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Mountains as barriers to gene flow in amphibians: quantifying the differential effect of a major mountain ridge on the genetic structure of four sympatric species with different life history traits

Sánchez-Montes, G.[1,2,3,5], Wang, J.[3], Ariño, A. H.[1], and Martínez-Solano, I.[2,4,5*]

[1] Department of Environmental Biology, Universidad de Navarra, 31008, Pamplona, Spain

[2] Museo Nacional de Ciencias Naturales, CSIC, c/ José Gutiérrez Abascal, 2, 28006 Madrid, Spain

[3] Institute of Zoology, Zoological Society of London, London NW1 4RY, UK

[4] Instituto de Investigación en Recursos Cinegéticos (IREC-CSIC-UCLM-JCCM), Ronda de Toledo, s/n, 13071 Ciudad Real, Spain

[5] Ecology, Evolution, and Development Group, Department of Wetland Ecology, Doñana Biological Station, CSIC, c/ Américo Vespucio, s/n, 41092 Seville, Spain

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*corresponding author: Íñigo Martínez-Solano

Current address: Department of Biodiversity and Evolutionary Biology, Museo Nacional de Ciencias Naturales, CSIC, c/ José Gutiérrez Abascal, 2, 28006 Madrid, Spain

E-mail: inigomsolano@gmail.com, Phone: (+34) 91 411 13 28, Fax: (+34) 91 564 50 78

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ABSTRACT

Aim: To test the role of mountains as barriers to gene flow in co-distributed taxa with different life history traits.

Location: *Sierra de Guadarrama*, Central Spain.

Methods: We used larval genotypes of four amphibian species (*Epidalea calamita*, *Hyla molleri*, *Pelophylax perezi* and *Pelobates cultripes*) sampled on northern and southern slopes of *Sierra de Guadarrama* to describe genetic structure with F_{ST}, migration rates per generation, clustering algorithms and resistance by elevation surfaces. We also recorded individual displacement events as a proxy of dispersal potential during a seven-year monitoring project based on capture-mark-recapture (CMR).

Results: All species travelled longer cumulative distances than those reported in the study area for *P. cultripes* (0.71 km). Individuals of *E. calamita* traveled up to 3.55 km, followed by *H. molleri* (2.84 km) and *P. perezi* (1.51 km). Pairwise F_{ST} estimates showed lower overall connectivity in *P. cultripes*. Average migration rates per generation were low in all species, with exceptions in same-slope populations of *H. molleri* and *P. cultripes*. Clustering algorithms consistently recovered well-differentiated population groups of *P. cultripes* in northern *vs* southern slopes, but widely admixed areas were observed in the other species, especially near mountain passes. Resistance by elevation surfaces showed a strong barrier effect of *Sierra de Guadarrama* in *P. cultripes* and suggested a potential role of topography in the genetic structure of *E. calamita* and *H. molleri*.

Main conclusions: *Sierra de Guadarrama* currently acts as a strong barrier to gene flow for *P. cultripes* and, to a lesser extent, for *E. calamita*, *H. molleri* and *P. perezi*. This differential effect can be partly explained by differences in life history traits, including dispersal potential. Our findings support the general role of the Central System as a key feature shaping population connectivity and genetic variation in amphibian communities.

INTRODUCTION

Mountains, along with rivers and oceans, are the main topographic factors associated with long-term barriers to gene flow (Zalewski et al., 2009; Wei et al., 2013; Pagacz, 2016). In amphibians, slope, elevation and mountain ridges have been shown to affect population connectivity (Lougheed et al., 1999; Funk et al., 2005; Martínez-Solano & Gonzalez, 2008; Richards-Zawacki, 2009, and their effect may have been especially intense during glacial maxima (Pereira et al., 2016). Nevertheless, mountains do not usually act as absolute barriers but rather as more or less permeable filters. Species with different dispersal potential, breeding behaviour or physiological traits are expected to respond differently to topography, and in consequence will show differences in their patterns of spatial genetic structure across shared landscapes (Steele et al., 2009). Studies comparing the genetic structure of species with different life history traits can thus provide comprehensive insights into the current and historical role of mountains as barriers to gene flow.

The Iberian Peninsula is one of the best examples of the “refugia within refugia” paradigm (Gomez & Lunt, 2007). Topographic features, in particular the orientation of major mountain ranges along west-east axes, have been hypothesized to constrain latitudinal population expansion/contraction events in response to climatic changes during the Pleistocene. Among these, the Central System mountains are thought to represent a historical barrier to gene flow across different taxonomic groups. The ranges of several amphibian species find their distributional limit in the Iberian Central System (Arntzen & Espregueira Themudo, 2008; Reino et al., 2017) and these mountains separate well differentiated intraspecific clades in other species (Gutiérrez-Rodríguez, Barbosa, et al., 2017).

Explicitly testing the differential role of a putative barrier in shaping genetic structure across taxa requires assessing: 1) whether the putative barrier acts as such in the present,

disrupting patterns of population connectivity, and 2) the consistency of the barrier effect across species with different life history traits (e.g. Richardson, 2012). Several molecular-based approaches allow testing the relative effect of different landscape features on regional patterns of gene flow (Cushman et al., 2006; Landguth et al., 2010; Blair et al., 2012). These approaches will provide robust inferences under a comparative approach, as species with differences in life history traits (e.g. size, activity patterns, dispersal capacity, longevity and so on) should exhibit different population dynamics and ecological requirements, responding differently to sharp ecological gradients such as those associated with high mountain ridges. In addition, direct field observations on individual spatial displacements in wild populations recorded in capture-mark-recapture (CMR) studies can provide key information to understand how local dynamics scale up to shape patterns of regional structure in different species (Frei et al., 2016).

Here we test the role of *Sierra de Guadarrama* (a segment of the Iberian Central System) as a major barrier to gene flow in four sympatric amphibian species: the natterjack toad *Epidalea calamita*, the Iberian treefrog *Hyla molleri*, Perez's frog *Pelophylax perezi* and the Western spadefoot toad *Pelobates cultripes*. These four species have different morphologies, life history traits, habitat preferences and altitudinal distribution limits (Table 1), and thus they are expected to be differentially affected by *Sierra de Guadarrama*. We complement a previous study providing information on dispersal potential in one of the species (*P. cultripes*, Gutiérrez-Rodríguez, Sánchez-Montes, et al., 2017) with new data on the other three species based on a seven-year CMR study (Fig. 1) and combined four genetic approaches to investigate interspecific differences in regional patterns of genetic structure.

MATERIALS AND METHODS

Study area and target species

The study was conducted in *Sierra de Guadarrama*, in the eastern end of the Iberian Central System (Fig. 2). This mountain range has 13 peaks above 2000 m.a.s.l., with the lowest elevations in the Alto del León (SW, 1510 m.a.s.l.) and Somosierra (NE, 1445 m.a.s.l.) passes (Fig. 2). Three additional passes are located in Navacerrada (1858 m.a.s.l.), Cotos (1829 m) and Navafría (1774 m, Fig. 2). Regional climate is Mediterranean with cold winters and mild dry summers, although the asymmetry of the massif results in heterogeneity of microclimates among different areas (López-Sáez et al., 2014).

Up to 15 amphibian species can be found in *Sierra de Guadarrama*, although many of them become rare above 1000-1500 m.a.s.l. (Martínez-Solano, 2006). We focused on four anurans that are widely distributed across both slopes of *Sierra de Guadarrama*: *E. calamita*, *H. molleri*, *P. perezi* and *P. cultripes*. Maximum reported elevations in *Sierra de Guadarrama* are 2200 m.a.s.l. for *E. calamita*, 2140 m for *H. molleri*, 2170 m for *P. perezi* and 1470 m for *P. cultripes* (Martínez-Solano, 2006). These four species show differences in life history traits (Table 1). Some of these, like larger size, increased longevity, facultative diurnal activity, fast larval development or high dispersal potential, might be advantageous for population connectivity in higher elevations, which should be reflected in regional patterns of genetic structure. Despite intersexual differences in reproductive behaviour, no evidence of sex-biased dispersal has been reported in any of the four species, although further research is required (Sinsch, 1992; Gutiérrez-Rodríguez, Sánchez-Montes, et al., 2017).

Dispersal potential

We recorded direct observations of individual movements during a seven-year (2010-2016) CMR monitoring of an assemblage of the four species near the locality of *Valdemanco*, Madrid (Fig. 1). *Laguna de Valdemanco* and other secondary breeding sites nearby were

surveyed on a yearly basis since 2010, with multiple CMR sessions performed every year. In each CMR session, all sexually mature individuals found during visual encounter surveys were captured, sexed based on morphological characters and marked with passive integrated transponder (PIT) tags (further details in Sánchez-Montes, Wang, et al., 2017). During this seven-year period we performed 219 CMR sessions, and marked 1086 adult *E. calamita* (427 of them were further recaptured in at least one subsequent CMR session, with a maximum of 23 recaptures per individual), 599 *H. molleri* (153 further recaptured, maximum: seven recaptures) and 662 *P. perezi* (325 further recaptured, maximum: 10 recaptures). Dispersal events of marked adults of the three species from *Laguna de Valdemanco* to nearby breeding sites were recorded from direct visual encounters (Fig. 1). The minimum cumulative distances covered by each individual were calculated by summing the distances between consecutive recorded locations. Cumulative distances only accounted for movements longer than the longitude of the main axis of the *Laguna de Valdemanco* flooding area (125 m). During the same seven-year period, 824 adult *P. cultripes* were marked in the study area (440 further recaptured, maximum: 17 recaptures); recorded displacements were reported in Gutiérrez-Rodríguez, Sánchez-Montes, et al. (2017).

Genotype dataset

We used larval genotypes of the four species (15-18 microsatellite loci per species, *n*=19-36 individuals per population) from 13-19 populations per species across both slopes of *Sierra de Guadarrama* (Table 2, Fig. 2). Genotypes of *E. calamita*, *H. molleri* and *P. perezi* were published in Sánchez-Montes, Ariño, et al. (2017). From that dataset we excluded sample localities containing less than six non-full sib individuals to avoid unreliable inferences derived from few full sib families in some genetic samples (Anderson & Dunham, 2008; Rodríguez-Ramilo & Wang, 2012; Sánchez-Montes, Ariño, et al.). We also excluded *Laguna de Valdemanco* from the dataset because tissue sampling in that locality was more

exhaustive than in other populations (Sánchez-Montes, Wang, et al., 2017). Additionally, we obtained larval samples of *P. cultripes* in 13 localities across the study area (total *n*=368, between 20-31 individuals per population, Table 2, Fig. 2) following the survey method described in Sánchez-Montes, Ariño, et al. We used 16 published microsatellite loci (Gutiérrez-Rodríguez & Martínez-Solano, 2013) to genotype the samples of *P. cultripes* following the laboratory and allele calling procedures described in Sánchez-Montes et al. (2016).

Genetic analyses

Pairwise population genetic distances and tests of IBD

We used the G-statistics subroutine in GENALEX (Peakall & Smouse, 2006) to estimate F_{ST} values (Wright, 1943, 1951) between all pairs of populations in each species and assessed their significance (9999 permutations) after applying the Bonferroni correction for multiple tests. We compared estimates of F_{ST} obtained either including or excluding full siblings (identified using COLONY, Jones & Wang, 2010) in each population for exploratory purposes (Sánchez-Montes, Ariño, et al.; Waples & Anderson, 2017). We then used CODIDI (Wang, 2015) to test for the utility of each marker set for unbiased F_{ST} or G_{ST} (Nei, 1973) estimation, by calculating the correlation between gene diversity and G_{ST}. Allele size permutation tests (1,000 permutations per locus) were performed in SPAGEDi v1.5 (Hardy & Vekemans, 2002) to check whether stepwise-like mutations contributed significantly to genetic differentiation, in which case R_{ST} measures (Slatkin, 1995) would be preferred over F_{ST} (Hardy et al., 2003). Finally, we used GENALEX to test for isolation by distance (IBD) patterns within each of the two slopes of the mountain range. For each species, we performed two Mantel tests (9999 permutations), each one including only the populations located either on the northern or on

the southern slope of *Sierra de Guadarrama*. Pairwise geographic distances were calculated from Latitude/Longitude coordinates using a modification of the Haversine formula (Sinnott, 1984).

Migration rates per generation

We estimated migration rates per generation between all pairs of populations in each species using BAYESASS (Wilson & Rannala, 2003) using five replicate analyses per species with 1,000,000 burn-in and 10,000,000 iteration steps. We adjusted mixing parameters for allele frequencies (Δ_A), inbreeding coefficients (Δ_F) and migration rates (Δ_M) to situate acceptance rates in the Markov chain Monte Carlo (MCMC) runs between 20-60% and checked the concordance of results by quantifying the differences among migration rate estimates across runs.

Clustering analyses

We employed three different clustering analyses to characterize the genetic structure of the four species. In all cases, we inferred the number of clusters (*K*) best explaining genetic data, but also focused on *K*=2 to assess whether this corresponded to a north-south break.

First, we performed unsupervised Bayesian clustering analyses in STRUCTURE (Pritchard et al., 2000). For each *K* value from one to the total number of sampled localities in each species, we performed ten replicates using an admixture model with correlated allele frequencies and 500,000 burn-in and 1,000,000 iteration steps (Pritchard et al., 2000; Falush et al., 2003). We summarized clustering results using CLUMPAK (Kopelman et al., 2015) and explored the likelihood of different *K* values using likelihood scores (Pritchard et al., 2000) and Δ*K* (Evanno et al., 2005) in STRUCTURE HARVESTER (Earl & vonHoldt, 2012). Second, we

206 performed discriminant analysis of principal components (DAPC, Jombart et al., 2010) using
207 the R package *adegenet* (Jombart, 2008; R Development Core Team, 2009). We selected
208 the minimum number of principal components required to account for at least 90% of the
209 variation contained in the data, explored the best value of *K* between one and 25
210 (encompassing the total number of populations in all species) and computed individual
211 membership probabilities to inferred clusters. Third, we used GENELAND (Guillot et al., 2005)
212 to perform spatially explicit clustering analyses. As in DAPC analyses, we explored the best
213 value of *K* between one and 25. Then, we performed ten different runs (allele frequencies:
214 correlated; 100,000 iterations; thinning=100; uncertainty in spatial locations=0.01) for each
215 species with *K*=2. .

216

217 *Landscape genetic analyses*

218 We employed a landscape genetics-based causal modelling approach (Cushman et al.,
219 2006, 2013) to test for barrier effects while accounting for elevation (resistance surfaces) and
220 geographical (Euclidean) distances on observed genetic distances among populations. To
221 construct elevation-based resistance measures, we obtained a digital elevation model of
222 *Sierra de Guadarrama* at 200m resolution (*Instituto Geográfico Nacional*, *Spain*,
223 http://centrodedescargas.cnig.es/CentroDescargas/). We then constructed four different
224 resistance surfaces, all assuming a linear relationship between elevation and resistance
225 (resistance = elevation), but with this linear effect starting at different minimum altitude
226 thresholds (0, 1000, 1500 and 2000 m.a.s.l.). The R package POPGENREPORT (Adamack &
227 Gruber, 2014) was used to: a) calculate least cost paths between all population pairs in each
228 species with the four elevation-based resistance models using an eight-pixel nearest-
229 neighbour approach; and b) construct genetic (based on Nei's G_{ST}) and Euclidean distance
230 matrices. The resistance matrix for the barrier effect was constructed by assigning a

resistance value of “0” to pairwise comparisons involving populations located on the same slope, and “1” to comparisons between populations on opposite slopes. We used the R package *ecodist* (Goslee & Urban, 2007) to assess the relative support for each model based on partial Mantel tests.

RESULTS

Dispersal potential

Long cumulative movements were recorded in some individuals of *E. calamita* (Fig. 3), but only two marked males were found in a breeding site >400 metres away from *Laguna de Valdemanco* (Fig. 1). These individuals moved at least two and five times, respectively, between *Laguna de Valdemanco* and a mining pond >700 metres away. These two and one additional male moved cumulative distances >1420m (maximum=3550m), highlighting the high dispersal capacity of this species (Fig. 3). We also obtained the first direct records of medium-distance dispersal across a terrestrial landscape matrix for individuals of *H. molleri* and *P. perezi*. Several marked individuals of both species were captured in different breeding sites >600m away from *Laguna de Valdemanco* and not connected by aquatic corridors (Fig. 1), either in the same season or in different years. One male of *H. molleri* moved at least four times between *Laguna de Valdemanco* and the mining pond in three years (cumulative distance: 2840m, Fig. 3). Several medium- and long-distance displacements (680-1510m) were also recorded in both male and female *P. perezi* (Fig. 3).

Genetic analyses

Pairwise population genetic distances and tests of IBD

We did not find negative correlations between gene diversity and G_{ST} in any species (not shown). Also, allele size permutation tests indicated no significant contribution of stepwise-like mutations on genetic differentiation in any species (average multilocus pairwise R_{ST}: *E. calamita*: 0.047, p=0.880; *H. molleri*: 0.107, p=0.564; *P. perezi*: 0.106, p=0.722; *P. cultripes*: 0.116, p=0.131). These results support the reliability of multilocus F_{ST} and G_{ST} estimates to estimate genetic distances between populations in the four species (Wang, 2012, 2015). Additionally, F_{ST} estimates were not affected by the presence of full sibs in the *P. perezi* samples, and only slight over- (in *E. calamita* and *P. cultripes*) or underestimations (in *H. molleri*) were detected in the other species (Fig. S1.1 in Appendix S1, Supporting Information).

Almost all pairwise F_{ST} estimates were significantly >0 after applying the Bonferroni correction (Fig. 4, Tables S1.1-S1.4 in Appendix S1). The highest values (>0.2) were obtained in *P. cultripes*, especially among populations located on different slopes of *Sierra de Guadarrama* (Fig. 4, Table S1.4 in Appendix S1). In *H. molleri*, comparisons involving TOR and COL scored the highest pairwise F_{ST} values (maximum F_{ST}= 0.147), whereas in *P. perezi* the most differentiated localities were BER and ARC (maximum F_{ST}=0.142). The maximum F_{ST} value in *E. calamita* was 0.082, and COL was the most differentiated population (Appendix S1).

We found significant evidence of IBD within the northern slope in *P. cultripes* (R=0.762, p=0.020), while *E. calamita* and *H. molleri* did not show evidences of IBD (*E. calamita*: R=-0.056, p=0.525; *H. molleri*: R=-0.302, p=0.166) and *P. perezi* showed a significant negative relationship between genetic and geographic distances (R=-0.403, p=0.025). In the southern slope, none of the four species showed evidences of IBD (*E. calamita*: R=0.323, p=0.094; *H. molleri*: R=0.271, p=0.088; *P. perezi*: R=0.239, p=0.234; *P. cultripes*: R=0.347, p=0.098), although removing the extreme southwestern population of CER from the analyses revealed significant IBD patterns in three of them (*E. calamita*:

R=0.593, *p*=0.001; *H. molleri*: R=0.339, *p*=0.044; *P. perezi*: R=0.411, *p*=0.131; *P. cultripes*: R=0.407, *p*=0.018, Fig. 4).

Migration rates per generation

Estimated migration rates per generation were concordant across replicate runs in all species. Mean (and maximum) differences in the estimated non-migrant proportion of each population across the five replicates were 0.031 (0.156) in *E. calamita*, 0.030 (0.133) in *H. molleri*, 0.012 (0.100) in *P. perezi*, and 0.047 (0.225) in *P. cultripes*. Average pairwise migration rates were low in all species (~0.01), except among some well-connected populations of *P. cultripes* in the northern (FUE, STO and TUR) and southern (CAB, COL, TEJ and ROB) slopes (mean=0.03, Tables S1.5-S1.8, Appendix S1). Migration rates dropped sharply beyond short geographic distances (*c.* 10 km) in *P. perezi* and, especially, in *E. calamita*. In contrast, *H. molleri* and *P. cultripes* maintained migration rates close to 0.2 between populations up to 40 km away, although high rates were only found among populations in the same slope (Fig. 5, Tables S1.5-S1.8, Appendix S1).

Clustering analyses

STRUCTURE analyses yielded increasing likelihood values with increasing *K* (Fig. S2.2, Appendix S2). The Δ*K* method yielded *K*=2 as the optimal partition for *E. calamita*, *P. perezi* and *P. cultripes* (Fig. S2.2 in Appendix S2). Two clearly differentiated clusters, with little to no genetic admixture, were recovered in *P. cultripes*, corresponding to different slopes of *Sierra de Guadarrama* (Figs. 2 and S2.9, Appendix S2). In *E. calamita*, *H. molleri* and *P. perezi*, northern and southern clusters were also recovered at *K*=2, with admixed populations near mountain passes (Fig. 2 and Appendix S2). The optimum partition in *H. molleri* was *K*=3 (Fig.

306 S2.5, Appendix S2). Further partitions with *K*=3 to 5 showed hierarchical structure in the four
307 species within each slope of *Sierra de Guadarrama*, but with little additional admixture across
308 opposite slopes (Figs. S2.3, S2.5, S2.7 and S2.9).

309 Best *K* values in DAPC analyses were between 4-7 in *E. calamita*, 8 in *H. molleri*, 7-8
310 in *P. perezi* and 10-11 in *P. cultripes* (not shown). High *K* values were in agreement with the
311 likelihood-based method in STRUCTURE (Fig. S2.2). Individual admixture results for *K*=2-5
312 were similar to those obtained with STRUCTURE in *P. perezi* and *P. cultripes* (Figs. S2.8,
313 S2.10, Appendix S2). In contrast, the strong genetic differentiation of PRA and TOR drove
314 the main clustering partitions in *H. molleri* (Fig. S2.6), and no strong structure was observed
315 in *E. calamita* (Fig. S2.4).

316 Best *K* values obtained with GENELAND were largely concordant with the total number
317 of populations in each species (not shown). These high *K* values were again consistent with
318 strong genetic structure. While the northern and southern clusters were clearly and
319 consistently discriminated at *K*=2 in the case of *P. cultripes*, results were more variable and
320 inconsistent in the other three species (Fig. S2.11).

321

322 *Landscape genetic analyses*

323 The causal modeling approach revealed a strong effect of *Sierra de Guadarrama* as a barrier
324 to gene flow for *P. cultripes*, since genetic distances showed highly significant correlations
325 with the barrier effect after partialling out the remaining candidate measures, while none of
326 the remaining models showed significant support (Table 3). Partial Mantel tests suggested a
327 potential role of elevation on the genetic structure of *E. calamita* and *H. molleri*, although this
328 effect was not fully supported based on the expectations of causal modeling (Cushman et al.,
329 2006, 2013). None of the models tested in *P. perezi* showed significant results (Table 3).

DISCUSSION

Our results indicate that *Sierra de Guadarrama* is acting as a current barrier to gene flow for *P. cultripes* and, to a lesser extent, for *E. calamita*, *H. molleri* and *P. perezi*. If this effect is significant in the present interglacial period, it is safe to assume that it was probably stronger during the Pleistocene, when glaciers covered large areas in *Sierra de Guadarrama* (Domínguez-Villar et al., 2013). This long-term effect could explain the phylogeographic breaks found in *P. cultripes* (Gutiérrez-Rodríguez, Barbosa, et al., 2017) and *H. molleri* (Sánchez-Montes & Martínez-Solano, unpublished), two species showing a clear north-south subdivision in the Iberian Peninsula and meeting at the Central System mountains.

All genetic approaches provided evidences of the current effect of *Sierra de Guadarrama* as a barrier to gene flow, but the four species showed different patterns of connectivity across the mountain ridge. Some of these differences can be explained in terms of variation in some key life history traits, particularly dispersal potential, with the less vagile species *(Pelobates cultripes*) showing the most pronounced genetic break. Gutiérrez-Rodríguez, Sánchez-Montes, et al. (2017) reported eight displacements of *P. cultripes* from *Laguna de Valdemanco* to nearby breeding sites, five of them covering a distance >700 metres (Table 1, Figs. 1, 3), which corresponds to the lowest cumulative distance recorded in *Laguna de Valdemanco* among the four species (Fig. 1). *Pelobates cultripes* is also a strictly nocturnal species with a long larval period and the narrowest altitudinal range among the study species (Table 1). This may reflect physiological constraints, although other factors, like dependence on soils adequate for its fossorial habits, probably play a role. Altogether, these traits could favour more pronounced phylopatric behaviour in this species, restricting regional connectivity.

Surprisingly, we obtained high migration rates per generation at larger geographic distances (up to 40 km) in populations of *P. cultripes* located on the same slope (Fig. 5 and Appendix S1). Although some migration rate estimates could be imprecise due to the high number of populations analyzed and the relatively low sample sizes, the estimated non-migrant fraction never switched between the bounds of the prior distribution, supporting the overall reliability of our inferences (Meirmans, 2014). High inferred migration rates per generation might result from a very low number of migrants per year in long-lived species, like *P. cultripes*, which can live up to 12 years in this area (Talavera, 1990, Table 1). Rare long dispersal events can easily pass unnoticed in CMR studies using PIT tags.

The strong barrier effect exerted by *Sierra de Guadarrama* on *P. cultripes* is well supported based on high overall population differentiation (Table S1.4) and results of the clustering and causal modeling approaches (Table 3, Figs. 2 and S2.9-S2.11). This barrier effect may explain the absence or rarity of this species above 1500 m (Cejudo, 1990) and the strong phylogeographic break at the Central System (Gutiérrez-Rodríguez, Barbosa, et al., 2017). Mountain passes in *Sierra de Guadarrama* are above the higher reported altitudes for this species except at Somosierra and Alto del León (Fig. 2).

We also found high migration rates per generation among some distant populations in *H. molleri*, although only within the southern slope (Fig. 5, Table S1.6), probably due to the fragmented distribution of this species in the northern slope (Márquez, 2002), reflected in the high differentiation of the PRA and TOR populations (Figs. S2.5, S2.6). Direct records of individual movements revealed the high dispersal potential of *H. molleri* (Figs. 1, 3), which probably favors regional population connectivity (Fig. 4, Table S1.2). However, causal modeling results suggest a potential effect of elevation on genetic distances, implying that topography may to some extent restrict across-slope gene flow in *H. molleri* (Table 3). These results are in agreement with a role of *Sierra de Guadarrama* as a semi-permeable barrier to

gene flow in this species, as also suggested by the widely connected areas identified among the two major (northern and southern) clusters recovered (Figs. 2 and S2.5-S2.6).

A similar role of *Sierra de Guadarrama* as a semi-permeable barrier to gene flow was inferred for *E. calamita* and *P. perezi*. These species showed high overall connectivity in the study area (especially *E. calamita*, Tables S1.1, S1.3) despite low inferred migration rates per generation (Tables S1.5, S1.7), and also show the broadest altitudinal range among the study species (Table 1). The high regional connectivity in *E. calamita* and *P. perezi* is in line with the high dispersal potential inferred in both species based on CMR data (Table 1, Figs. 1, 3). Two life history traits related to breeding site selection may also contribute to regional connectivity in the two species. On the one hand, *E. calamita* successfully exploits ephemeral ponds for breeding, thanks to their extremely fast larval development (by far the shortest among the four species, Table 1), thus avoiding competion because of the high mortality risk associated with early pond drying. This trait allows *E. calamita* to successfully exploit extremely small and shallow (but also widely available, even above the treeline at high altitudes) breeding sites, which probably contributes to maintain high levels of population connectivity. On the other hand, tadpoles of *P. perezi* require longer hydroperiod ponds to complete their development (Table 1), but this species uses a wider variety of breeding sites including streams, natural or artificial ponds, water troughs and urban, degraded, salty or polluted areas (Egea-Serrano, 2014). This ecological breadth probably helps maintaining high levels of regional connectivity.

Overall, our integrative approach combining field-based and molecular approaches to estimate population connectivity in four co-distributed anurans allowed explicitly testing the role of *Sierra de Guadarrama* as a barrier to gene flow. Our results show that these mountains have played a major role in disrupting historical and current connectivity across populations on different slopes, but differently so depending on life history traits such as breeding strategy and dispersal capacity. These results highlight the major role of the Central

System Mountains as a key feature shaping historical patterns of population connectivity across taxa, promoting population divergence and the evolution and accumulation of endemicity.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Appendix S1. Pairwise F_{ST} and migration rates.

Appendix S2. Results of clustering analyses.

Appendix S3. New microsatellite genotype data for *P. cultripes*

BIOSKETCHES

This work is part of G.S.-M.'s PhD thesis. The research group has an interest in developing and applying model-based genetic analyses to address the study of demographic processes and assess their role in driving biodiversity patterns. Author contributions: G.S.-M., J.W., A.H.A., and I.M.-S. designed the research. G.S.-M. and I.M.-S. conducted field work and sample collection. G.S.-M. conducted laboratory work. G.S.-M. and J.W. performed the

genetic analyses. G.S.-M. and I.M.-S. wrote the manuscript and J.W. and A.H.A. provided edits to the manuscript.

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679 Table 1. Differences in morphology, life history traits, habitat preferences, movement capabilities and topographic distributional limits among *E.*
680 *calamita*, *H. molleri*, *P. perezi* and *P. cultripes*. SVL: snout-to-vent length; Longv.: longevity; Matur.: age of sexual maturation; Veg. cover prefer.:
681 vegetation cover preference; Disp.: maximum recorded dispersal; Mig.: maximum recorded migration; Alt.: Maximum recorded elevation across the
682 species' range of distribution (in metres above sea level).

Species	SVL range (mm)	Activity	Longv. (years)	Matur. (years)	Breeding site selection	Length of larval period	Veg. cover prefer.	Disp. (m)	Mig. (m)	Alt.	References
E. calamita	31.3-98	nocturnal	10-17	2-3	lentic	24-54 days	grassland	4,411	2,600	2,500	(Beebee, 1983; Boomsma & Arntzen, 1985; Banks & Beebee, 1987; Banks et al., 1993; Denton & Beebee, 1993; Tejedo et al., 1997; Gómez-Mestre & Tejedo, 2002; García-París et al., 2004; Leskovar et al., 2006; Sinsch et al., 2010; Oromi et al., 2012; Trochet et al., 2014).
H. molleri	35-45	preferentially nocturnal	-	-	lentic	3 months	forest, shrubland, grassland	-	-	2,140	Barbadillo (1987), García et al. (1987), Márquez-M. de Orense & Tejedo-Madueño (1990), García-París et al. (2004), Márquez et al. (2005), Martínez-Solano (2006).
P. perezi	41.6-110	diurnal and nocturnal	4-6	1-3	lotic and lentic	2-4 months	forest, shrubland, grassland	-	-	2,380	Díaz-Paniagua (1986), Lizana et al. (1987), Docampo & Milagrosa-Vega (1988, 1991), Patón et al. (1991), Real & Antúnez (1991), Báez & Luis (1994), Esteban et al. (1996), Fernández-Cardenete et al. (2000), Díaz-Paniagua et al. (2005), Trochet et al. (2014).
P. cultripes	36.8-125	nocturnal	12	2	lotic and lentic	3-4 months	shrubland, grassland	710	-	1,770	Salvador et al. (1986), Álvarez et al. (1990), Cejudo (1990), Talavera (1990), Lizana et al. (1994), Díaz-Paniagua et al. (2005), Leclair et al. (2005), Marangoni & Tejedo (2007), Trochet et al. (2014), Gutiérrez-Rodríguez, Sánchez-Montes, et al. (2017).

683

Table 2. List of sampled localities for each species (Ecal: *E. calamita*, Hmol: *H. molleri*, Pper: *P. perezi* and Pcul: *P. cultripes*), with their abbreviations (Abr), geographic coordinates (Coord), elevation in m.a.s.l. (Elev), and the number of tadpole tissue samples obtained in each locality (Sample size). Further information about the *E. calamita*, *H. molleri* and *P. perezi* samples can be found in Sánchez-Montes, Ariño, et al. (2017).

Locality	**Abr**	**Coord**	**Elev**	**Sample size**			
				Ecal	*Hmol*	*Pper*	*Pcul*
Alameda del Valle	ALA	40.91º N 3.85º W	1104	24	-	-	-
Arcones	ARC	41.13º N 3.73º W	1142	-	30	19	-
Arroyo Tejada	TEJ	40.67º N 3.74º W	850	-	-	-	30
Berrocal	BRC	41.06º N 3.98º W	1098	30	-	-	-
Bustarviejo	BUS	40.85º N 3.68º W	1092	28	30	30	21
Cabanillas de la Sierra	CAB	40.85º N 3.65º W	1009	30	22	27	27
Cerceda	CER	40.72º N 3.96º W	1031	30	20	23	30
Collado Hermoso	HER	41.05º N 3.93º W	1193	-	23	32	20
Colmenar Viejo	COL	40.69º N 3.83º W	854	30	21	-	30
Dehesa de Roblellano	ROB	40.86º N 3.63º W	1072	36	30	-	29
El Berrueco	BER	40.93º N 3.57º W	927	-	21	20	30
Fuenterrebollo	FUE	41.33º N 3.93º W	909	-	20	20	31
Gargantilla del Lozoya	GAR	40.95º N 3.72º W	1074	30	-	-	-
Gascones	GAS	41.01º N 3.65º W	1035	-	21	-	-
La Pradera de Navalhorno	PRA	40.88º N 4.03º W	1192	30	22	23	30
Lozoyuela	LOZ	40.92º N 3.65º W	1107	28	-	-	-
Medianillos	MED	40.76º N 3.68º W	933	-	21	25	-
Muñoveros	MUN	41.20º N 3.95º W	906	32	-	-	-
Navafría	NAV	41.06º N 3.83º W	1180	30	-	-	-
Puerto de Canencia	CAN	40.81º N 3.68º W	1477	28	25	22	-
Puerto de La Morcuera	MOR	40.87º N 3.76º W	1720	20	30	22	-
Puerto del Medio Celemín	CEL	40.84º N 3.83º W	1248	30	-	-	-
Rascafría	RAS	40.88º N 3.66º W	1516	-	20	22	-
Santo Tomé del Puerto	STO	40.85º N 3.91º W	1121	30	-	21	30
Sauquillo de Cabezas	SAU	41.19º N 3.59º W	911	-	20	22	-
Soto del Real	SOT	41.19º N 4.06º W	936	30	20	-	30
Torrecaballeros	TOR	40.76º N 3.80º W	1127	-	34	-	-
Turrubuelo	TUR	41.00º N 4.02º W	1042	-	21	21	30

690 Table 3. Results of the landscape genetic causal modeling approach. Partial Mantel tests evaluate the
691 effects of four different elevation-based resistance surfaces (Elev, Elev1000, Elev1500 and Elev2000,
692 with the linear relationship between elevation and resistance starting at 0, 1000, 1500 and 2000
693 m.a.s.l., respectively), a barrier effect (Bar) and Euclidean distances (Eucl) on observed genetic
694 distances (Gen). Models are named after the dependent variable (Gen) ~ the tested effect | and the
695 partialled out covariable. Significant results at the 0.05 level are marked in bold.

Model	*E. calamita*		*H. molleri*		*P. perezi*		*P. cultripes*	
	R	***p***	**R**	***p***	**R**	***p***	**R**	***p***
Gen~Bar | Eucl	0.086	*0.242*	**0.207**	***0.017***	0.125	*0.081*	**0.471**	***<0.001***
Gen~Eucl | Bar	0.169	*0.169*	0.025	*0.419*	-0.076	*0.653*	-0.031	*0.552*
Gen~Elev | Eucl	0.131	*0.229*	**0.281**	***0.026***	-0.026	*0.568*	0.209	*0.118*
Gen~Elev | Bar	0.188	*0.116*	0.069	*0.308*	-0.109	*0.738*	-0.079	*0.680*
Gen~ Eucl | Elev	-0.060	*0.623*	-0.232	*0.923*	0.022	*0.449*	-0.131	*0.786*
Gen~ Bar | Elev	0.030	*0.404*	0.145	*0.059*	0.147	*0.060*	**0.452**	***0.001***
Gen~Elev1000 | Eucl	0.181	*0.141*	**0.317**	***0.011***	-0.041	*0.604*	0.218	*0.105*
Gen~Elev1000 | Bar	0.201	*0.100*	0.075	*0.296*	-0.110	*0.747*	-0.069	*0.654*
Gen~ Eucl | Elev1000	-0.119	*0.738*	-0.275	*0.961*	0.037	*0.420*	-0.150	*0.817*
Gen~ Bar | Elev1000	0.026	*0.420*	0.146	*0.064*	0.147	*0.065*	**0.452**	***0.001***
Gen~Elev1500 | Eucl	**0.250**	***0.002***	**0.175**	***0.038***	-0.029	*0.595*	0.062	*0.330*
Gen~Elev1500 | Bar	0.187	*0.146*	0.033	*0.392*	-0.083	*0.670*	-0.044	*0.577*
Gen~ Eucl | Elev1500	-0.231	*0.995*	-0.162	*0.942*	0.028	*0.415*	-0.039	*0.609*
Gen~ Bar | Elev1500	0.067	*0.300*	**0.197**	***0.022***	0.130	*0.085*	**0.470**	***0.001***
Gen~Elev2000 | Eucl	0.120	*0.158*	-0.010	*0.541*	0.045	*0.339*	-0.020	*0.556*
Gen~Elev2000 | Bar	0.179	*0.153*	0.021	*0.431*	-0.075	*0.641*	-0.039	*0.562*
Gen~ Eucl | Elev 2000	-0.098	*0.785*	0.024	*0.400*	-0.046	*0.657*	0.043	*0.371*
Gen~ Bar | Elev2000	0.078	*0.275*	**0.208**	***0.018***	0.125	*0.090*	**0.473**	***0.001***

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Figure 1. Map of the *Valdemanco* area (Madrid, Spain, see inset) showing the location of the main breeding site (A: *Laguna de Valdemanco*, photograph in the lower left corner) and four secondary breeding sites (B: a water trough 230 m away from A, C: a quarry with ephemeral ponds 395 m away from A, D: an abandoned swimming pool 680 m away from A, and E: a mining pond 710 m away from A). The pie chart in *Laguna de Valdemanco* (A) shows the number of individuals of each species (white: *E. calamita*, black: *H. molleri*, light grey: *P. perezi*, dark grey: *P. cultripes*) that were marked and recaptured only in A. Photographs of these species are shown on the right, with *E. calamita*, *H. molleri*, *P. perezi* and *P. cultripes* from top to bottom, respectively. Pie charts in B, C, D and E show the number of individuals of each species for which the longest recorded displacement was from A to B, C, D or E, respectively (i.e., every individual is represented in only one pie chart: the chart corresponding to the most distant breeding site from A where it was captured). Recorded displacements of *P. cultripes* are summarized from Gutiérrez-Rodríguez, Sánchez-Montes, et al. (2017).

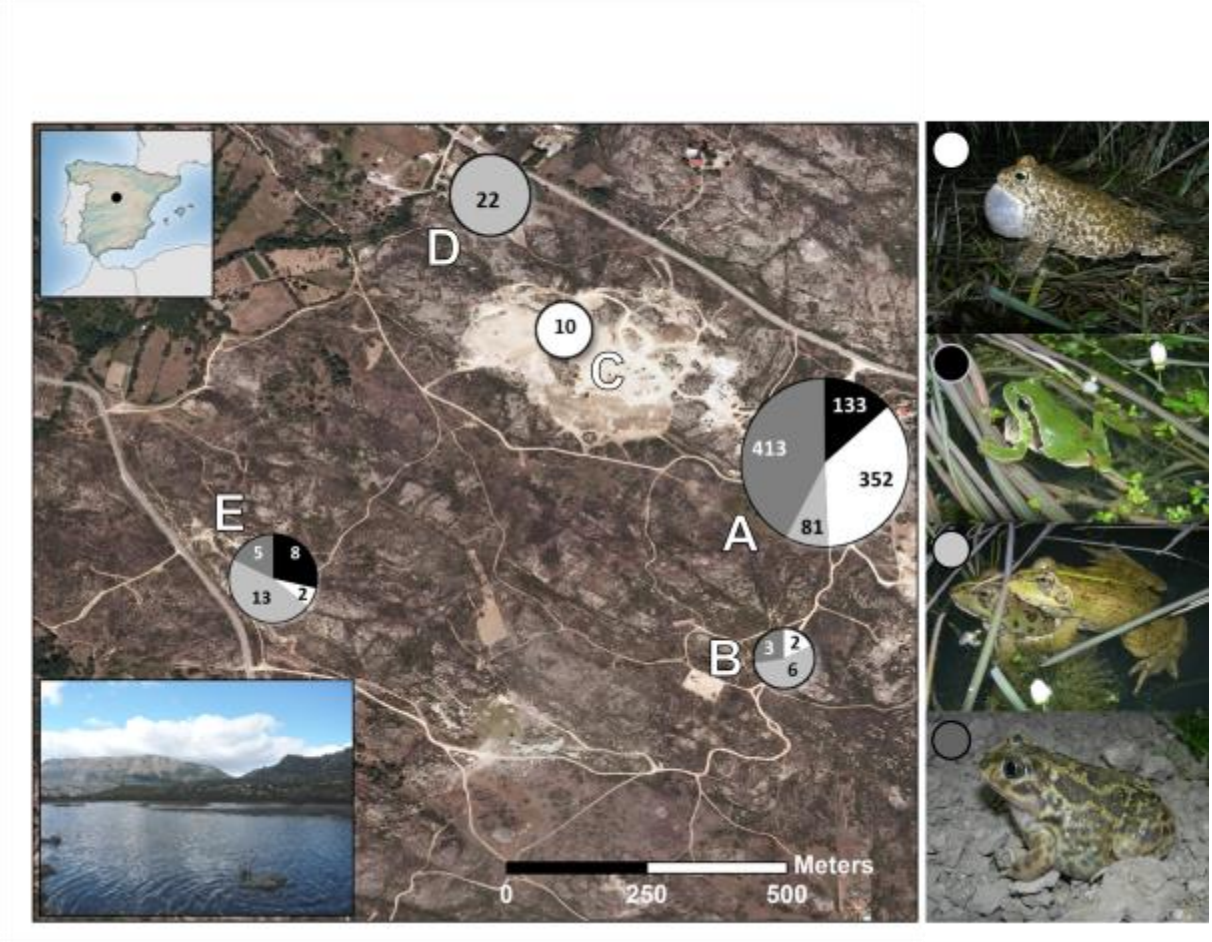
Figure 2. Patterns of genetic structure obtained in STRUCTURE with *K*=2 for the four species in *Sierra de Guadarrama*. For each sampled population (see Table 2 for abbreviations), colours of pie charts represent the proportion of alleles corresponding to each of the two inferred clusters (represented by black and white colours, respectively) obtained in admixture analyses. The locations of the five lowest mountain passes are indicated with a star. Background colours represent altitudinal ranges and the highest reported limits for *P. cultripes* (1770 m), *H. molleri* (2140 m) and *P. perezi* (2380 m).

Figure 3. Recorded cumulative distances covered by individuals of the four species in the *Valdemanco* area (see Fig. 1). The number of individuals only recaptured at less than 100 meters from the marking location (i.e., *E. calamita*: 400 individuals, *H. molleri*: 145, *P. perezi*: 269, *P. cultripes*: 419) was much higher than the number of dispersers in all species, so the lowest distance category of each histogram (0-100 m) has been truncated for clarity (dashed line). Recorded displacements of *P. cultripes* are summarized from Gutiérrez-Rodríguez, Sánchez-Montes, et al. (2017).

Figure 4. Relation between genetic (F_{ST}) and geographic distances among all pairs of populations located on the southern (dark circles) or the northern slope (white circles) of *Sierra de Guadarrama*. Pairwise distances involving CER and the remaining populations in the southern slope are represented by black triangles.

Figure 5. Estimated migration rates as a function of geographic distance between populations located in the same (dark circles) or on different slopes (white circles) of *Sierra de Guadarrama*.

736 Figure 1



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739 Figure 2

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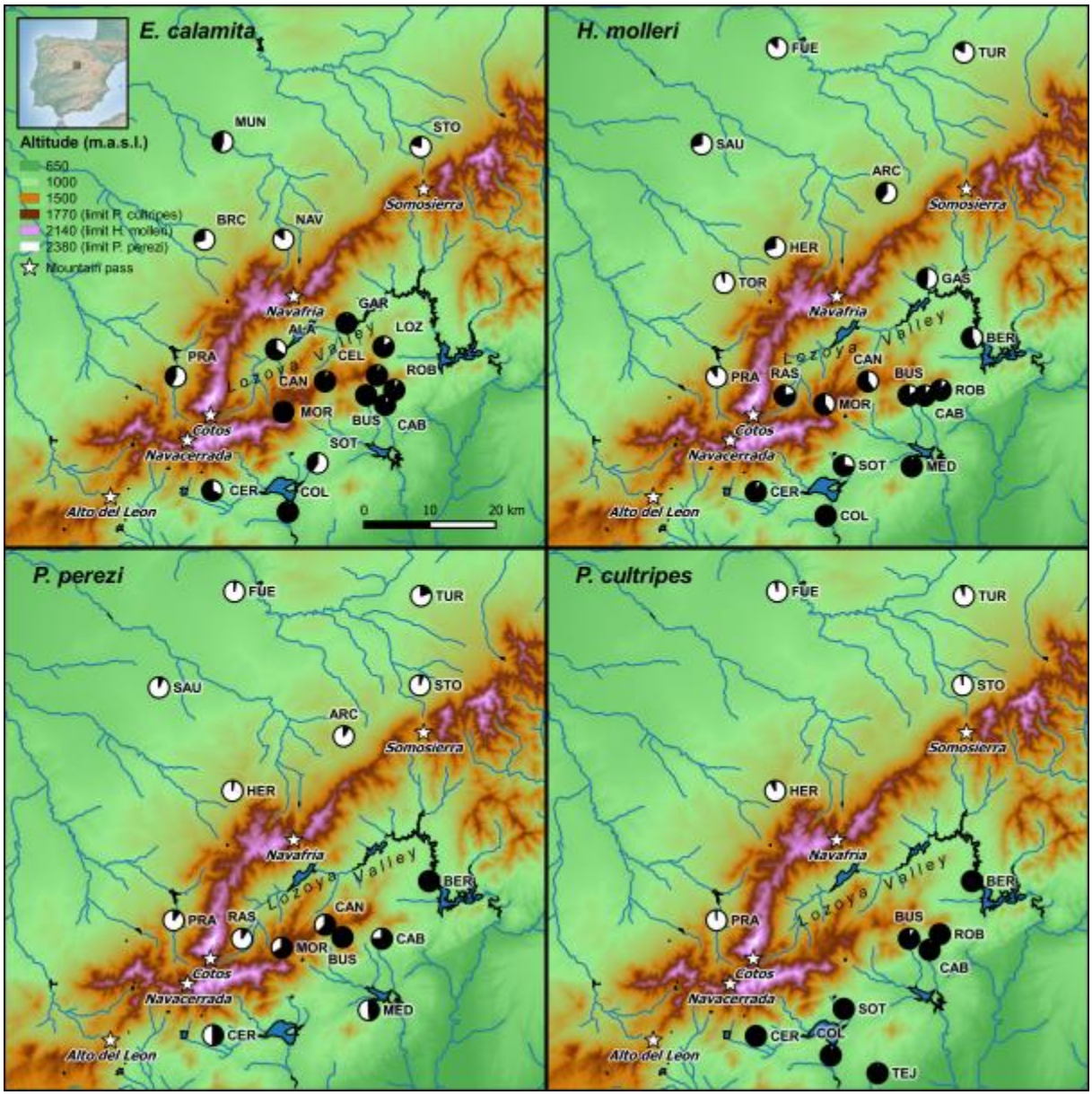

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743 Figure 3

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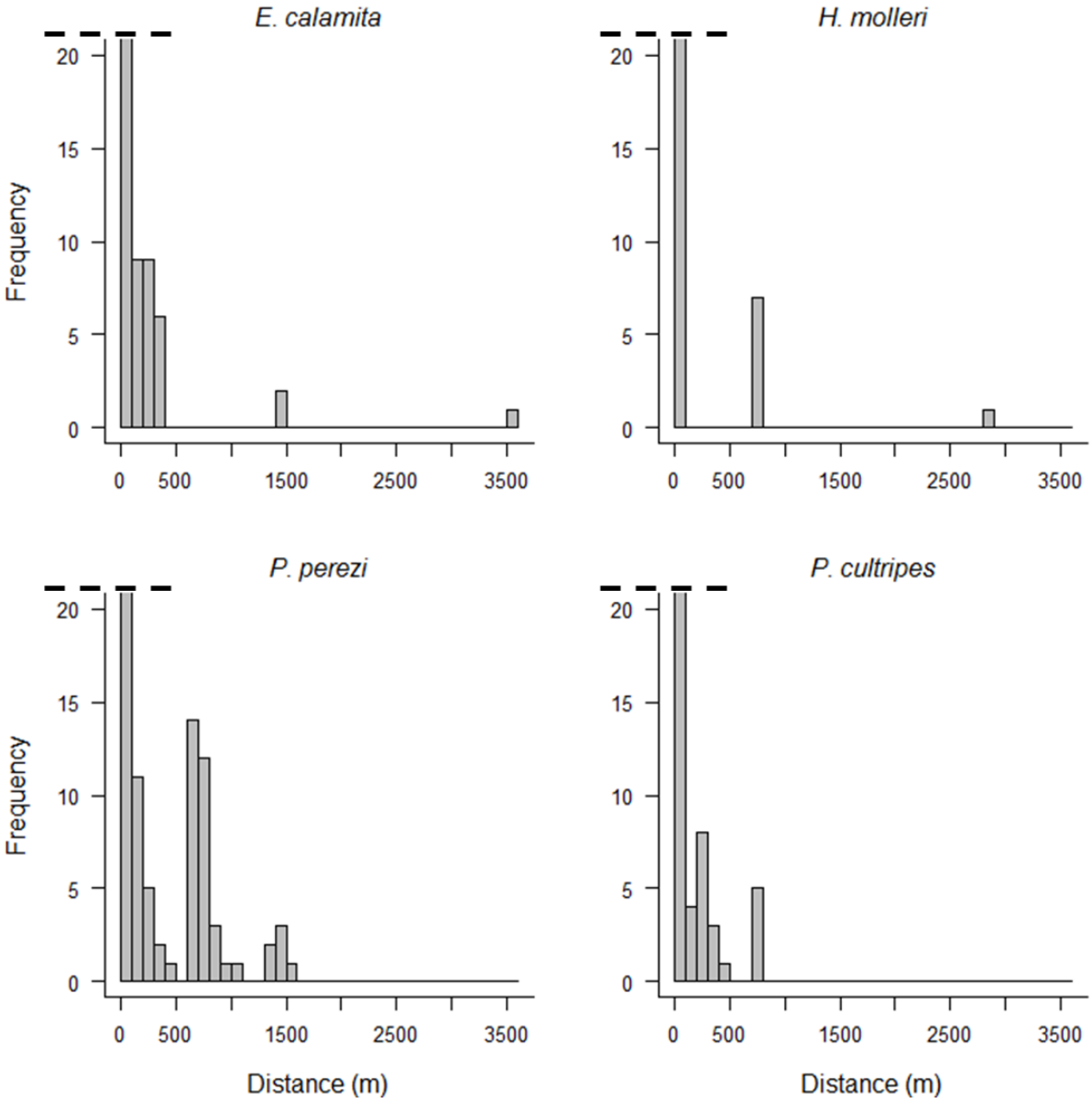

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748 Figure 4

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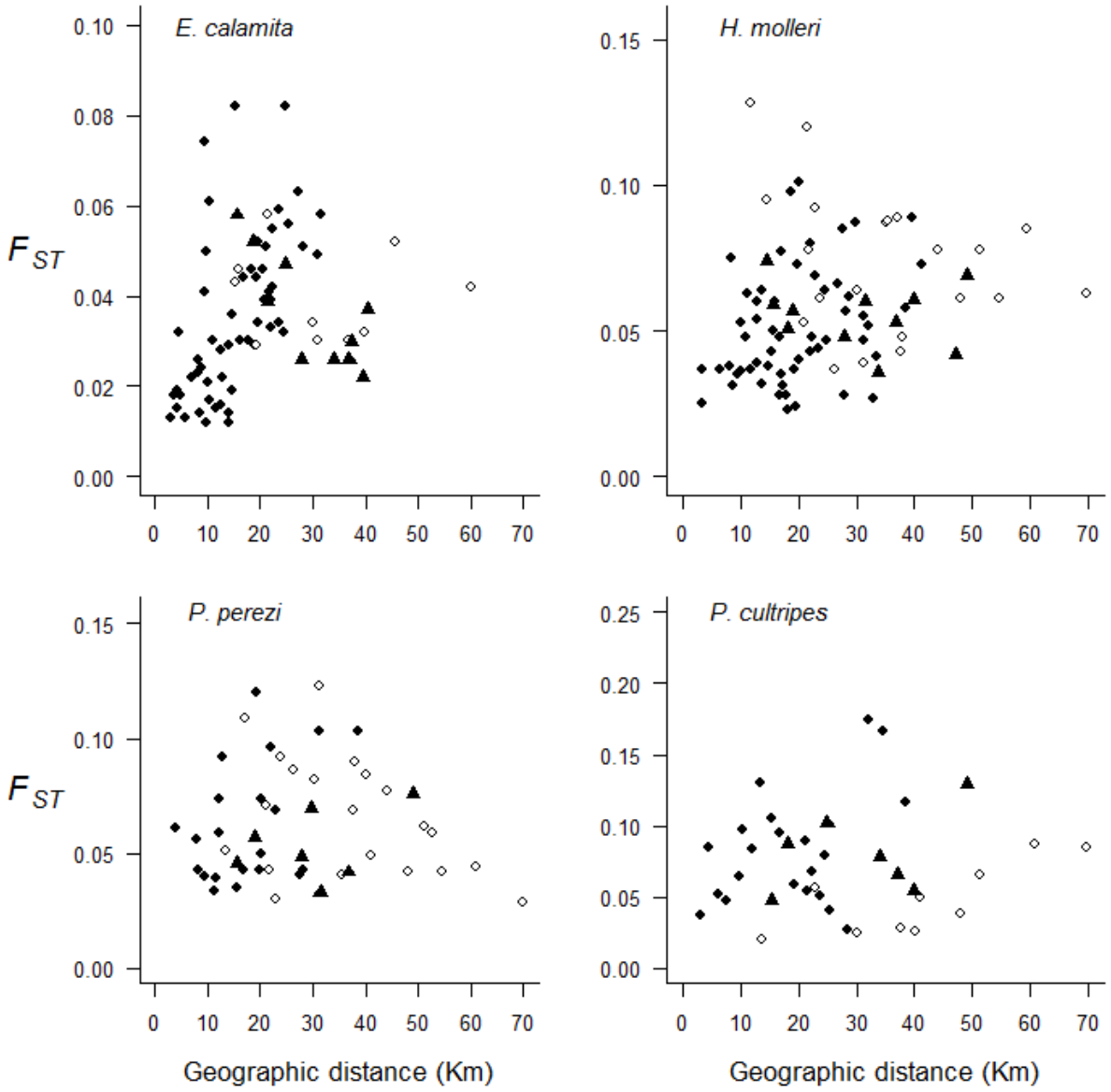

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753 Figure 5

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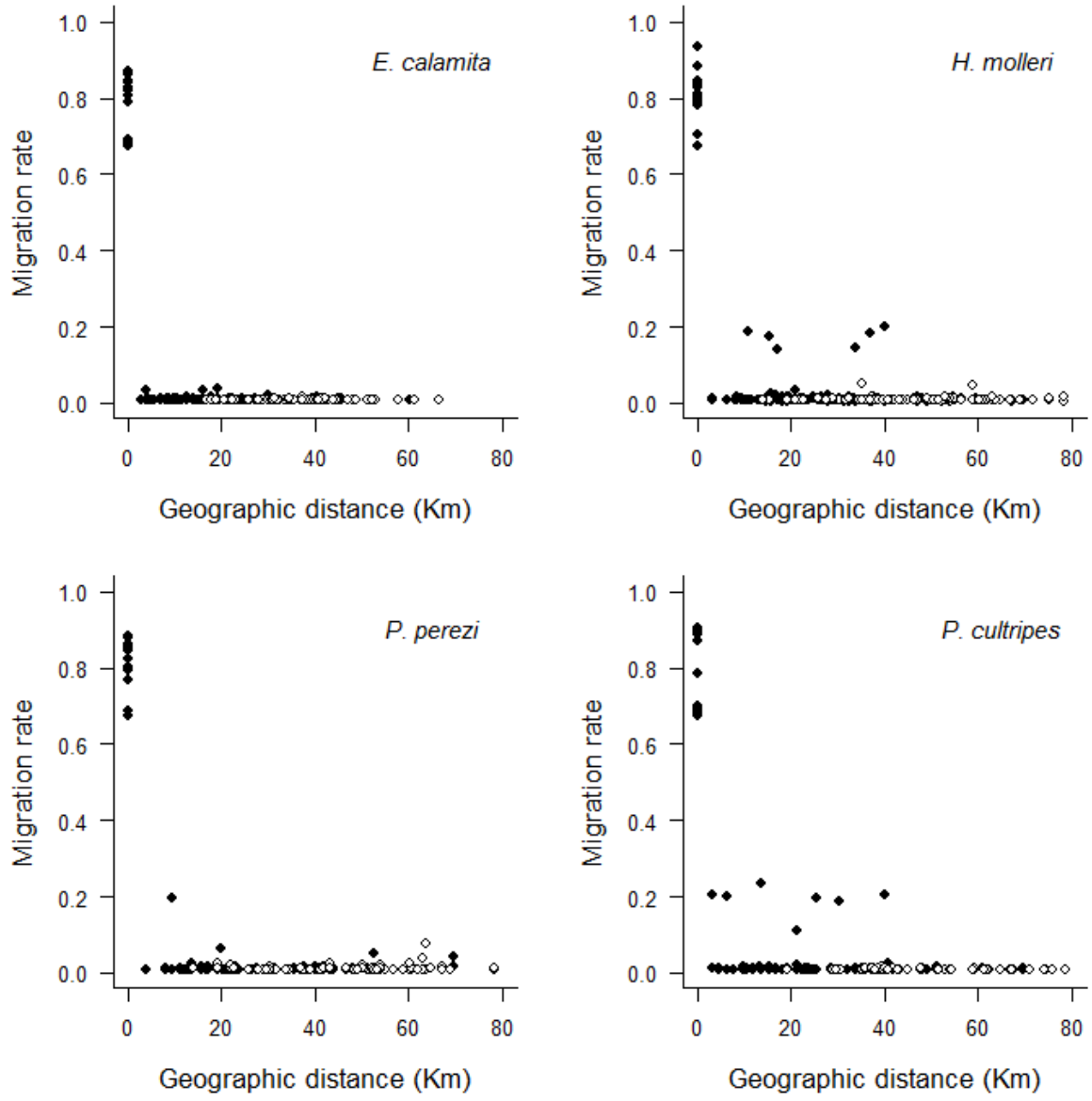

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