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Can human-made structures enhance the genetic diversity and population dynamics of midwife toads (Alytes dickhilleni)?

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Abstract

The Betic midwife toad (*Alytes dickhilleni*) is a threatened amphibian endemic to southeastern Spain. Its populations are threatened by current climate change, desertification, habitat fragmentation, and chytridiomycosis. This study evaluates the role of artificial water sources (e.g., livestock ponds and fire ponds) as permanent habitats that support higer genetic diversity and population connectivity than natural temporary ponds. We genotyped 539 individuals from 20 locations in Sierra de Cazorla, Segura y Las Villas Natural Park using 12 microsatellite markers. Genetic diversity (observed heterozygosity, allelic richness) and the inbreeding coefficient (FIS) were similar between artificial and natural ponds. However, we observed genetic structure along the main mountain system, with a Bayesian cluster analysis identifying two main genetic groups. Populations showed a strong pattern of isolation by distance, indicating that geographical distance is a key factor driving genetic differentiation. Migration analyses revealed limited but asymmetric gene flow, with certain populations acting as sources and sinks. Our results suggest that artificial ponds can maintain levels of genetic diversity as high as natural ponds, but not higher, and may play a crucial role in helping populations persist in fragmented landscapes. Pond size and distance to the nearest pond, more than being natural or artificial, are key factors to explain their genetic diversity. Small ponds suffer a rapid decline in genetic diversity with increasing isolation, whereas large ponds maintain stable diversity even when geographically isolated. Conservation strategies should focus on maintaining a network of interconnected ponds, both natural and artificial, to ensure genetic flow and the long-term viability of *A. dickhilleni* populations.

Introduction

Genetic variation within wild species acts as a driver of evolutionary change. It allows species to adapt in response to environmental changes and makes them less susceptible to human disturbance (Frankham, 1996). Small population sizes, low connectivity, and habitat fragmentation are some of the most frequent causes for the loss of genetic diversity and population extinction (Elliott et al., 2000; Epps et al., 2005; Frankham, 1996).

Habitat fragmentation generates ecological "islands," i.e., patches of suitable habitat surrounded by a hostile matrix that limits the movement of individuals between populations (Watling & Donnelly, 2006). This spatial isolation disrupts gene flow, favors genetic drift, and promotes inbreeding, especially in species with low dispersal capacity, such as many amphibians (Wang et al., 2014). As a result, many small populations have reduced levels of genetic diversity and are usually inbred. Thus, these populations lose the ability to adapt to climate change and reduce their capacity to survive in long-term. Furthermore, in small and confined populations the effects of gene flow is more pronounced (Frankham et al., 2009). In many parts of the world, amphibians are increasingly threatened by habitat destruction and environmental change which raise fears about their long-term viability (Meléndez-Cal-y-Mayor et al., 2025; Stuart et al., 2004).

Due to climate change, the center and south of the Iberian Peninsula may suffer a particular increase in aridity by the end of this century, making species that need water points for reproduction more vulnerable (Gao & Giorgi, 2008). The Betic midwife toad (*Alytes dickhilleni*) is endemic to southeastern Spain, where its populations are largely confined to fragmented water bodies in mountainous areas (Albert, 2012; Arntzen & García-París, 1995). According to the IUCN, *A. dickhilleni* is Endangered (EN) due to an observed population decline of more than 50% over three generations between 2010–2020. This marked decline was due to the impact of chytridiomycosis, a fungal disease, and the lack of favorable habitats, the desiccation of breeding ponds and also because the high level of population fragmentation (Salvador et al., 2023).

Alytes dickhilleni adults, are commonly found in crevices and fissures of ravines, under stones near streams, springs, and pools, as well as in loose soil banks along the edges of paths and roads (Pleguezuelos et al., 2002). Its distribution is closely linked to the presence of clean and clear water, which is essential for both survival and reproduction. Although the genus *Alytes* is known for its terrestrial oviposition and early development of eggs on land, the larvae complete their development in aquatic habitats (Márquez, 1992). Males of this species carry the egg strings attached to their hind limbs (Duellman & Trueb, 1994). However, the successful completion of the larval stage depends on the long-term availability of aquatic habitats (Salvador & Garcia-Paris, 2001). In fact, the persistence of water in a given water body is the only necessary condition for *A. dickhilleni* to complete its larval development (Egea-Serrano et al., 2006). Even in intermittently dry landscapes as Mediterranean (Gao & Giorgi, 2008), certain habitat structures such as streambeds may serve as movement corridors for amphibians, offering moist microclimates and herbaceous cover that facilitate dispersal, despite being dry most of the year (Gibbs, 1998). This emphasizes the ecological value of ephemeral or artificial ponds in fragmented environments.

Amphibian demography is highly sensitive to climatic variability, with juvenile and subadult stages particularly affected by weather fluctuations, while adult survival tends to be more independent of these changes (Cayuela et al., 2016). Typically, the primary breeding habitats for *A. dickhilleni* are ephemeral natural water bodies (Fig. 1). *Sierra de Cazorla, Segura y Las Villas Natural Park* is one of the most important centers for the conservation of *A. dickhilleni and* is considered the area with the best health of this species' populations (Albert, 2012).

Table 1

Allelic frequencies per population. N (number of individuals sampled), observed heterozygosity (Ho), expected heterozygosity (He), private alleles (PA), allelic richness (AR), inbreeding coefficient (FIS). Natural ponds are highlighted in gray. In the PA column, the numbers before the parentheses correspond to the number of private alleles found in each population, while the number in parentheses () indicates the number of loci in which private alleles were found.

Pop	Code	Name	N	He	Ho	PA	AR	FIS
1	CCF	Campamento Cañada Fuentes	11	0.610	0.664	1	5.250	0.010
2	CCC	Casa Control Chozuelas	16	0.584	0.599	2(2)	4.417	0.008
3	CCR	Casa Control Rasos	22	0.669	0.655	5(5)	5.917	0.008
4	AGH	Arroyo Guadahornillos	37	0.644	0.667	2(2)	7.833	0.012
5	PRH	Piscina Roblehondo	15	0.598	0.644	0	4.917	0.010
6	AGE	Arroyo de la Gracea Este	16	0.662	0.672	2(2)	7.000	0.009
7	ARB	Arroyo del Rio Borosa	9	0.692	0.722	3(2)	5.333	0.009
8	CPJ	Cañada Pajarera	16	0.628	0.664	0	4.750	0.009
9	BRS	Bebederos de Rambla Seca	12	0.660	0.632	0	5.667	0.007
10	CPN	Cortijo de Pinar Negro	11	0.628	0.705	0	5.250	0.011
11	CVP	Cueva Paria	30	0.667	0.653	0	5.750	0.007
12	RCR	Refugio Cañada Rincón	31	0.674	0.682	2(2)	7.000	0.009
13	PNV	Pozo Nuevo	20	0.691	0.695	2(2)	6.417	0.009
14	CÑR	Cañada Rincón	12	0.622	0.648	0	5.250	0.009
15	PPR	Pozo Purga	24	0.649	0.667	1	7.000	0.010
16	PLA	Pozo la Losilla	34	0.691	0.667	1	8.083	0.006
17	BEP	Bebederos Espinos	20	0.654	0.654	1	6.333	0.008
18	ADF	Abrevadero Don Fernando	16	0.685	0.677	1	5.583	0.008
19	PPG	Pozo Prado Gonzalo	20	0.643	0.667	2(2)	6.333	0.010
20	FSB	Fuente del Borbotón	20	0.668	0.679	1	6.417	0.010
21	FCH	Fuente de la Chaparra	16	0.585	0.672	0	3.917	0.013
22	LMS	Loma de los Muertos	13	0.650	0.718	0	4.500	0.011
23	TCS	Tiná de las Cruces	16	0.636	0.682	2(1)	5.083	0.011
24	AJF	Arroyo de la Juan Fria	20	0.652	0.658	1	6.083	0.009
25	PNS	Pozo de los Navarros	16	0.642	0.588	0	5.500	0.006

Pop	Code	Name	N	He	Ho	PA	AR	FIS
26	FSZ	Fuente de la Zarza	19	0.593	0.623	0	4.417	0.011
27	FSA	Fuentesaltas	15	0.653	0.671	3(3)	5.750	0.009
28	FSE	Fuente del Engarbo	16	0.617	0.646	1	5.333	0.010
29	HYV	Hoya de la Viga	16	0.682	0.771	2(2)	6.000	0.013

However, a large proportion of the extant water bodies in most of the species' distribution are of human origin (Fig. 1). These artificial structures are not all the same; some are small watering holes for livestock, while others are large pools used as fire ponds. These infrastructures ensure a much more constant water supply during the dry season, which could allow a more constant water availability and could favor the conservation of genetic diversity and population connectivity for *A. dickhilleni*.

In this study we explore the population structure of *A. dickhilleni* across an important part of its distribution range, Sierra de Cazorla, Segura y Las Villas Natural Park, and assess whether man-made water bodies foster population genetic diversity, connectivity and viability. If such the role of the structures is understood, it would be possible to investigate new conservation strategies that would incorporate man-made structures as potential refugia for amphibian species in highly fragmented environments.

Material and methods

Study area and field sampling

A total of 539 individuals of *Alytes dickhilleni* were sampled from 29 localities from the *Sierra de Cazorla Segura y las Villas between 2010 and 2015* (Fig. 2). The samples were collected during the breeding season, and it consists mainly in tissue from tail tips of larvae. All samples were preserved in 95% ethanol, and the tissues were stored in a freezer at -80 degrees until to be extracted. We selected larvae from different places in each pond to avoid siblings in order to sample maximum genetic diversity (O'Connell et al., 2019). We registered the location of all ponds using a GPS unit. The permits for sampling were provided from the regional authorities (Junta de Andalucía) after ethical approval by the Spanish National Research Council.

The *Sierra de Cazorla, Segura y Las Villas Natural Park*, located in the northeast of the province of Jaén (Andalusia, Spain), is the largest protected area in the Iberian Peninsula. It covers an area of approximately 209,920 hectares and has an altitude range between 600 and 2,107 m above sea level (Junta de Andalucía, 2008). The climate is predominantly Mediterranean mountainous, with cold winters and dry summers, and highly variable thermal range and rainfall. The temperature ranges from an average of -1°C in the cold season to an average maximum temperature of 34°C in the dry season., while annual rainfall ranges from 500 mm in the drier areas to 2,000 mm in the higher and more exposed areas

(Ballesteros, 2008; Junta de Andalucía, 2008). Seasonality is marked, with rainfall concentrated in autumn and winter, and a dry season of between 3 and 5 months in summer.

Lab work

DNA was extracted with the QIAGEN DNeasy Tissue Extraction kit. We used 10 polymorphic microsatellites designed for *Alytes dickhilleni* (Albert et al., 2011) and two microsatellites designed for the sister species *Alytes muletensis* (Kraaijeveld-Smit et al., 2003). We used a Quiagen multiplex kit with standard conditions and a Q-solution to generate multiplex PCR with 0.25 µM of primer, 3 µL of genomic DNA and 62º C of annealing temperature. We analyzed amplified fragments using an ABI 3130xl Genetic Analyser with a LIZ 500 like size standard. Genotype size determination was performed using GeneMapper 4.0 (Applied Biosystems). Negative and positive control were included per extraction and PCR to ensure genotyping accuracy. In addition, at least 10% of all samples were reamplified to detect genotyping errors.

Genetic diversity analysis

Population diversity was assessed using observed heterozygosity (Ho) and expected heterozygosity (He), assuming random mating, using GENETIX 4.05. (Belkhir K. et al., 1996). Allelic richness (AR) and the population inbreeding coefficient (FIS) were calculated using FSTAT 2.9.4 (Goudet, 2003). Privates' alleles (PA) per population were calculated using GENALEX 6.5 (Smouse et al., 2017). We used the statistical software R (R Core Team, 2025) to represent the results and compare genetic diversity in natural and artificial ponds.

Genetic differentiation between all populations was quantified by estimating Wright's FST statistic using FSTAT 2.9.4 (Goudet, 2003). To assess the robustness of the overall FST estimates, we performed both jackknifing and bootstrapping on the loci.

In order to evaluate the effect of pond size and isolation on genetic diversity, we modeled observed heterozygosity (Ho) as a function of pond area in m² and distance to the nearest pond (km)established a model with interaction between both predictor variables to evaluate whether the effect of isolation depends on pond size. The betareg package in R (Cribari-Neto & Zeileis, 2010) was used to perform the analyses.

Spatial analysis

Spatial analyses were performed using QGIS 3.40.1 software. The GPS coordinates of the populations studied were added as a .kml layer of points for the different analyses. Images in .tif format were used to create elevation (DEM) and vegetation index (NDVI) layers, which were obtained from the USGS EarthExplorer platform (U.S. Geological Survey, 2025). A 1000 m buffer was created around each of the

populations, allowing us to calculate the average, minimum, and maximum values for elevation and NDVI at each of the points studied (Table S1). The surface area of the ponds was measured using photometry tools. To distinguish between livestock watering holes and larger irrigation ponds, the ponds were classified into two groups according to their surface area, using a threshold of 20 m².

Population structure analysis

The pattern of genetic differentiation was represented by correspondence factor analysis using GENETIX 4.05. (Belkhir K. et al., 1996) and visualized using the GRACE graphics tool. (Stambulchik, 1998)

To evaluate the pattern of isolation by distance, a Mantel test was performed comparing the geographic distance and genetic distance matrices between pairs of populations. We constructed the geographic distance matrix from the GPS coordinates of each population using the haversine formula from the geosphere package in R. The Mantel test was performed using the mantel function from the R vegan v2.6-4 package, which applies Pearson's correlation method with 999 permutations to evaluate statistical significance.

To infer genetic structure and define the number of clusters genetically different, we used software STRUCTURE 2.3, a model-based Bayesian clustering approach (Pritchard et al., 2010). Data were tested using an admixture model and indicating sampling sites. STRUCTURE was run for K ranging from 1 to 10 with a burn-in of 100,000 steps followed by 200,000 Markov chain-Monte Carlo (MCMC) iterations. The optimum number of distinct clusters was estimated using the web server StructureSelector (Li & Liu, 2018) applying the fastSTRUCTURE method (Raj et al., 2014).

In order to estimate simultaneous migration rates between populations, we used BA3 v3.0.5 (BAYESASS), a Bayesian software that infers recent migration patterns that have occurred in the last 2–3 generations. We configured the Markov Chain Monte Carlo (MCMC) simulation with 3,000,000 iterations, discarded the first 1,000,000 as burn-in, and sampled every 200 iterations to obtain 1,000 samples. To ensure reproducibiliy, a fixed random seed of 10 was used. In addition, the Metropolis-Hastings update scheme was applied with delta values set at 0.10 for migration rates, inbreeding coefficients, and allele frequencies, keeping acceptance rates within the recommended range of 20–60%. To visualize the resulting migration patterns, we constructed a string diagram using RawGraphs 2.0 (Mauri et al., 2017), in which the thickness of the arc corresponds to the estimated migration rate between pairs of populations.

Results

Genetic diversity analysis

Table 1 summarizes genetic diversity estimates for each population. Observed heterozygosity (Ho) shows considerable variation across populations, ranging from 0.588 (Population 25) to 0.771

(Population 29) with a mean value of 0.667. Expected heterozygosity (He) ranged between 0.584 and 0.692, with an average value of 0.644. The allelic richness (AR) varied between 3.917 and 8.083. The average allelic richness across all populations was approximately 6.07 alleles per locus. The analysis of private alleles revealed limited population-specific variation. The majority of populations hosted between 0 and 2 private alleles; However, it is noteworthy that Pop3 contained 5 private alleles, well above average. The inbreeding coefficient (FIS) suggests limited levels of inbreeding values across populations, ranging from 0.006 to 0.013, with a mean FIS of 0.009 across all sampled populations.

Wright's Fst statistic ranged between 0.00169 (between Pop19 and Pop23) and 0.20388 (Pop8 and Pop20; Table S2). Jackknifing produced an average global FST of 0.075 (standard error = 0.007), while bootstrapping generated a 95% confidence interval of 0.062–0.088 for θ (an estimator of FST).

Genetic diversity (Ho) and allelic richness (AR) did not differ between natural and artificial ponds; there are no significant differences between both groups suggesting that genetic diversity is not conditioned by the status of pond. Furthermore, in Figure S1, to obtain a broader view, other genetic and environmental parameters that could be key were compared. As with the observed heterozygosity, no significant differences were found for the inbreeding coefficient (FIS). Nor were any found for environmental values related to NDVI and average altitude, or for pond size alone.

To identify the factors that determine genetic diversity, we compared different generalized linear models that included environmental variables individually and in combination (Table 2). The univariate models with each predictor separately (pond size, distance to nearest pond, altitude, NDVI, and pond type) did not show significant effects ($p > 0.05$). Similarly, an additive model that included all variables simultaneously was also not significant. We also included an additive model with size and distance as the only predictor variables. Only the model that incorporated the interaction between pond size and distance to the nearest pond showed a significant fit ($p < 0.001$) and obtained the lowest AICc value, indicating that it is the best model to explain the variation in heterozygosity observed.

Table 2
Comparison of GLM models explaining observed heterozygosity. The Akaike information criterion (AIC), coefficient of determination (R^2), and statistical significance (p-value) are shown for each of the variables studied, used individually and additively.

Model	AIC	R^2	p-value
Null	-106.21	0.000	None
Status	-107.42	0.040	p = 0.300
Distance	-106.39	0.005	p = 0.721
Size	-106.36	0.004	p = 0.745
Altitude	-106.36	0.004	p = 0.743
NDVI	-106.54	0.010	p = 0.604
Additive	-100.52	0.075	all p > 0.25
Size + Distance	-104.52	0.009	size: p = 0.737, distance: p = 0.714
Size * Distance	**-118.17**	**0.422**	**All terms significant p < 0.01**

To better model the proportional nature of observed heterozygosity (limited between 0 and 1), we readjusted this interaction using a beta regression. The results of our beta regression model explaining the observed heterozygosity (Ho) as a function of pond size and distance to the nearest pond show a high level of significance (Pseudo R2 = 0.562). The analysis revealed a significant interaction effect between both variables (*p* < 0.001), indicating that Ho depends on the distance effect depends on pond size, and vice versa. All effects are highly significant; the coefficients are summarized in Table 3.

Table 3
Results of the beta regression model evaluating the effects of pond size and distance to the nearest pond on observed heterozygosity (Ho). The model of means (logit linkage) is shown. Pseudo R^2 = 0.562. Precision parameter (Phi) = 313.38 (SE = 78.23, *p* < 0.001).

Predictor	Coefficient(β)	Std. error	z-value	*p*-valor
(Intercept)	0.774	0.035	22.14	< 0.001
Nearest pond (km)	-0.054	0.014	-3.87	< 0.001
Pond size (m^2)	-0.001	0.0002	-4.65	< 0.001
Distance × Size	0.001	0.0002	5.81	< 0.001

We can observe the visualization of these opposing interaction effects in Fig. 3. For small ponds (< 20 m^2), as the distance to the nearest pond increases, a sharp decline in genetic diversity is observed. In contrast, in ponds larger than 20 m^2, genetic diversity remained high and stable even at greater distances of isolation.

Population structure and contemporary gene flow

A Mantel test was performed comparing pairwise geographic distances with genetic distances between populations. The analysis revealed a significant positive correlation (Mantel r = 0.5631, p = 0.001), indicating a clear pattern of isolation by distance. Genetic differentiation increases with greater geographic separation (Fig. 4).

Bayesian genetic assignment analysis performed using STRUCTURE software revealed the existence of two main genetic clusters among our sampled populations. Figure 5 shows the population membership graph, where each bar represents a population, and the colors indicate the proportion of ancestry assigned to each cluster. Some populations exhibit a partial admixture pattern; however, genetic differentiation is evident in at least two clearly detectable groups.

BAYESASS software was used to detect migration patterns between populations. The results are visualized using a chord diagram (Fig. 6) constructed using RawGraphs 2.0, which represents the magnitude of migration between each pair of populations. In most populations, the migration rates detected were low (< 1%), although some flows with higher values stand out, such as those detected from Pop1 and Pop3 to Pop4 or Pop24 to Pop11, all with rates above 10%. This data can be found in Table S3 of the supplementary material. This pattern suggests that connectivity in the study area is limited; however, there are certain cores that act as sources of migrants to adjacent populations. These results are consistent with the pattern of isolation by distance detected from the Mantel test and with the genetic structure inferred using STRUCTURE.

Discussion

In this study, we analyzed the genetic diversity and population structure of the Iberian midwife toad within the Sierra de Cazorla, Segura y las Villas Natural Park. We characterized two types of ponds within the study area: temporary ponds, which are only present during the rainy season and disappear in summer, and artificial ponds, which are watering holes for livestock and fire ponds.

Our results indicate that there are no significant differences in genetic diversity between natural and artificial ponds. Although many natural ponds are temporary and tend to dry up seasonally, whereas artificial ponds usually persist year-round, our dataset does not allow us to disentangle whether the observed patterns are directly attributable to drying events or to other environmental and ecological factors associated with pond type. *Alytes dickhilleni* populations show moderately high genetic diversity (average Ho = 0.67) despite habitat fragmentation and the small population size of some of the ponds. These results are comparable to those described in other studies on *Alytes cisternasii* or *Alytes muletensis*, in which genetic diversity is high despite fragmentation (Gonçalves et al., 2009; Kraaijeveld-Smit et al., 2005). This is also the case in other Mediterranean amphibians, such as *Triturus pygmaeus* in Doñana, whose Ho values are 0.74 and 0.70 in the north and south, respectively (Albert & García-Navas,

2022). The populations studied appear to maintain sufficiently high variability to avoid problems of inbreeding depression in the short term (FIS < 0.013) (Wright, 1965) .

However, analysis of population differentiation revealed a complex genetic structure. The average Fst analysis for each pair of ponds revealed that several populations are genetically distinct. The ponds Pop20 (Fst = 0.144), Pop24 (0.126), Pop27 (0.123), and Pop28 (0.121) consistently show greater average genetic differentiation from all others. This pattern suggests that these populations may be experiencing strong isolation due to geographical barriers, ecological constraints, or low connectivity, a concept that fits within the framework of landscape genetics (Manel et al., 2003). Our results show that the anthropogenic or natural origin of a pond is less decisive for genetic diversity than its physical characteristics, especially its size and connectivity with other ponds. Our findings corroborate previous studies that identify the density of occupied ponds within a radius of ~ 0.5 km as a critical factor for the persistence of amphibians in highly fragmented landscapes (Moor et al., 2024). The enormous decline in genetic diversity in small, isolated ponds is consistent with the metapopulation theory, according to which these ponds act as demographic and genetic “sinks” (Levins, 1969), being more prone to genetic drift and inbreeding due to their small effective size and lack of migrants to compensate for genetic loss (Gilpin, 1991). However, in larger ponds, the population size is quite high, cushioning the possible effects of genetic drift. This effect allows them to maintain high genetic diversity even in isolation scenarios, functioning as key population “sources” for maintaining the total metapopulation (Pulliam, 1988). It should be noted that many of these ‘source’ ponds are artificial (large irrigation ponds), so their maintenance is invaluable as a conservation tool in fragmented landscapes.

Despite the high overall genetic diversity, the genetic structuring underscores that ecological factors other than pond type (natural vs. artificial) are responsible for population divergence in our system. It is possible that the mountainous terrain is facilitating connectivity between some ponds while at the same time imposing barriers to gene flow for others, resulting in a mosaic of genetically distinct groups. One of the objectives of this study was to evaluate the capacity of artificial ponds to act as suitable habitats that maintain genetic diversity in comparison with natural ponds. Box plots showed that there are no significant differences between natural and artificial ponds. These results show that anthropogenic ponds can support populations with levels of genetic diversity similar to those found in natural ponds. Similar results have been reported in other studies, where artificial ponds have provided valid reproductive habitats and increased connectivity within highly fragmented landscapes (Albero et al., 2025; Albert et al., 2013; Moor et al., 2022).

However, similar genetic diversity values do not necessarily reflect similar ecological conditions. Other factors, such as connectivity between populations, the age of the streams and the presence of microhabitats, may compromise the long-term viability of these populations (Keyghobadi, 2007). Since the pond type factor does not seem to explain genetic diversity, we decided to explore other factors such as geographical distance. We used Mantel's test to compare the geographical distance and genetic distance matrices, the results of which indicate clear isolation by distance ($r = 0.56$, $p = 0.001$) (Wright, 1943). These results show us how, as the geographical distance between populations increases, they

become more different as gene flow between them decreases. This pattern of isolation by distance is consistent with that documented in other amphibian species with limited dispersal capacity, where geographic distance was a significant predictor of genetic differentiation, such as the natterjack toad in Ireland and others (Alex Smith & M. Green, 2005; Reyne et al., 2023).

The presence of this gradual isolation highlights the importance of landscape connectivity for the viability of *Alytes* populations. Although artificial ponds maintain high levels of genetic diversity and act as suitable reproductive habitats, it is important that they are distributed in such a way as to contribute to the exchange of individuals between populations. This is particularly important from a conservation perspective, as it is necessary to maintain a network of breeding sites that are not only suitable for the species but also close enough to each other to promote gene flow.

Based on Bayesian clustering analyses performed with STRUCTURE, we were able to identify a clear pattern of genetic structuring into two main clusters, suggesting that there is a genetic division between the individuals sampled. Each of these subdivisions largely coincides with the environmental differences observed between the wetter and drier areas of the Sierra (Fig. 6). The dispersion of *Alytes dickhilleni* appears to be conditioned by topography and the availability of humid corridors, which act as connecting routes. Although separation is evident, there appears to be some genetic overlap in intermediate areas, suggesting that there is no absolute barrier to gene flow. These results could indicate that local ecological conditions play an important role in shaping population structure.

In this sense, the BayesAss results provide us with a complementary view of gene flow. The string diagram constructed based on migration rates between populations indicates that there is some gene flow between almost all populations. However, the intensity of this flow is not homogeneous, with some population nuclei exhibiting a greater amount of exchange. Population 4 (Arroyo Guadahornillos) is a natural pond with a very short hydroperiod; it dries up quickly, which probably causes high mortality among recruits and traps individuals that arrive. In contrast, population 11 (Cueva Paria) is an artificial pond that has been heavily manipulated by humans and also functions as a sink. From a conservation perspective, this pattern is critical. These sinks act as demographic traps, where immigrating individuals face high mortality or reproductive failure, preventing further dispersal. In addition, there appears to be a certain division into gene flow groups between populations 1 to 10 and beyond, coinciding with the results of STRUCTURE and the hydrology of the Sierra. Even so, *Alytes* populations continue to maintain sufficient levels of gene flow between all populations to prevent complete differentiation.

Conclusions

The results of this study show that artificial ponds used as watering holes for livestock and fire ponds are valuable habitats for the reproduction and viability of populations of the Betic midwife toad (*Alytes dickhilleni*) in the Sierra de Cazorla, Segura y las Villas. Genetic analyses reveal that these structures can maintain levels of genetic diversity (observed heterozygosity and allelic richness) and inbreeding coefficient (FIS) similar to those of natural ponds.

The determining factor for genetic diversity does not seem to be related to the origin of the pond (natural or artificial) but rather to the physical and landscape characteristics of the pond. A beta regression model identified a highly significant interaction between pond size and distance to the nearest pond (isolation). Small ponds experience a drastic loss of genetic diversity as the distance to the nearest pond increases, acting as genetic sinks vulnerable to gene drift. In contrast, larger ponds function as resilient genetic reservoirs, maintaining high diversity even in isolated situations. At the landscape scale, a clear genetic structure was identified with two main clusters, corresponding to the orographic and hydrological division of the mountain range between a wetter northwestern region and a drier southeastern region. This pattern, together with the significant isolation by distance detected, indicates that the complex mountainous topography plays a key role in structuring genetic diversity, facilitating connectivity in some areas and imposing barriers to gene flow in others.

In conclusion, this study highlights that the long-term conservation of *A. dickhilleni* must focus on ensuring the persistence of a network of ponds that guarantee good landscape connectivity, especially for smaller and more isolated populations. Far from being negative elements, artificial ponds are essential active conservation tools for mitigating the effects of habitat fragmentation and climate change on this threatened species in the Sierra de Cazorla, Segura y las Villas Natural Park.

Conservation implications

Based on the findings of this study, we propose recognizing human-made structures (watering troughs and irrigation ponds) as key elements for the conservation of the species. Management programs for the Natural Park should include their periodic maintenance, ensuring the permanence of water during the dry season, which is critical for larval development.

Conservation actions should prioritize the creation, expansion, and maintenance of large ponds. These structures appear to function as genetic reservoirs that cushion the negative effects of isolation and ensure the long-term persistence of viable populations. In addition, the planning of new artificial ponds should be carried out strategically to improve connectivity between existing populations. The location of new ponds should prioritize the connection between identified genetic clusters and act as a bridge between the wet and dry areas of the mountains. It is essential to conserve the quality of surrounding terrestrial habitats and wet micro-corridors such as seasonal streams and riparian areas that facilitate the movement of adult individuals between ponds and thus genetic flow. It is also necessary to establish a long-term monitoring program that combines periodic genetic assessments to detect loss of diversity with demographic monitoring of variables such as reproductive success and population size. Good management will allow us to continue evaluating the effects of artificial ponds andenable us to respond quickly to potential threats to the species. Further ecological studies are needed to better understand the species' habitat requirements, life cycle characteristics, and responses to environmental changes, which will provide a more robust scientific basis for conservation and management measures.

Finally, cooperation and outreach are essential to involve local stakeholders such as livestock farmers in the conservation of the species. Promoting management practices compatible with their activities can make a difference and ensure the availability of water for *Alytes dickhilleni* and many other species in the Natural Park.

Declarations

Competing interests

The authors declare no conflicts of interests.

Authors contribution

The methodological design and conceptualization of the project was done by all the authors, J.M.A. C.V., and E.M.A. Sample collection and laboratory work were performed by E.M.A. The data analysis, the creation of figures and the writing of the first draft were carried out by J.M.A. The final reading, review, and editing of the manuscript was done by all the authors, J.M.A. C.V., and E.M.A., and J.M.A.

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Data availability: All genetics (genotypes) and related information areavailable upon request to the authors.

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Figures

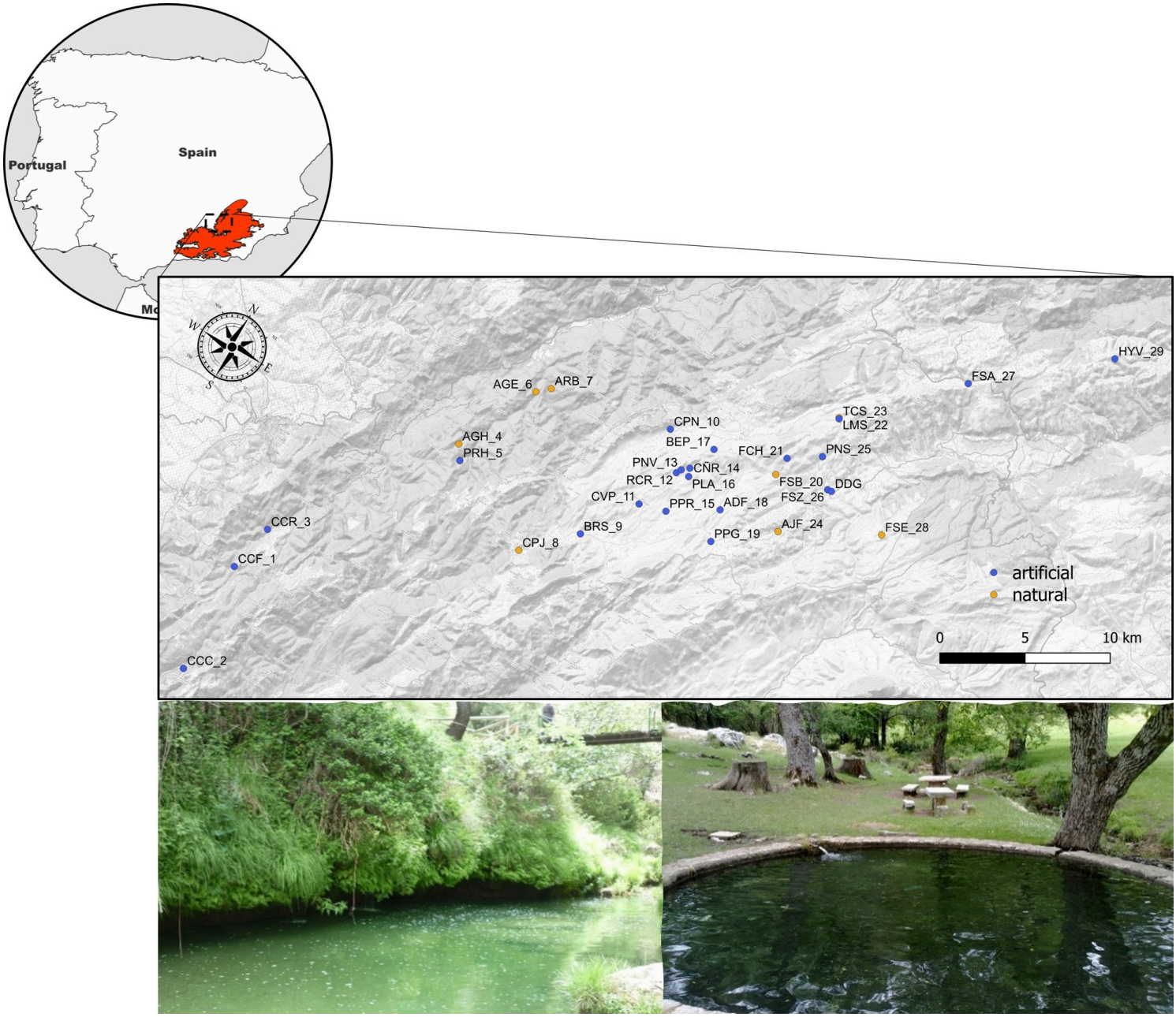


Figure 1

Geographical distribution of the *Alytes dickhilleni* populations analyzed. On the left is the location of the study area on the Iberian Peninsula (black box) and the total distribution of the species. On the right is an enlarged map showing the 29 locations sampled, coded by their abbreviations (see Table 1). Orange dots are natural ponds, and blue dots are artificial ponds. The figures below show the contrast between the habitats sampled: temporary natural pond (bottom left) and permanent artificial pond (bottom right).

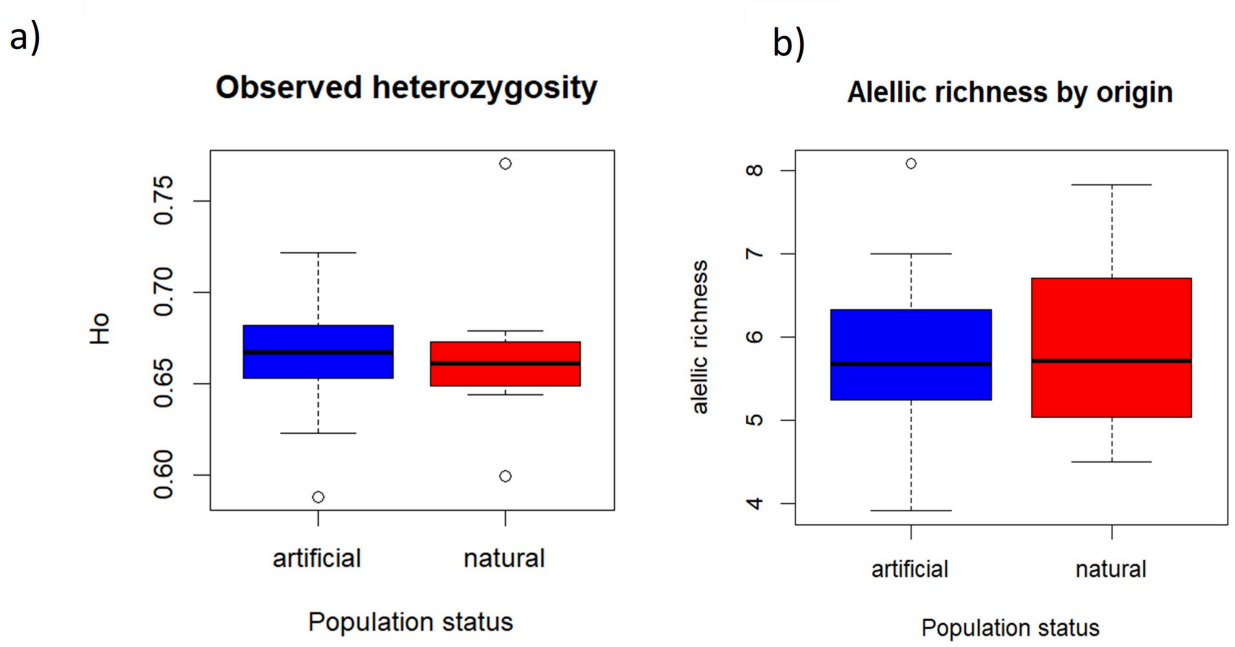


Figure 2

(a) Box plot comparing observed heterozygosity (Hobs.) values between natural and artificial populations. (b) Box plot comparing allelic richness (AR) values between natural and artificial populations. No statistically significant differences were detected between groups ($p > 0.05$).

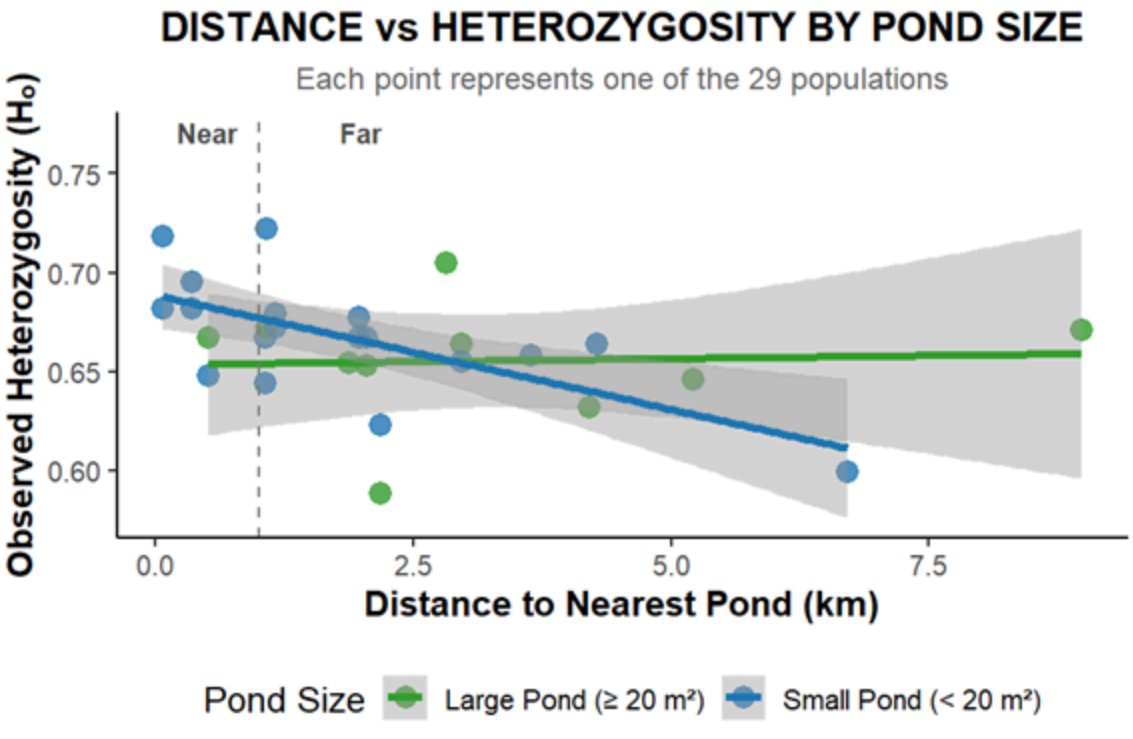


Figure 3

Effect of the interaction between pond size and distance to the nearest pond on observed heterozygosity (Ho) in Alytes dickhilleni. The regression lines predict Ho for small ponds (25th percentile, in blue) and large ponds (75th percentile, in green). The shaded area represents the 95% confidence interval.

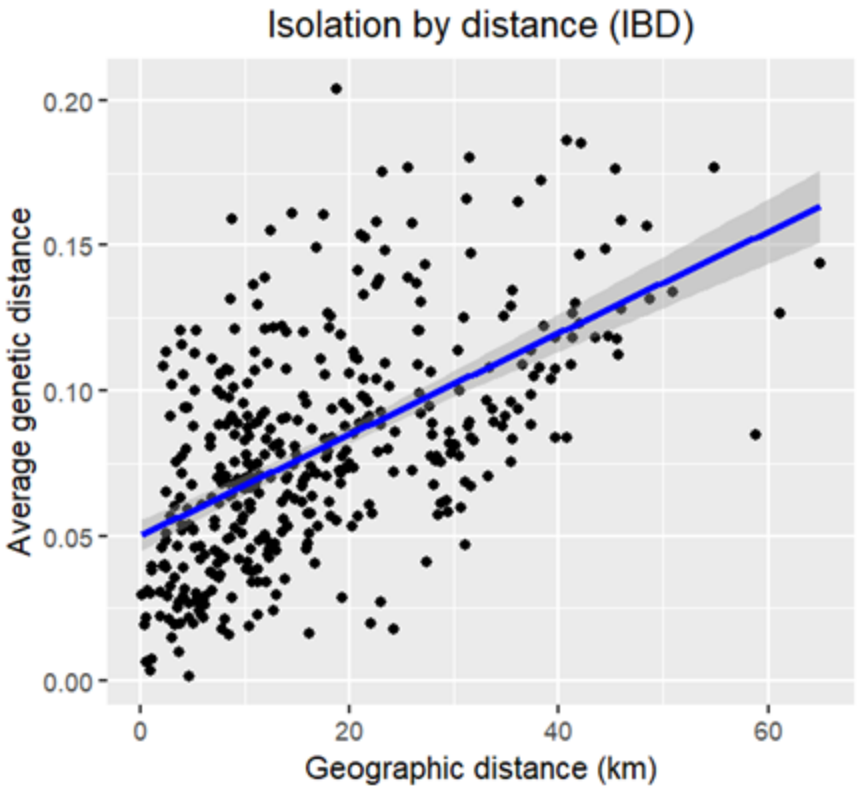


Figure 4

Relationship between genetic differentiation (Fst) and geographic distance between populations (Mantel test, r = 0.5631, p = 0.001.

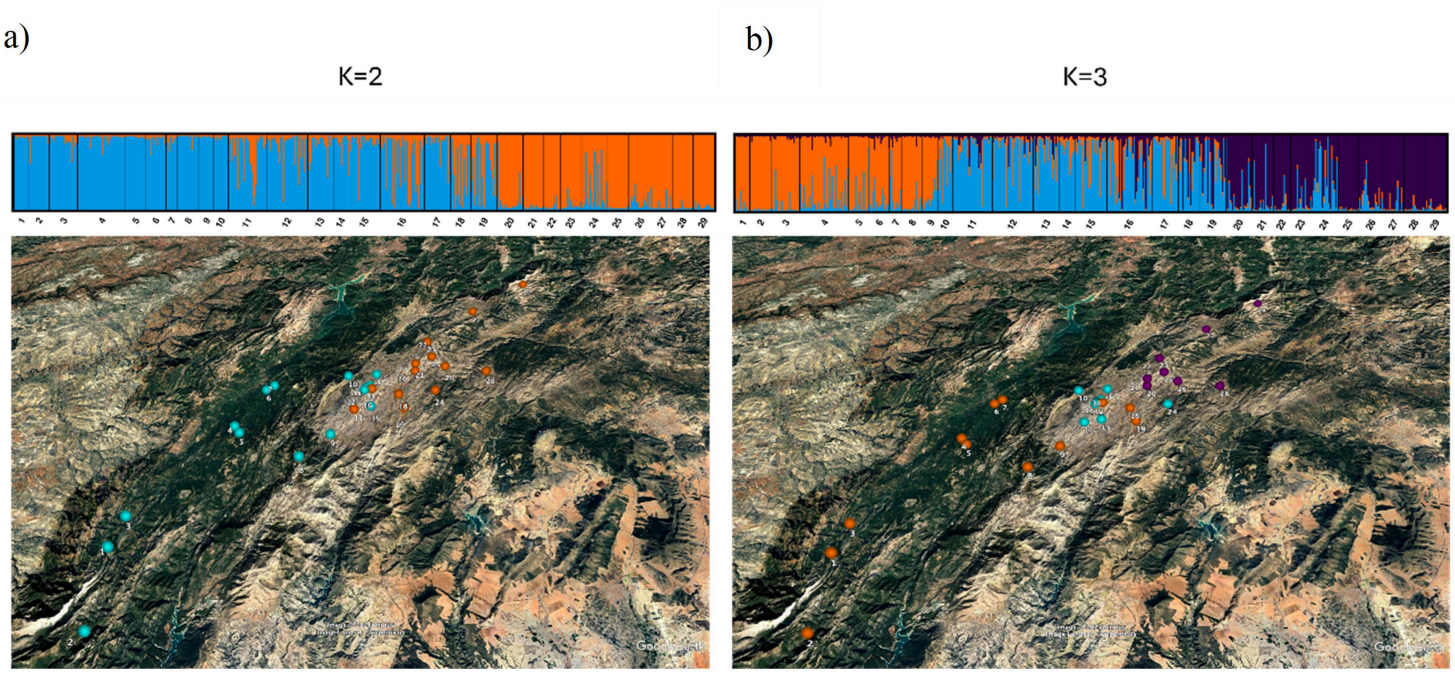


Figure 5

Relationship between genetic structure and the orography of the Sierra de Cazorla. (a) Map of the study area showing the location of the 29 sampled populations, color-coded according to their assignment to the main genetic cluster (K=2). The blue and orange dots represent the populations belonging to the humid (south) and dry (north) regions of the mountain range, respectively. (b) Results for K=3 show an additional partition, with blue dots corresponding to a transition zone and purple dots to the dry zone.

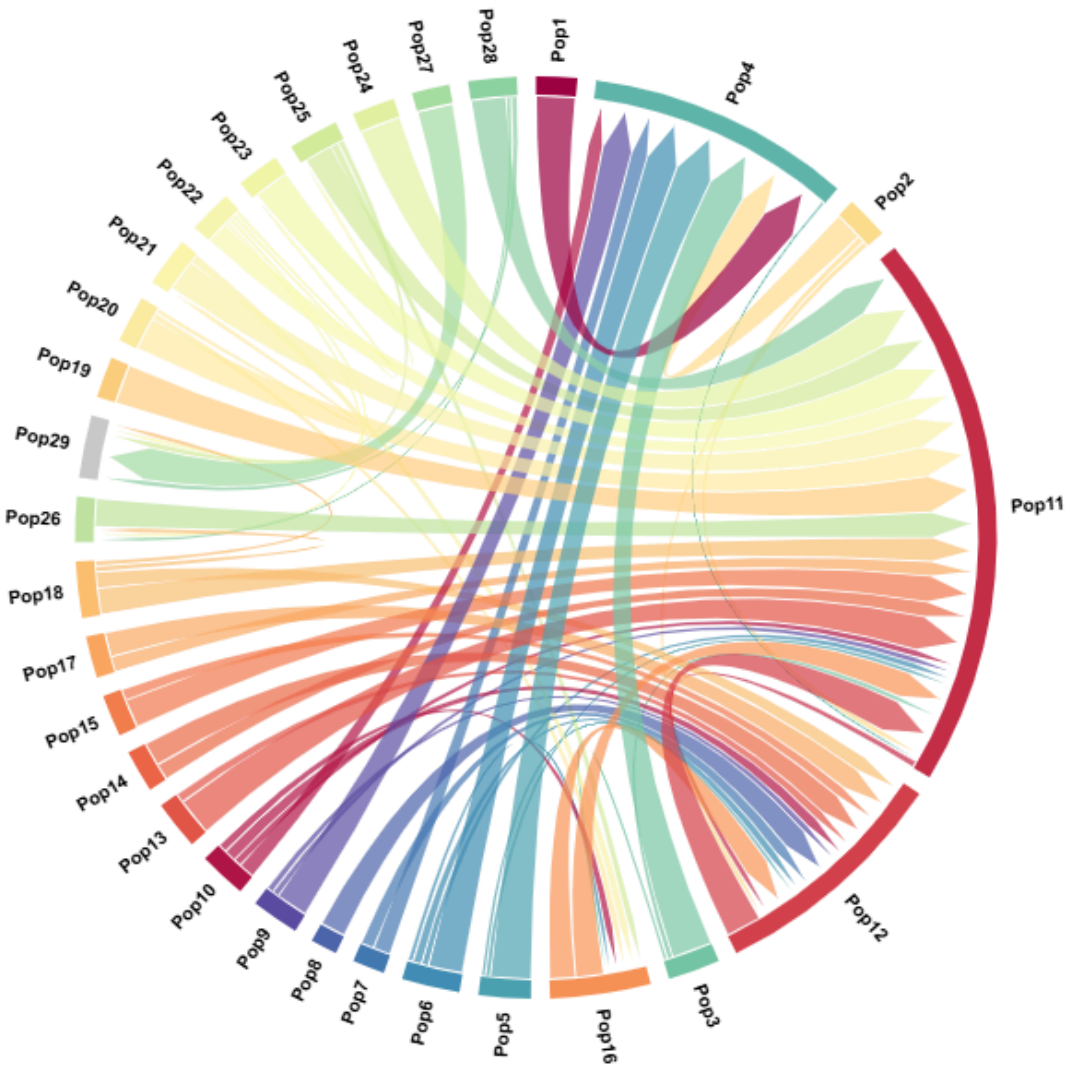


Figure 6

Chord diagram showing the estimated migration spats between populations of *Alytes dickhilleni*, calculated using Bayesian equations. The arcs represent the direction and magnitude of migrant flow between each pair of populations; their thickness is proportional to the migration rate. The table only shows pairs of populations with migration rates above 1%.

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