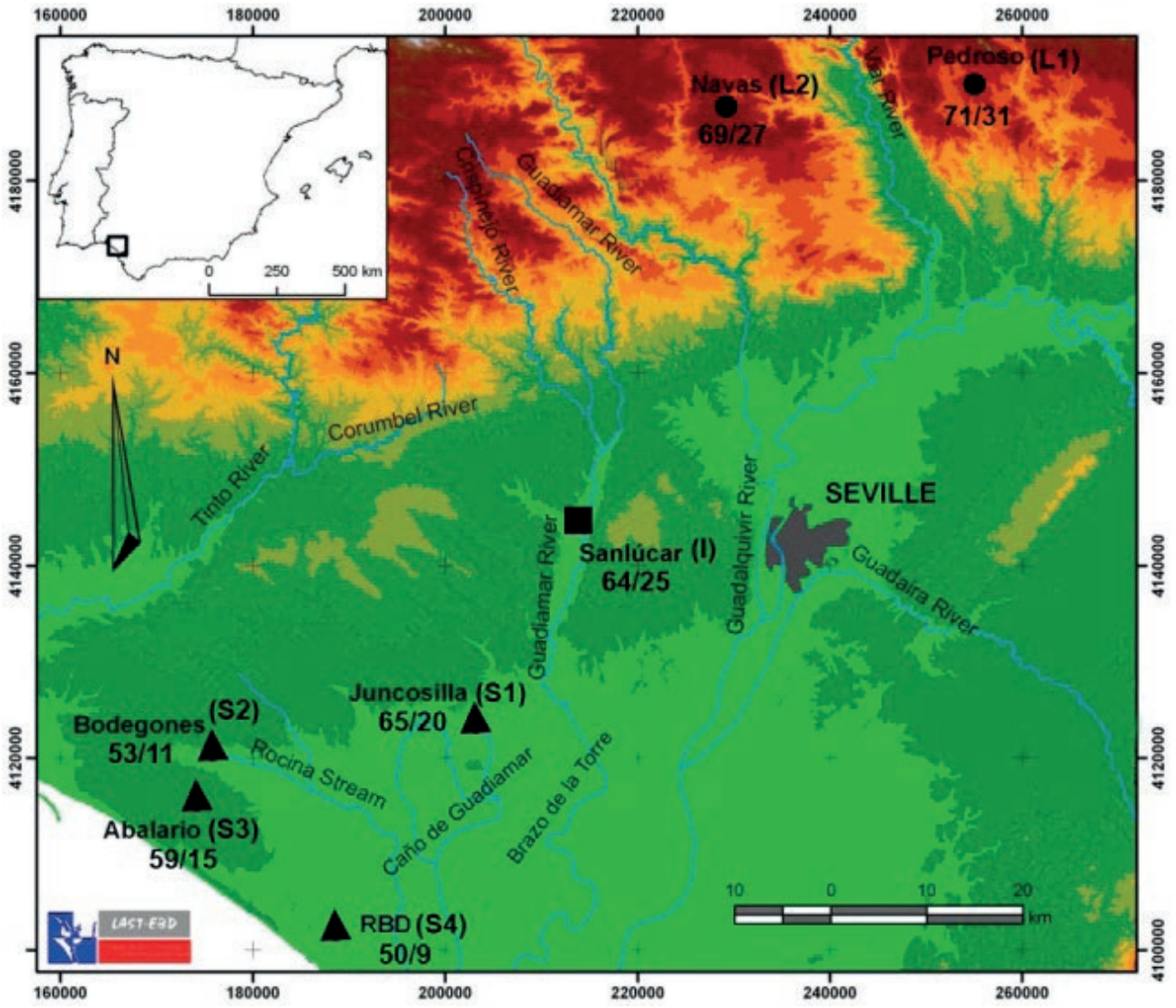

Fig. 1. Location and geological substrate of the studied *Epidalea calamita* populations. Abbreviated names of sampling localities, geographic coordinates (Coordinates UTM x/y in meters, Datum European 1950, Spain and Portugal, Zone: 30), elevation, and sample size are as follows: L1, 255170/4190574, 395 m, n = (39); L2, 229255/4187617, 420 m, n = (43); I, 213349/4144548, 34 m, n (22); S1, 203208/4124509, 23 m, n = (44); S2, 175577/4120711, 32 m, n = (45); S3, 174267/4115417, 63 m, n = (35); S4, 188450/4102197, 24 m, n = (42). Circles: Sierra (Paleozoic, Granite + Schist rocks), square: Intermediate (Miocene-Pliocene, Clay + Sandy), triangle: Doñana (Holocene, Sandy soil). Below each abbreviated names of sampling localities are indicated the mean body size (snout-vent length in mm/weight in g) from Marangoni et al. (2008).

tion, Juncosilla (S1), Bodegones (S2), Abalario (S3) and Reserva Biológica de Doñana (S4), from the Doñana area (quaternary sandy eolian deposits): and one population with intermediate body size, Sanlúcar (I), hereafter), geographically located between Sierra and Doñana (mixture of clays and sand) (Fig. 1). A more detailed description of the environments and the life history traits (body size, age, growth patterns and reproductive output) of the populations selected, is available in Marangoni and Tejedo (2008) and in Marangoni et al. (2008, 2021).

Sampling and molecular genetic analysis

Twenty recently laid clutches of similar larval stage (Gosner stage 10, Gosner, 1960) from each of the seven population of *Epidalea calamita* were sampled during the breeding season (January 2003). Each clutch (full-sib families) were brought to the laboratory and kept separately in plastic trays filled with dechlorinated tap water until tadpoles reached Gosner's stage 25 (Gosner, 1960), to be included in a common garden experi-

ments (Marangoni, 2006). Tissue samples for the present genetic study were obtained by cutting the tip of the tail of 15-20 tadpoles from each population. They were randomly taken from a sample in which the twenty clutches from each population were previously mixed, to maximize the chances of sampling unrelated tadpoles. In present study were used the same eight microsatellite loci that previously were analyzed by Gomez-Mestre (2001) and Gomez-Mestre and Tejedo (2004), which included *Bcal* 1, *Bcal 2*, *Bcal 3*, *Bcal 4*, *Bcal 5*, *Bcal 7* (Rowe et al. 1997), *Bcal 10*, and *Bcal 11* (Rowe et al., 2000). The 5' to 3'-primers were labeled with a color fluorophore, either HEX, TET, or FAM (Gomez-Mestre, 2001). DNA was obtained using an DNA Dneasy Tissue Extraction Kit (QIAGEN). The DNA was extracted by digesting each sample, approximately 25 mg of tissue minced into small pieces, to which 180 µl of ATL buffer and 20 µl of proteinase K were added. The samples incubated for 12 hours at 55 °C. Afterwards, if the tissue was not completely digested, an additional 20 µl of proteinase K was added and incubated for another 3 hours. Once the samples were fully digested, they were shaken for 15 seconds, 200 µl of buffer AL was added and incubated at 70 °C for 10 minutes. Subsequently, after adding 200 µl of ethanol, the mixture was centrifuged for 1 minute at 8000 rpm using the columns provided by the kit. Transferring the columns to other 2 ml tubes, 500 µl of buffer AW1 was added and centrifuged once more. This last step was repeated one more time, but adding 500 µl of buffer AW2, and centrifuged for 3 min at 15,000 rpm. Finally, once the columns were transferred to 1.5 ml Eppendorf tubes, 200 µl of AE buffer was added, incubated at room temperature for 1 min and centrifuged at 8000 rpm. This step was repeated twice, obtaining a final volume of 400 µl of buffer and the DNA extracted from the sample, which was stored at –20 °C until further procedures. Loci amplification was conducted using polymerase chain reactions (PCRs) of 1.5 ml total volume with 5 ml of DNA. The PCR amplifications followed a 66-50 °C touchdown procedure. PCR products of each of the eight loci for each individual sampled were aliquoted and mixed according to their abundance. An aliquot of 1.5 ml of the resulting mix was added to 13 ml of formamide plus 0.3 ml of Tamra 500 (Applied Biosystems, Foster City, CA) standard. Samples were analyzed in a fluorescence-based automatic fragment analyzer (ABI-PRISM 310 Genetic Analyzer, Applied Biosystems). Allele sizes for each locus were resolved by comparison of the peaks obtained to those yielded by the Tamra 500 standard using the software GeneScan version 3.1.2 (Applied Biosystems). All procedures described were performed in the Laboratorio de Ecología Molecular, at the Estación Biológica de Doñana (EBD-CSIC), Seville, Spain. Permits for capture and sampling of *E. calamita*, including all ethical considerations, were acquired from the regional authorities.

Statistics

Arlequin software packages (Schneider et al., 2000) was used to perform a nested molecular analysis of variance (nested AMOVA) comparing genetic diversity between and within populations, as well as between ecological environments population groups (Large-bodied populations from Sierra and Small-bodied populations from Doñana). This analysis provides genetic structure parameters in the form of F statistics (Wright, 1951; Excoffier et al., 1992). The significance of these statistics is assessed with the null distributions of the variance components, since both are highly correlated (Excoffier et al., 1992). Using the Arlequin software packages, were analyzed the allele frequencies, mean number of alleles per locus, and observed and expected heterozygosity. The significance of each of the variance components in subsequent analysis was estimated using 10,000 permutations. Genepop (version 3.4 online; Raymond and Rousset, 1995) was used to: i) analyze the population differentiation computing F_{ST} estimators (Weir and Cockerham, 1984) and their confidence intervals, ii) tests for linkage disequilibrium between loci using a Fisher exact test using Markov chains, and iii) analyze the existence of isolation-by-distance through Mantel tests carried out between matrices of log-transformed geographic distances and odds-transformed genetic distances ($F_{ST}/[1-F_{ST}]$; Rousset, 1997). Mantel Tests were also performed using *Isolation By Distance Web Service* (IBDWS) Version 2.5 (Jensen et al., 2005). Departures of the allelic frequencies from Hardy-Weinberg expectations were performed with the GENETIX Version 4.04 program (Belkhir et al., 2000) and allelic richness was estimated using the FSTAT program (Goudet, 1995). Finally, was conducted the analysis at a significance level of $\alpha = 0.05$, and applied the Dunn-Sidak sequential correction of the level of significance for multiple tests (Sokal and Rohlf, 1995) when necessary.

RESULTS

No large differences between populations were found regarding genetic diversity and allelic richness (Table 1). Moreover, no allele had a frequency greater than 95%, which indicates that all analyzed loci were polymorphic. The mean number of alleles per locus ranged between 8 and 25 (*Bcal 2* and *Bcal 4*, respectively), while in the remaining loci were: *Bcal 1* = 23, *Bcal 3* = 17, *Bcal 4* = 25,

Table 1. Molecular variation in populations of *Epidalea calamita* from southern Spain. Populations (mean number of alleles), number of alleles per loci (NA), expected (H_e) and observed (H_o) heterozygosity, genetic diversity (GD) and allelic richness (AR). Significant deviations from Hardy-Weinberg equilibrium are marked with an asterisk.

Locus	L1 (11.1)			L2 (13)			I (9.7)			S1 (12)			S2 (12.4)			S3 (12.5)			S4 (15.2)		
	NA	H_e	H_o	NA	H_e	H_o	NA	H_e	H_o	NA	H_e	H_o	NA	H_e	H_o	NA	H_e	H_o	NA	H_e	H_o
Bcal μ1	17	0.901	0.897	18	0.891	0.837	14	0.888	0.818	15	0.846	0.704*	14	0.884	0.777	13	0.863	0.885	14	0.855	0.785*
Bcal μ2	7	0.762	0.575*	5	0.500	0.138*	5	0.555	0.200*	5	0.651	0.232*	6	0.507	0.157*	6	0.527	0.416*	6	0.559	0.114*
Bcal μ3	11	0.871	0.281*	14	0.878	0.333*	10	0.879	0.272*	13	0.886	0.405*	15	0.902	0.342*	14	0.875	0.428*	14	0.898	0.323*
Bcal μ4	16	0.889	0.550*	17	0.880	0.555*	12	0.885	0.381*	16	0.862	0.780	15	0.894	0.522*	17	0.859	0.512*	18	0.801	0.642*
Bcal μ5	8	0.735	0.525*	12	0.752	0.767	10	0.792	0.736	12	0.818	0.697*	11	0.803	0.681	11	0.836	0.666*	14	0.860	0.795
Bcal μ7	11	0.807	0.717	13	0.792	0.613*	6	0.420	0.450	11	0.616	0.613*	14	0.752	0.600	13	0.717	0.717	13	0.773	0.744
Bcal μ10	9	0.854	0.906	11	0.840	0.795	9	0.807	0.727*	9	0.839	0.846*	11	0.832	0.750	10	0.810	0.815	8	0.817	0.825
Bcal μ11	10	0.756	0.240*	14	0.826	0.282*	12	0.883	0.181*	14	0.887	0.620*	13	0.851	0.410*	16	0.897	0.552*	15	0.920	0.700*

continuation:	GD	AR	GD	AR	GD	AR	GD	AR	GD	AR	GD	AR	GD	AR
Bcal μ1	0.913	12.948	0.903	12.308	0.911	12.298	0.858	10.063	0.895	10.496	0.876	10.558	0.867	9.970
Bcal μ2	0.777	5.825	0.513	4.019	0.588	5.000	0.664	4.831	0.519	5.255	0.537	4.709	0.574	5.641
Bcal μ3	0.895	9.396	0.901	11.591	0.915	9.538	0.906	10.917	0.922	12.125	0.895	10.935	0.921	11.889
Bcal μ4	0.905	11.969	0.897	11.400	0.920	10.959	0.874	11.265	0.910	12.094	0.875	11.283	0.813	11.268
Bcal μ5	0.748	6.127	0.761	8.076	0.816	9.241	0.830	8.996	0.814	8.535	0.850	8.457	0.872	10.435
Bcal μ7	0.820	8.420	0.804	9.089	0.430	5.236	0.623	6.924	0.763	8.291	0.727	8.643	0.783	8.468
Bcal μ10	0.867	8.088	0.851	8.565	0.829	7.853	0.851	8.097	0.843	8.521	0.821	7.685	0.828	7.464
Bcal μ11	0.783	8.652	0.845	9.845	0.921	11.274	0.908	12.174	0.868	10.248	0.914	11.952	0.935	12.912

Bcal 5 = 19 and *Bcal 7* = 18. Additionally, the mean number of alleles per locus in each studied population ranged between 9.75 (I) and 12.75 (S4). No private alleles were found, indicating that each allele was found in two or more populations. However, allele frequencies significantly differed across populations for all loci. The analysis of linkage disequilibrium showed that only 19 of all possible comparisons between pairs of loci in each population (28 × 7 = 119) were significant for α = 0.05, which represented 9.6%. However, all of them lost their significance after applying the Dunn-Sidak significance level correction for multiple comparisons. Genepop estimates for pairs of loci taking all populations together did not yield any significant evidence of linkage disequilibrium (for α = 0.05), so the eight loci were treated as independent henceforth. Furthermore, were detected significant departures from Hardy-Weinberg expectations in different loci across all populations (Table 1). *Bcal 11* and *Bcal 2* were departed in all analyzed populations, while *Bcal 4* in six of them. The population with the highest number of loci (7) outside the H-W expectations was S1, while in S2 we found the lowest (4). The rest of the populations presented five loci outside the H-W balance. Observed heterozygosity in the studied loci was generally lower than expected under Hardy-Weinberg equilibrium, indicating a deficiency of heterozygotes.

Regarding the relation between geographic distance (km) and, estimated distance genetics (F_{st}) and gene flow (Nm) of the pairwise comparisons between populations are shown in Table 2. There were significant differences at 13 of the 21 pairwise comparison between populations, before Bonferroni sequential correction (shown in bold in the Table 2). Nevertheless, all of them lost statistical significance after the Bonferroni correction was applied (critical level of significance was P = 0.0023; Sokal and Rohlf, 1995). Neutral genetic distance among populations was not correlated with geographical distance (r = 0.140, P = 0.217), rejecting the hypothesis of isolation by distance (Fig. 2). Two groups were clearly differentiated, one containing the pairwise comparisons between nearby populations (SS or LL), and another group containing the pairwise comparisons of distant populations (SL or LS), however these two groups did not show significant differences in the mean values of genetic distances (Fig. 2). For example, the S4 population had similar genetic distances when compared with both its most distant population (L2, F_{st} = 0.056/110 km) and its closest one (S3, Fst = 0.042/19 km). I also did not find any significant covariation between geographic distance and gene flow (y = 2.027 - 0,003*x; r = -0.129; P = 0.578; r^2 = 0.017).

The two AMOVA performed showed that the 95.4% of overall variation was held within populations, where-

Table 2. Geographical distance (km), distance genetic (*Fst)* and gene flow (*Nm)* between populations of *E. calamita*. Significant values before Bonferroni sequential correction are shown in bold.

Pairwise comparison	Geographic distance	Genetic distance	Gene flow
S2-S3	5.5	**0.006**	41.7
S3-S4	19.4	**0.042**	5.86
S2-4	22.5	**0.039**	6.35
S1-I	22.5	0.094	2.65
L1-L2	26.1	**0.042**	5.88
S1-S4	26.8	0.06	4.14
S1-S2	27.9	**0.02**	12.13
S1-S3	30.3	**0.025**	9.66
S2-I	44.7	0.064	3.86
L2-I	45.9	**0.021**	11.39
S3-I	48.7	0.079	3.13
S4-I	49.1	**0.024**	10.18
L1-I	62.2	0.07	3.56
L2-S1	68.3	0.068	3.63
L1-S1	84.1	**0.032**	7.76
L2-S2	85.8	**0.046**	5.41
L2-S3	90.8	0.062	4.03
L2-S4	94.7	**0.025**	9.64
L1-S2	105.9	**0.015**	16.31
L1-S3	110.4	**0.038**	6.49
L1-S4	110.7	0.056	4.41

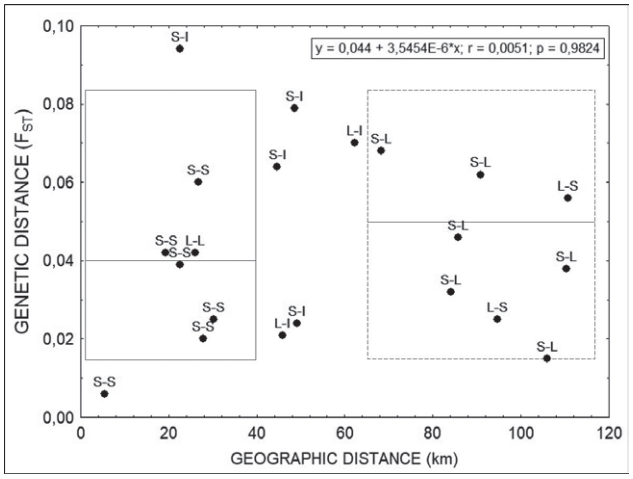

Figure 2. Pairwise comparisons between populations from the three environments studied, relating the geographic distance and estimated genetic distance. Two groups are clearly differentiated, one (inner square with continues lines) containing the pairwise comparisons between nearby populations (SS or LL), and another group (inner square with broken lines) containing the pairwise comparisons of distant populations (SL or LS). Nevertheless, mean values of genetic distances (middle lines) are not significantly different. L = large-bodied population (Sierra), I = intermediate and S = small-bodied population (Doñana).

Table 3. Nested molecular analysis of variance, where df stands for degrees of freedom, SS for sum of squares, Varcomp for variance components, and %Var for proportion of total variance accounted for by each source. Environments are Hercinic (L1 and L2) and Sandy soils (S2-S4).

Source of variation	df	SS	Varcomp	% Var
Between environments	1	19.985	0.023	0.69
Among populations within environments	4	58.991	0.133	3.91
Within populations	508	1660.179	3.268	95.4
Total	513	1739.156	3.425	100

as the lowest variance components were associated to the differences between environments (Sierra Morena vs Doñana), which suggests a lack of population substructuring (Table 3).

DISCUSSION

This study showed the highest allelic diversity found in *Epidalea calamita* populations. This was greater than that previously found by Gomez-Mestre and Tejedo (2004) in Spanish populations, and even greater than that found in the northernmost populations of the specie distribution (Rowe et al., 1998; Beebee and Rowe, 2000) (Table 4). The differences in the allelic diversity between this study and that of Gómez-Mestre and Tejedo (2004), particularly in the S1 population (Fresh 1 in Gómez-Mestre and Tejedo, 2004), could be attributed to a significant increase in the sample size in this study. However, I found no difference in the L1 population (Fresh 3 in Gómez-Mestre and Tejedo, 2004) (Table 4). The lower allelic diversity found in the British populations also supports the hypothesis that the Iberian Peninsula constituted a Pleistocene refuge for *E. calamita* (as it was for other species in other Mediterranean peninsulas; Hewitt, 1996; Taberlet et al., 1998). From this refuge, the species would have expanded rapidly to north and east during the post-glacial stage (Beebee and Rowe, 2000), resulting in a pattern of high levels of genetic diversity in populations derived from southern refuge and a progressive loss of diversity in recolonized areas to the north (Avise, 1994).

Despite the high variability, there were significant departures from Hardy-Weinberg expectations in different loci across all populations, due to deficiency of heterozygotes. It is possible that there is a certain degree of variability in the primer pairing regions, so that their sequence would not be completely homologous and would fail to amplify some alleles. In this case, by amplifying only one of the two alleles present, the proportion

Table 4. Genetic diversity. Mean number of alleles per locus (MAPL), percentage of polymorphic loci (P^{95}), and expected (He) and observed (Ho) heterozygosity of *E. calamita* throughout its distribution range. PS (in bold): present study, 1: Gómez-Mestre and Tejedo (2004), 2: Beebee and Rowe (2000). * and #, large and small-bodied population respectively, are showing the same population studied in PS and 1, using the same eight microsatellite loci.

Population	N	MAPL	P^{95}	He	Ho	Source
L1 (Spain)*	39	11.12	100	0.822	0.586	PS.
Fresh 3 (Spain)*	22	11.25	100	0.767	0.677	1
S4 (Spain)	**42**	**12.75**	**100**	**0.810**	**0.616**	**PS.**
S2 (Spain)	**45**	**12.37**	**100**	**0.803**	**0.530**	**PS.**
S3 (Spain)	**35**	**12.5**	**100**	**0.798**	**0.624**	**PS.**
S1 (Spain)#	**44**	**12**	**100**	**0.801**	**0.612**	**PS.**
Fresh 1 (Spain)#	22	10.5	100	0.698	0.581	1
L2 (Spain)	**43**	**13**	**100**	**0.795**	**0.540**	**PS.**
I (Spain)	**22**	**9.75**	**100**	**0.764**	**0.471**	**PS.**
Saline 1 (Spain)	23	9.38	100	0.664	0.626	1
Fresh 2 (Spain)	20	7.5	100	0.629	0.534	1
Velez (Spain)	11	4.88	100	0.689	0.607	2
Brittany (France)	32	4.38	87.5	0.491	0.355	2
Boulogne (France)	15	3.88	75	0.461	0.455	2
Ooy-Polder (The Netherlands)	40	5.13	87.5	0.520	0.466	2
Kerry (Ireland)	40	2.38	62.5	0.344	0.335	2
Merseyside (England)	40	2.63	62.5	0.294	0.295	2
Cumbria (England)	40	3.75	75	0.391	0.344	2
E/SE (England)	40	2.50	75	0.352	0.289	2
Texel (The Netherlands)	40	2.63	62.5	0.367	0.430	2
Sweden	40	1.63	25	0.119	0.144	2
Poland	40	2.00	62.5	0.245	0.283	2

of heterozygotes may have been underestimated, which would move the observed frequencies away from those expected for a Hardy-Weinberg equilibrium situation (Gómez-Mestre, 2001). However, we cannot rule out the homogenizing effect that gene flow (see below) may have in the departures from Hardy-Weinberg expectations observed. In addition, we could consider that the genes responsible for the expression of body size are potentially under selection and need to be studied.

Considering that some estimates of gene flow between populations were quite high, the lack of a relation between geographical distance and the degree of genetic differentiation (Fig. 2) is well exemplified by the L1 population. This population is genetically more similar to the S2 population, located 105 km apart, than to the L2 population, located only 26 km apart. As most of the observed variability corresponds to within-population differences (95.4%), this suggests that the populations are not structured. In addition neutral genetic differentiation cannot explain the geographical variation in body size observed, since only 0.6% of the total variance could be attributed to differences between the two environments.

The evolution and geographic variation of the Mediterranean herpetofauna has been influenced by a succession of geographic barriers to faunistic exchange over the last 23 myr (López-Martínez, 1989). The Guadalquivir River basin in the present study area has been suggested as major factor in the speciation processes in amphibians and as a barrier to dispersal of *Salamandra salamandra* (García-París et al., 1998). This intracontinental barrier has also been suggested as responsible of the geographic variation pattern in water salinity tolerance among *Epidalea calamita* populations in southern Spain (Gomez-Mestre, 2001). Nevertheless, since all our studied populations are all geographically located on the west bank of the Guadalquivir River, without population in the east, we cannot evaluate the hypothesis that considers the river as a barrier to gene flow.

A possible explanation for the lack of isolation-by-distance could be associated with the high dispersive capability that Bufonidae species can potentially present (e.g., 1.3 km/night, in *Rhinella marina*, Leblois et al., 2000), which could result in high gene flow despite populations being geographically distant. However, although this hypothesis is probable, it does not explain the high difference between closer populations (e.g., L1 and L2, or S3 and S4, Table 2). Alternatively, other less conspicuous physical barriers could exist between closer populations and need to be evaluated in futures studies. So, given the pattern of the gene flow observed in our study, there are some possible explanations for the maintenance of the geographical variation in the body size of *Epidalea calamita*. Populations subject to selection in two or more environmental patches, completely connected by gene flow, may develop phenotypic plasticity or adaptive reaction norms (Schmalhausen, 1949; Bradshaw, 1965), such that genetically similar individuals express different phenotypes in each environment. Then, it could be expected that the alleles that cause different phenotypes in *E. calamita* between Sierra and Doñana environments can evolve by natural selection, if the plastic response of the phenotype produces an increase in biological fitness (Via and Lande, 1985), as was demonstrated in *Pelobates cultripes* using reciprocal transplant experiments (Marangoni, 2006). This and other studies made in *E. calamita* and newts have shown that the dwarfism could be involved in response to some common environmental factor in Doñana (Díaz-Paniagua et al., 1996; Díaz-Paniagua and Mateo, 1999). It is clear that sandy soil substrates, directly or indirectly impose a strong effect (e.g., by imposing

high energetic costs of maintaining water balance or limiting availability of food resources; Marangoni, 2023), on adult body size, age and growth pattern (Marangoni et al. 2008, 2021). In addition, it could also be possible that gene flow would have prevented differentiation of these *E. calamita* populations for neutral loci, while intense selection would have maintained differences in adaptive traits of size and reproduction as has been suggested for other processes of adaptive divergence (Bensch et al., 1999; Gómez-Mestre and Tejedo, 2004). Recently, in the first study reporting genetic diversity estimates in the Iberian endemic pygmy newt (*Triturus pygmaeus*) from Doñana environment, Albert and García-Navas (2022) showed differences in genetic variability between temporary and permanent ponds. The authors suggested that the pond connectivity may constitute a more important factor than hydroperiod length in determining the genetic diversity and viability of pygmy newt populations. Moreover, given the geologically recent formation of the Guadalquivir basin, which may have occurred in mid-Holocene (about 5,000 years ago), it is possible that current genetic diversity patterns still reflect the historical distribution and gene flow among populations and that the effect of current gene flow (or lack thereof) is still not visible.

In conclusion, the neutral genetic differentiation cannot explain the geographical variation in the body size of natterjack toad, in accordance with the previous study by Gomez-Mestre and Tejedo (2004). Therefore, it is suggested that future studies are needed on the interaction between the genetic component with the environmental factors, and life history traits (e.g., age and growth pattern, Marangoni et al., 2021; food resources, Marangoni, 2023) at both larval and juvenile stages.

Finally, it is considered necessary to investigate other potential source of both within- and between-population components of variance, in addition to purely additive genetic variance, since that preliminary analyses showed that maternal effects may potentially contribute to the origin and evolution of the geographical variation in body size observed in *E. calamita* (Marangoni, unpublished data), and others amphibians (Bernardo, 1996; Mousseau and Fox, 1998; Räsänen et al., 2003, 2005).

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