Received: 14 October 2016 | Revised: 12 June 2017 | Accepted: 12 July 2017

DOI: 10.1111/mec.14272

ORIGINAL ARTICLE

WILEY MOLECULAR ECOLOGY

Comparative landscape genetics of pond-breeding amphibians in Mediterranean temporal wetlands: The positive role of structural heterogeneity in promoting gene flow

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Funding information
Secretaría de Estado de Investigación, Desarrollo e Innovación, Grant/Award Number: CGL2011-28300; Consejo Superior de Investigaciones Científicas of Spain (CSIC); European Social Fund (ESF); FCT, Grant/Award Number: SFRH/BD/90112/2012, SFRH/BPD/109182/2015; POPH-QREN; CSF; Spanish "Ramón y Cajal", Grant/Award Number: RYC-2007-01668; Severo Ochoa, Grant/Award Number: SEV-2012-0262

Abstract

Comparative landscape genetics studies can provide key information to implement cost-effective conservation measures favouring a broad set of taxa. These studies are scarce, particularly in Mediterranean areas, which include diverse but threatened biological communities. Here, we focus on Mediterranean wetlands in central Iberia and perform a multi-level, comparative study of two endemic pond-breeding amphibians, a salamander (*Pleurodeles waltl*) and a toad (*Pelobates cultripes*). We genotyped 411 salamanders from 20 populations and 306 toads from 16 populations at 18 and 16 microsatellite loci, respectively, and identified major factors associated with population connectivity through the analysis of three sets of variables potentially affecting gene flow at increasingly finer levels of spatial resolution. Topographic, land use/cover, and remotely sensed vegetation/moisture indices were used to derive optimized resistance surfaces for the two species. We found contrasting patterns of genetic structure, with stronger, finer scale genetic differentiation in *Pleurodeles waltl*, and notable differences in the role of fine-scale patterns of heterogeneity in vegetation cover and water content in shaping patterns of regional genetic structure in the two species. Overall, our results suggest a positive role of structural heterogeneity in population connectivity in pond-breeding amphibians, with habitat patches of Mediterranean scrubland and open oak woodlands ("*dehesas*") facilitating gene flow. Our study highlights the usefulness of remotely sensed continuous variables of land cover, vegetation and water content (e.g., NDVI, NDMI) in conservation-oriented studies aimed at identifying major drivers of population connectivity.

KEYWORDS
conservation, gene flow, Iberian Peninsula, NDMI, NDVI, population connectivity

1 | INTRODUCTION

Comparative landscape genetics studies on codistributed species have great potential to design cost-effective conservation plans focusing on measures favouring a wider set of taxa, but are still relatively scarce. So far, comparative studies have focused primarily on vertebrates, including amphibians (Coster, Babbitt, Cooper, & Kovach, 2015; Richardson, 2012; Zancolli, Rödel, Steffan-Dewenter, & Storfer, 2014), mammals (Dudaniec et al., 2016; Frantz et al., 2012; Muscarella, Murray, Ortt, Russell, & Fleming, 2011), and fishes (Olsen et al., 2011) and more occasionally on invertebrates (Engler, Balkenhol, Filz, Habel, & Rödder, 2014; Ortego, García-Navas,

Noguerales, & Cordero, 2015; Phillipsen et al., 2015). These multi-species studies may allow identifying interspecific differences in the way landscape features influence connectivity and gene flow and provide general guidelines for land management programmes aimed at protecting biological communities or ecosystems (Goldberg & Waits, 2010a; Keller, Holderegger, Strien, & Bolliger, 2014; Nicholson & Possingham, 2006; Schwenk & Donovan, 2011). Additionally, by comparing spatial genetic patterns across syntopic species in a shared landscape, important yet often obscure aspects about species life history traits can be inferred (Coster et al., 2015; Goldberg & Waits, 2010a; Igawa, Oumi, Katsuren, & Sumida, 2013; Kurz, Nowakowski, Tingley, Donnelly, & Wilcove, 2014; Richardson, 2012; Steele, Baumsteiger, & Storfer, 2009).

Pond-breeding amphibians are a good study system for comparative landscape genetics studies because they form communities including species that use to different extents both terrestrial and aquatic habitats. Due to their low dispersal abilities (Bowne & Bowers, 2004; Graeter, Rothermel, & Gibbons, 2008), small effective population sizes (Funk, Tallmon, & Allendorf, 1999) and the discontinuous distribution of their preferred breeding habitats (Jehle, Burke, & Arntzen, 2005), they tend to form "patchy" breeding assemblages. Dispersal and the maintenance of gene flow between breeding ponds depends on the composition and configuration of the landscape (Coster et al., 2015), and studies focusing on functional connectivity have described different species responses to landscape features such as differences in soil moisture, clear-cut habitats, agriculture lands, riparian network, as well as differences in metamorphosis, philopatry, dispersal, selection of breeding sites and post-breeding behaviour (Coster et al., 2015; Goldberg & Waits, 2010a; Peterman et al., 2015; Richardson, 2012; Steele et al., 2009). Identification of the factors promoting or reducing gene flow is key to prevent local and regional extinctions in the long-term and to inform conservation management.

Most comparative amphibian landscape genetic studies have focused on North American communities with continental (Coster et al., 2015; Goldberg & Waits, 2010a; Richardson, 2012), temperate (Steele et al., 2009) or humid subtropical climates (Peterman et al., 2015), but little is known about factors shaping regional patterns of gene flow across taxa in Mediterranean wetlands, which are diverse and highly threatened ecosystems (Beja & Alcazar, 2003; Blondel & Aronson, 1999). The effects of unpredictability in hydroperiod, coupled with a continued decline in the number and extent of these wetlands (Doulgeris, Papadimos, & Kapsomenakis, 2016), challenge the survival of their rich biotic communities (Green, Bustamante, Janss, Fernández-Zamudio, & Díaz Paniagua, 2016). Assessing comparative patterns of regional gene flow can help in developing integrative management practices that preserve pond-breeding communities with explicit consideration of functional connectivity.

Landscape analysis methods allow simultaneously testing for the relative influence of different factors acting as potential barriers to dispersal at different spatial scales, at an increasingly finer resolution (Greenberg, Dobrowski, & Ustin, 2005; Wulder, Hall, Coops, & Franklin, 2004). This allows linking genetic data with different potential response variables and explicitly quantifying the effects of landscape composition, configuration and matrix quality on gene flow and spatial genetic variation (Storfer et al., 2007). Here, we perform a multi-level, comparative study focusing on two endemic pond-breeding amphibian species that are characteristic of Mediterranean wetlands in the Iberian Peninsula, *Pleurodeles waltl* Michahelles, 1830 and *Pelobates cultripes* (Cuvier, 1829). Both are usually syntopic but probably differ in their use of the available terrestrial and aquatic habitats, although much of their life history remains little studied. In addition, both are facing a slow but continued decline mostly associated with habitat loss, invasive species and road mortality, and as a consequence, they are listed with "Near Threatened" (NT) status by the IUCN (Beja et al., 2009, 2016). We compared patterns of genetic diversity and structure and identified the key factors associated with gene flow and population connectivity in both species through the analysis of genotypic data from a suite of highly polymorphic microsatellites in a large sample of individuals of both species in a shared geographic setting.

We explored the relative contribution of landscape features in shaping regional patterns of genetic structure in the two species. We anticipated land cover-related variables to have a strong impact on population genetic structure in both species, as the study region (near the city of Madrid, central Spain) has experienced major land use changes in the last century (Hewitt & Escobar, 2011). Thus, to derive optimized resistance surfaces, besides maps with discrete land use/cover categories, we incorporated continuous remotely sensed vegetation/moisture indices, which allow for detailed characterization of components of terrestrial habitats at scales relevant for low-dispersing taxa. A model ranking approach was then used to identify variables with greater influence on regional patterns of genetic structure.

2 | METHODS

2.1 | Study area and sampling

The study area is bounded by −4.136° to −3.296° longitude and 40.131° to 41.416° latitude (Figure 1). Elevation ranges from 482 m up to 2,403 m a.s.l. The two species are widely distributed in the study area, but their populations are fragmented due to the continued loss of terrestrial and aquatic habitats (Martínez-Solano, 2006).

We sampled 306 individuals of *P. cultripes* and 411 of *P. waltl* at 16 and 20 ponds, respectively (Figure 1, Table 1). Average geographic distances between populations for both species were similar and ranged from 0.7 to 40 km between the closest sampled populations (Figure 1; Tables S1 and S2). The scale of analysis reflects uncertainty about actual dispersal potential in both species but includes minimum estimates of adult dispersal (700 m) based on capture–mark–recapture studies (Gutiérrez-Rodríguez, Sánchez-Montes, & Martínez-Solano, 2017). Tissues were obtained from tail tips of larvae (most samples) and toe clips of adults (only the *P. waltl* samples from populations W4 and W17). All sampled individuals were released back in their place of capture after sample collection.

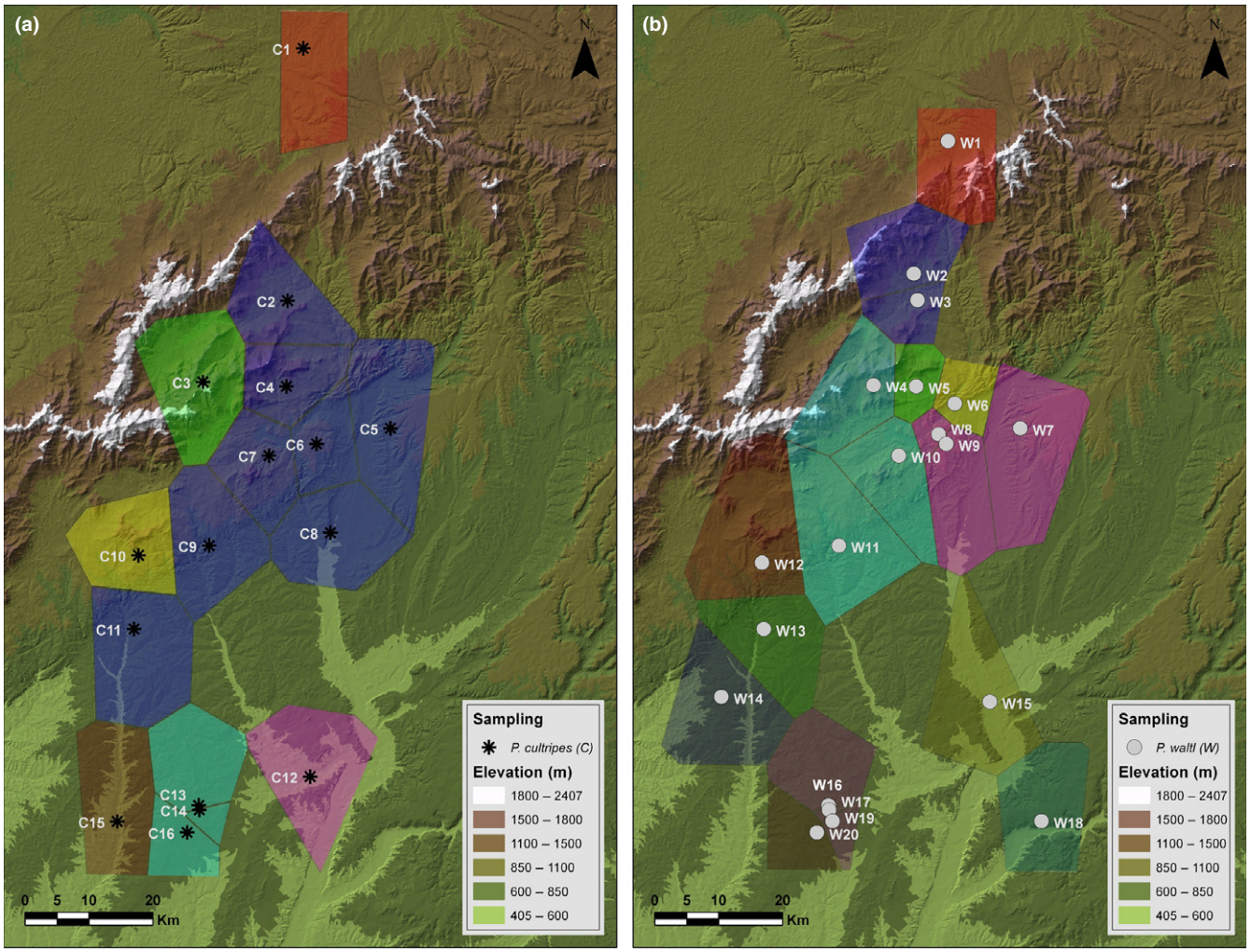


FIGURE 1 Sampling locations for the two study species: *Pelobates cultripes* (a) and *Pleurodeles waltl* (b). Codes as in Table 1. Results of the optimal number of clusters for each species according to BAPS are also shown [Colour figure can be viewed at wileyonlinelibrary.com]

2.2 | Genetic analyses

Genomic DNA was extracted using NucleoSpin Tissue-Kits (Macherey-Nagel). A total of 16 previously characterized microsatellite markers for *P. cultripes* were amplified following the PCR conditions described in Gutiérrez-Rodríguez and Martínez-Solano (2013). For *P. waltl*, 18 previously published loci (Gutiérrez-Rodríguez, Gonzalez, & Martínez-Solano, 2014; van de Vliet, Diekmann, Serrão, & Beja, 2009) were grouped into six multiplex reactions (multiplex 1: *Pleu2.3*, *Pleu2.19*, *Pleu2.34*; multiplex 2: *Pleu2.16*, *Pleu2.31*, *Pleu3.2*; multiplex 3: *Pleu3.5*, *Pleu4.1*; multiplex 4: *Ppl2*, *Ppl3*, *Ppl5*; multiplex 5: *Ppl1*, *Ppl12*, *Ppl13*, *Ppl14*; and multiplex 6: *Ppl6*, *Ppl7*, *Ppl10*). PCR conditions and genotype calling followed Gutiérrez-Rodríguez et al. (2014).

We tested for the presence of possible null alleles, stuttering and large allele dropout in microsatellite markers using MICROCHECKER v2.2.3 (van Oosterhout, Hutchinson, Wills, & Shipley, 2004). As including sibs and half-sibs in population samples can introduce undesired biases in the analyses (Goldberg & Waits, 2010b), we conducted genetic parentage analyses with the software COLONY v2.0.5.1 (Jones & Wang, 2010), assuming a monogamous mating system for both sexes, with the full-likelihood method (Wang, 2004). Analyses consisted of 10 independent runs, with medium-length likelihood precision and updated allele frequencies, and individuals identified as full-siblings with a probability of .8 or higher were discarded from subsequent analyses.

We tested for deviations from Hardy–Weinberg equilibrium (HWE) and evidence of linkage disequilibrium (LD) with the software GENEPOP (Raymond & Rousset, 1995; Rousset, 2008), applying the sequential Bonferroni correction (Rice, 1989) to adjust significance values for multiple tests. We calculated different estimates of genetic diversity for each population using GENALEX v6.5b5 (Peakall & Smouse, 2012), including the number of alleles (N_A), observed (H_O) and expected heterozygosity (H_E). We also calculated the inbreeding coefficient (F_{IS}) because it is an indirect measure of philopatry (more philopatric species will in principle have higher F_{IS} values).

We used BAPS v6 (Cheng, Connor, Sirén, Aanensen, & Corander, 2013; Corander, Sirén, & Arjas, 2008) to characterize population

TABLE 1 Locality information and genetic diversity estimates for sampled *Pelobates cultripes* (C) and *Pleurodeles waltl* (W) populations: N = sample size; N_C = sample size after exclusion of potential siblings from the sample; N_A = mean number of alleles per locus; H_O: observed heterozygosity; H_E = expected heterozygosity; F_{IS} = inbreeding coefficient

ID	Locality	Longitude	Latitude	N	N_C	N_A	H_O	H_E	F_{IS}
C1	Segovia, Boceguillas	−3.618	41.330	10	9	2.857	0.341	0.364	0.121
C2	Madrid, Buitrago de Lozoya	−3.644	40.974	20	16	2.857	0.341	0.362	0.088
C3	Madrid, Canencia, Prado Toril	−3.800	40.859	20	12	1.429	0.190	0.159	−0.154
C4	Madrid, Valdemanco	−3.645	40.853	19	18	3.786	0.437	0.460	0.080
C5	Guadalajara, El Cubillo de Uceda	−3.451	40.795	19	13	3.571	0.455	0.419	−0.043
C6	Madrid, El Vellón	−3.586	40.773	20	18	3.714	0.452	0.447	0.017
C7	Madrid, Guadalix de la Sierra, Medianillos	−3.676	40.756	20	15	3.786	0.367	0.380	0.070
C8	Madrid, Algete, Salomón	−3.561	40.648	18	14	3.286	0.436	0.410	−0.027
C9	Madrid, Colmenar Viejo	−3.786	40.628	20	14	3.857	0.413	0.407	0.021
C10	Madrid, Hoyo de Manzanares	−3.916	40.613	20	14	1.786	0.352	0.296	−0.151
C11	Madrid, Las Rozas	−3.923	40.509	20	16	3.786	0.424	0.427	0.039
C12	Madrid, Getafe, Camino de Preresa	−3.596	40.303	20	14	2.643	0.352	0.358	0.054
C13	Madrid, Fuenlabrada, Valdehondillo	−3.801	40.261	20	16	3.714	0.531	0.493	−0.045
C14	Madrid, Fuenlabrada, Camino de las Panaderas	−3.800	40.255	20	17	3.643	0.492	0.480	0.006
C15	Madrid, Batres, Soto del Endrinal	−3.950	40.238	20	16	2.286	0.330	0.323	0.009
C16	Madrid, Griñón, Cerro del Rayo	−3.821	40.223	20	18	4.143	0.552	0.526	−0.020
W1	Segovia, Santo Tomé del Puerto	−3.589	41.200	20	20	2.824	0.476	0.478	0.028
W2	Madrid, Gascones	−3.651	41.012	20	20	3.235	0.529	0.518	0.003
W3	Madrid, Buitrago de Lozoya	−3.644	40.975	20	9	2.941	0.458	0.439	0.017
W4	Madrid, Bustarviejo, Fuente del Collado	−3.724	40.855	20	17	3.765	0.550	0.517	−0.034
W5	Madrid, Valdemanco	−3.645	40.853	33	28	5.118	0.610	0.583	−0.028
W6	Madrid, Torrelaguna	−3.573	40.830	20	12	2.647	0.490	0.456	−0.030
W7	Guadalajara, El Cubillo de Uceda	−3.451	40.795	20	17	4.059	0.578	0.596	0.060
W8	Madrid, El Vellón, Cotos de Monterrey	−3.603	40.786	20	14	4.059	0.558	0.595	0.101
W9	Madrid, El Vellón	−3.586	40.773	20	16	3.765	0.563	0.547	0.004
W10	Madrid, Guadalix de la Sierra, Medianillos	−3.676	40.756	18	16	4.588	0.652	0.593	−0.067
W11	Madrid, Colmenar Viejo	−3.786	40.628	20	17	4.353	0.612	0.603	0.015
W12	Madrid, Hoyo de Manzanares, La Berzosa	−3.928	40.603	20	18	4.471	0.592	0.573	−0.004
W13	Madrid, Las Rozas	−3.923	40.509	20	18	3.824	0.526	0.526	0.028
W14	Madrid, Brunete	−4.000	40.413	20	16	3.765	0.500	0.533	0.094
W15	Madrid, San Fernando de Henares, La Guindalera	−3.504	40.409	20	17	3.059	0.554	0.504	−0.068
W16	Madrid, Fuenlabrada, Valdehondillo	−3.801	40.261	20	17	3.941	0.522	0.542	0.067
W17	Madrid, Fuenlabrada, Camino de las Panaderas	−3.800	40.255	20	19	4.000	0.563	0.560	0.021
W18	Madrid, Morata de Tajuña	−3.408	40.241	20	17	2.059	0.353	0.320	−0.066
W19	Madrid, Parla, Sancha Barca	−3.793	40.239	20	19	4.235	0.554	0.569	0.052
W20	Madrid, Griñón, Cerro del Rayo	−3.821	40.223	20	19	3.353	0.495	0.489	0.013

genetic structure for both species data sets. We ran spatial genetic mixture analyses, with ten independent runs and a maximum number of groups equal to the number of sampled localities. We compared clusters resulting from each replicate run based on their likelihood score and identified the optimal clustering level based on a stochastic optimization algorithm (Corander et al., 2008).

We calculated three estimates of genetic differentiation between populations, F_{ST} (Weir & Cockerham, 1984), G''_{ST} (Meirmans & Hedrick, 2011) and D_{EST} (Jost, 2008) using GENEPOP, GENODIVE v2.0b23 (Meirmans & van Tienderen, 2004) and SMOGD v1.2.5 (Crawford, 2010), respectively, to compare patterns of regional genetic structure across species and characterize genetic differentiation between populations. We used software CODIDI v1.0 (Wang, 2015) (100,000 permutations) to determine whether G''_{ST} provides an accurate estimate of genetic differentiation in the two species. We also estimated recent migration rates between localities using the software BAYESASS v3.0 (Wilson & Rannala, 2003). We ran three different replicates for each species with 50,000,000 iterations, a burn-in period of

2,000,000 and sampling frequency each 2,000. We assessed convergence of results across runs and used those with the best likelihood in subsequent analyses.

2.3 | Input data used for resistance surfaces and preprocessing

During preprocessing, we aggregated all raster variables to a spatial resolution of 100 m to make the optimization of resistance surfaces tractable and with the same raster support characteristics. We obtained elevation data from ASTER Global Digital Elevation Model (GDEM v2; Tachikawa, Hato, Kaku, & Iwasaki, 2011) with a spatial resolution of 30 m later resampled to 100 m (bilinear method). Then, from elevation data, we calculated per cent slope, describing surface roughness and the Topographic Wetness Index (TWI) describing the tendency of a cell to accumulate water (Quinn, Beven, & Lamb, 1995). We hypothesized elevation, slope and TWI as potential factors for explaining gene flow and genetic differences between sampled populations at a coarser regional scale (see Table 2 listing all factors).

Land use/cover (LUC) considering both composition (the amount of certain land cover categories) and configuration (referring mainly to the spatial distribution and diversity of land cover types) has been hypothesized to impact connectivity, species movements and gene flow (Pérez-Espona et al., 2008) at the landscape level. To assess this, we used two land use/cover data sets to derive resistance surfaces and test the effect of landscape matrix composition. We used the first data set, Corine Land Cover (CLC) (spatial resolution: 100 m), as a categorical map in optimization procedures to derive a resistance surface accounting for the different resistance of each land cover category. Then, we reclassified this data set into a lower number of categories (Table S3) to make it tractable by the RESISTANCEGA package (Peterman, 2014; Peterman, Connette, Semlitsch, & Eggert, 2014). We also used a second fine-scale LUC data set (SIOSE) for deriving a continuous resistance surface. Due to the large number of initial classes in the data set, a reclassification was also necessary (Table S4). We initially attributed an aprioristic resistance weight to each class reflecting the increased resistance to amphibian movement and higher mortality rates associated with artificial (urban habitats, roads) vs. natural land cover/use classes observed in our study area; these weights were subsequently optimized (see initial weights in Table S5).

Roads have also been linked to species mortality, movements and gene flow (Pérez-Espona et al., 2008). Considering this, we obtained linear road data by Spanish Provinces from the *Centro Nacional de Información Geográfica* (CNIG; URL: http://centrodedescargas.cnig.es) to calculate total road density for each 100 m pixel (m/ha) in the study area.

Finally, we hypothesized that the amount and spatial heterogeneity of vegetation cover and vegetation water content (VWC) might also affect genetic differentiation. We downloaded preprocessed remote sensing image data for the Landsat 5 TM sensor, with a spatial resolution of 30 m, from the USGS/ESPA service (URL: http://espa.cr.usgs.gov/). We used one scene for each month of June, August and September 2009, coinciding with field surveys, to generate a single average composite image. The Normalized Difference Vegetation Index (NDVI), extensively used in ecological applications (Nagendra et al., 2013; Turner et al., 2003), provides a continuous measure related to vegetation canopy characteristics such as biomass, leaf area index and percentage of vegetation cover.

TABLE 2 Input variables by level and data source used to explain gene flow and calculate optimized resistance surfaces. Summary statistics in brackets: minimum (min), average (avg), maximum (max) and standard deviation (*SD*)

Level	Variable acronym	Description	Source
Regional	*elev*	Elevation (metres a.s.l; min: 482.0, avg: 935.0, max: 2403.0, *SD*: 326.1)	USGS/ASTER GDEM
	slope	Slope (%; min: 0.0, avg: 9.4, max: 121.2, *SD*: 10.4)	
	twi	Topographic wetness index (unitless; min: 2.9, avg: 7.0, max: 12.6, *SD*: 1.2)	
Local	*clc06*	Corine Land Cover class for year 2006 (15 classes)	EEA
	siose05	SIOSE 2005 Land cover class (resistance weights, unitless; min: 0.0, avg: 12.7, max: 100.0, *SD*: 26.3)	CNIG/Spain
	rdens	Road density (m/ha; min: 0, avg: 27.5, max: 1272.5, *SD*: 26.3)	
Habitat	*ndvi_avg*	Normalized Difference Vegetation Index (spatial average from 30 to 100 m; vegetation biomass/greenness amount; $\times 10^{-4}$, unitless, min: −3,013, avg: 3,652, max: 8,971, *SD*: 1,723)	Landsat 5 TM USGS/ESPA
	ndvi_std	Normalized Difference Vegetation Index (spatial standard deviation from 30 to 100 m; vegetation heterogeneity; $\times 10^{-4}$, unitless, min: 0, avg: 429, max: 5,503, *SD*: 310)	
	ndmi_avg	Normalized Difference Moisture Index (spatial average from 30 to 100 m; vegetation water content; $\times 10^{-4}$, unitless, min: −4,976, avg: −335, max: 5,613, *SD*: 1,413)	
	ndmi_std	Normalized Difference Moisture Index (spatial standard deviation from 30 to 100 m; vegetation water content heterogeneity; $\times 10^{-4}$, unitless, min: 10, avg: 385, max: 3,862, *SD*: 277)	

This index varies from −1 (nonvegetated/artificial surfaces) to 1 (densely vegetated areas). Complementarily, we employed the Normalized Difference Moisture Index (NDMI) to characterize vegetation water content (VWC; Gao, 1996). NDMI also varies from −1 (indicative of low VWC) to 1 (high VWC). Image data were aggregated to 100 m to match the same spatial resolution of topographic and land cover data using the average (defining the amount of vegetation cover and VWC) and the standard deviation (translating the spatial heterogeneity of vegetation cover and VWC).

2.4 | Statistical modelling and resistance surface optimization

To assess the relative support of each variable to explain differences in patterns of genetic structure between sampled populations of *P. cultripes* and *P. waltl*, we used the RESISTANCEGA package in R v3.0.3 (R Core Team 2014). This package implements a genetic algorithm that optimizes a set of equation parameters used to transform the initial values of each variable into resistance surfaces. This optimized surface is then used to calculate cost-based distances between sampled locations through least-cost path analyses using the GDISTANCE package (van Etten, 2015). We used a linear mixed effects model with the maximum-likelihood population effects (MLPE) parameterization (Clarke, Rothery, & Raybould, 2002) to relate the genetic and the cost-based distance matrices and to calculate the model Akaike information criterion (with a correction for finite sample size; AICc). The AICc value is the fitness function output used for iteratively improving the genetic algorithm. The MLPE allows accounting for the nonindependence of values within pairwise distance matrices (Clarke et al., 2002; van Strien, Keller, & Holderegger, 2012) and was fitted by maximum-likelihood using R package LME4 (Bates, Maechler, Bolker, & Walker, 2014).

We used pairwise genetic distances (measured by F_{ST}, G''_{ST} , and D_{EST}), as well as migration rates between populations as the response variable, while scaled and centred effective cost-based resistance distances between populations were considered the independent variable. The genetic algorithm to determine the best set of parameters for optimizing the resistance surfaces used a maximum number of 250 rounds, or 20 rounds without performance improvement.

To evaluate model performance, we compared AICc values between models generated with each optimized resistance surface to two baseline null-models: one based on a single intercept term (*IntOnly*) and other including only isolation-by-distance (*IBD*) effects. Then, we ranked each model based on delta AICc values (ΔAICc) considering the confidence set determined by models with substantial or good support, that is, with $\Delta AICc \leq 4$. We calculated Akaike weights (w_i) to quantitatively represent the strength of evidence or support of each tested model.

3 | RESULTS

We amplified genotypes with a success rate of >99% for both species. Based on MICROCHECKER results, we discarded two microsatellite loci (*Pc4.7* and *Pc4.11*) in *P. cultripes*, because of the possible presence of null alleles. In *P. waltl*, locus *Pleu2.3* was monomorphic in all samples analysed and was thus discarded. Sibship analyses in COLONY detected a low proportion of full-sibs in both species (see Table 1), which were subsequently removed from the data set, leaving one representative per sibship group. We did not detect significant deviations from HWE and LD, except in locus *Pleu3.5* in Pop14 (Brunete) in *P.waltl*, which showed evidence of heterozygote deficit.

Descriptive statistics of genetic diversity for *P. cultripes* and *P. waltl* are presented in Table 1. Estimates of genetic diversity were slightly higher in *P. waltl*. The mean number of alleles per population ranged from 1.43 (Pop3) to 4.14 (Pop16) in *P. cultripes* and from 2.06 (Pop18) to 5.12 (Pop5) in *P. waltl*. The observed heterozygosity ranged from 0.19 (Pop3) to 0.55 (Pop16) in *P. cultripes* and from 0.35 (Pop18) to 0.65 (Pop10) in *P. waltl*. Average population inbreeding coefficients (F_{IS}) were higher in *P. waltl* (0.01) than in *P. cultripes* (0.004).

The results of BAPS analyses supported optimal clustering levels at $K = 13$ and $K = 7$ for *P. waltl* and *P. cultripes*, respectively (Figure 1). The number of clusters was consistent across replicate runs. In *P. waltl*, inferred clusters included geographically close sampling locations, such as clusters (W2 + W3), (W4 + W10 + W11), (W7 + W8 + W9) and (W16 + W17 + W19), whereas in *P. cultripes* two clusters included larger groups of populations, one cluster including locations: (C2 + C4 + C5 + C6 + C7 + C8 + C9 + C11), distributed across medium elevations at the foothills of *Sierra de Guadarrama*, and another cluster including locations (C13 + C14 + C16) in the south of our study area. This is consistent with resistance surfaces, where these areas generally show low resistance/higher connectivity. Overall, there are clear differences between species, with a stronger signal of geographic structure in *P. waltl* (Figure 1).

Pairwise estimates of F_{ST}, G''_{ST} and D_{EST} between populations of each species are presented in Tables S6–S11. Lower average values, indicating greater population connectivity, were found in *P. cultripes* ($F_{ST} = 0.117$; $G''_{ST} = 0.192$; $D_{EST} = 0.044$) than in *P. waltl* ($F_{ST} = 0.187$; $G''_{ST} = 0.402$; $D_{EST} = 0.184$). Analyses with CODIDI did not detect significant negative correlations between diversity (*Hs*) and G''_{ST} in either species (*P. cultripes*: $r = .20$, $p = .73$; *P. waltl*: $r = -.21$, $p = .21$), indicating G''_{ST} estimates accurately describe population differentiation and are comparable across species.

BAYESASS results indicate low migration rates across sites, except among geographically close localities (Tables S12 and S13), with higher average migration rates between populations of *P. cultripes* (mean = 0.0179) than in populations of *P. waltl* (mean = 0.0126).

3.1 | Multi-model selection and ranking

For *Pelobates cultripes*, optimization and model selection showed similar and congruent results with low model uncertainty when comparing rankings obtained for distance matrices based on D_{EST}, F_{ST} and G''_{ST} (Table 3). For this species, average NDVI (related to vegetation amount) was the most frequently selected variable for all

TABLE 3 Model selection table for *Pelobates cultripes* (left) and *Pleurodeles waltl* (right). Models highlighted in bold attained the highest relative support and were included in the confidence set (ΔAICc ≤ 4). AICc—Akaike information criterion value (with a correction for finite sample size), ΔAICc—delta AIC value, w_i—Akaike weights. *IBD*—isolation-by-distance model. *IntOnly*—Intercept-only/null model

	Pelobates cultripes				*Pleurodeles waltl*			
Genetic distance metric	Variable	AICc	ΔAIC	w_i	Variable	AICc	ΔAIC	w_i
D_{EST}	***elev***	**−578.90**	**0.00**	**0.47**	***ndvi_std***	**−522.71**	**0.00**	**0.52**
	ndvi_avg	**−578.72**	**0.18**	**0.43**	***elev***	**−521.95**	**0.75**	**0.36**
	ndmi_avg	−573.38	5.52	0.03	***ndmi_std***	**−519.76**	**2.95**	**0.12**
	slope	−573.37	5.54	0.03	*rdens*	−511.89	10.81	0.00
	twi	−570.84	8.06	0.01	*slope*	−511.23	11.47	0.00
	ndmi_std	−570.48	8.43	0.01	*twi*	−509.96	12.74	0.00
	ndvi_std	−570.09	8.82	0.01	*ndvi_avg*	−508.57	14.14	0.00
	siose05	−569.66	9.24	0.00	*siose05*	−502.84	19.87	0.00
	rdens	−569.58	9.33	0.00	*IBD*	−502.24	20.46	0.00
	IntOnly	−569.46	9.44	0.00	*ndmi_avg*	−502.09	20.62	0.00
	clc06	−568.84	10.06	0.00	*clc06*	−488.00	34.70	0.00
	IBD	−567.70	11.20	0.00	*IntOnly*	−387.81	134.90	0.00
F_{ST}	***ndvi_avg***	**−495.08**	**0.00**	**0.59**	***ndvi_std***	**−646.58**	**0.00**	**0.63**
	slope	**−492.48**	**2.60**	**0.16**	***slope***	**−643.24**	**3.35**	**0.12**
	twi	−490.29	4.79	0.05	***ndmi_std***	**−642.69**	**3.89**	**0.09**
	siose05	−490.29	4.80	0.05	***elev***	**−642.45**	**4.14**	**0.08**
	elev	−489.90	5.18	0.04	*rdens*	−641.74	4.84	0.06
	ndmi_std	−489.85	5.23	0.04	*twi*	−638.87	7.72	0.01
	rdens	−488.20	6.88	0.02	*ndvi_avg*	−638.44	8.14	0.01
	ndvi_std	−488.10	6.98	0.02	*siose05*	−636.99	9.60	0.01
	ndmi_avg	−487.73	7.35	0.01	*clc06*	−629.92	16.66	0.00
	IBD	−484.04	11.04	0.00	*ndmi_avg*	−624.61	21.98	0.00
	IntOnly	−482.72	12.36	0.00	*IBD*	−624.42	22.16	0.00
	clc06	−479.98	15.10	0.00	*IntOnly*	−516.03	130.55	0.00
G''_{ST}	***ndvi_avg***	**−383.78**	**0.00**	**0.77**	***ndvi_std***	**−364.56**	**0.00**	**0.74**
	slope	**−381.21**	**2.57**	**0.21**	***ndmi_std***	**−361.18**	**3.38**	**0.14**
	twi	−375.35	8.44	0.01	*rdens*	−358.92	5.64	0.04
	ndmi_std	−372.81	10.97	0.00	*slope*	−358.32	6.24	0.03
	elev	−372.58	11.20	0.00	*elev*	−357.48	7.08	0.02
	ndvi_std	−372.54	11.24	0.00	*twi*	−355.91	8.64	0.01
	ndmi_avg	−371.50	12.28	0.00	*siose05*	−355.15	9.41	0.01
	rdens	−369.93	13.85	0.00	*ndvi_avg*	−354.98	9.58	0.01
	siose05	−368.80	14.98	0.00	*IBD*	−343.12	21.44	0.00
	IBD	−364.43	19.35	0.00	*ndmi_avg*	−342.98	21.58	0.00
	clc06	−363.11	20.67	0.00	*clc06*	−340.58	23.98	0.00
	IntOnly	−360.67	23.11	0.00	*IntOnly*	−230.69	133.86	0.00

distance measures. Topographic variables related to elevation (selected for D_{EST}) and slope (selected for F_{ST} and G''_{ST}) were also important to explain genetic differentiation in *P. cultripes*.

For *Pleurodeles waltl* model selection also showed similar and coherent results with low model uncertainty across different genetic distances. For this species, spatial heterogeneity of vegetation (NDVI std.-dev.) and spatial heterogeneity in vegetation water content (NDMI std.-dev.) were the most frequently selected variables. These attained very high model support being selected for all genetic distances (Table 3). Topographic resistance surfaces related to elevation (selected for D_{EST} and F_{ST}) and slope (selected for F_{ST}) were also important to explain genetic differentiation for *P. waltl*.

For both species, models related to landscape composition and structure (siose05) or road density showed very little to no support

in explaining genetic differentiation (Table 3). Overall, for both species and considering all genetic distance matrices, the IBD and the intercept-only models attained no relative support, showing a good relative performance of resistance surfaces to explain gene flow patterns.

3.2 | Resistance surfaces

Resistance surface optimization revealed several nonlinearities regarding the way landscape features influence gene flow (Figure 2). Also, when considering model-selected variables (Table 3), the transformations used for generating optimized resistance surfaces showed a very strong similarity of responses across different genetic distances translating a certain degree of stability between optimization rounds.

For *Pelobates cultripes*, we found lower resistance areas for below median elevations (with a minimum resistance around 870 m) and moderate slopes (minimum 11.4%) corresponding to low- and midland areas. Vegetated areas with above median NDVI values had low resistance (minimum around 0.42; Figure 3) showing both an avoidance for artificial areas (e.g., urban settlements, roads; typically, with very low or negative NDVI values) and strongly vegetated areas mostly related to elevated mountainous zones with forest cover.

For *Pleurodeles waltl*, we found two different (and complementary) transformations for elevation. When considering F_{ST} and G''_{ST}, the relationship was almost linear, that is, with increasing resistance following the increase in altitude (Figure 3), while for D_{EST}, strong resistance values were related to lowland areas. In addition, low-resistance landscapes had very high NDVI/NDMI standard deviation, corresponding to highly heterogeneous and moderately disturbed areas with natural and/or semi-natural vegetation, frequently with water surfaces or lines.

4 | DISCUSSION

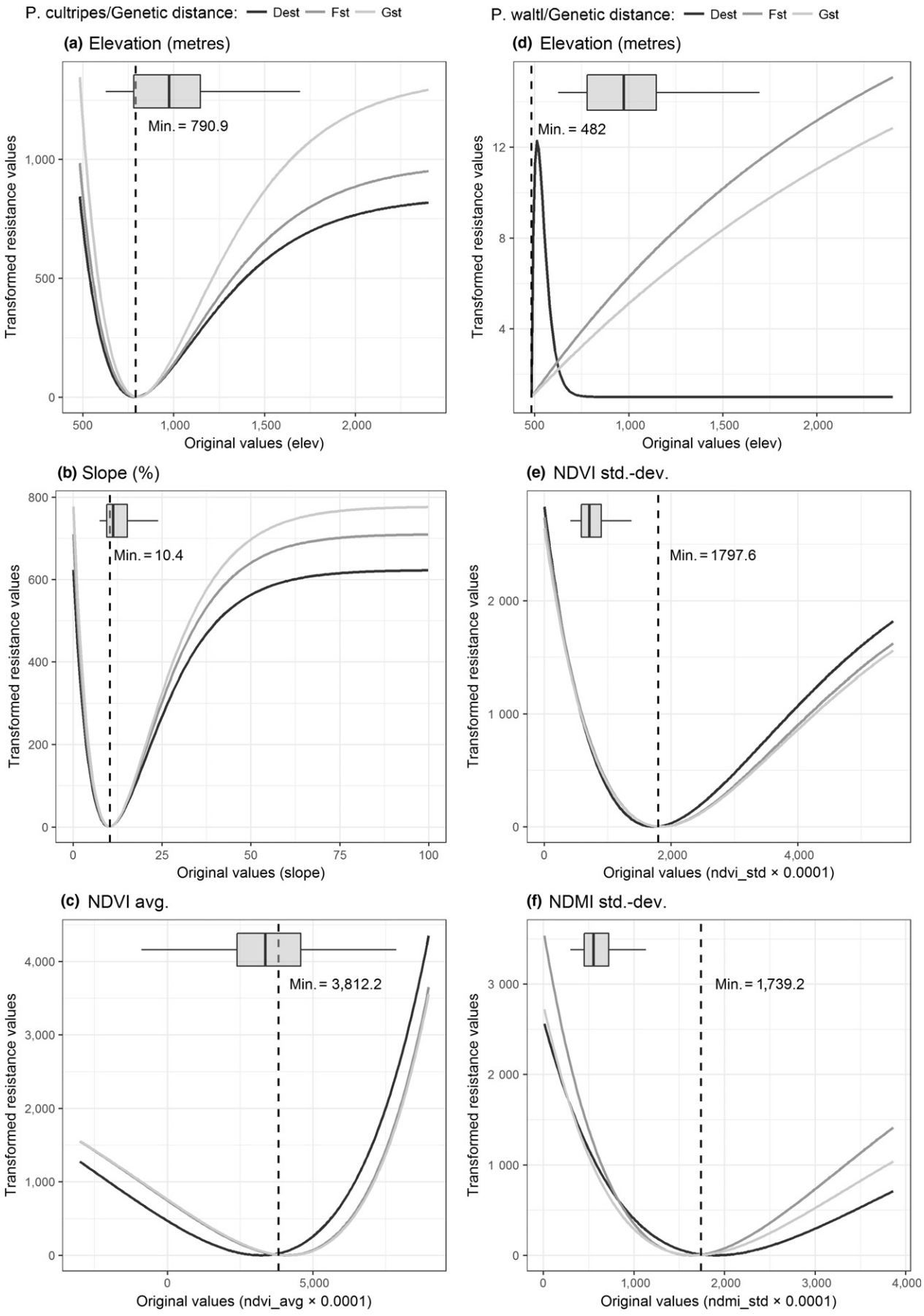
Comparative studies based on molecular approaches hold great potential to improve our understanding on the effect of different landscape features on regional patterns of population connectivity across taxa. Here, we anticipated land cover-related variables to have the most impact on genetic structure and hypothesized remotely sensed data would provide a more accurate description of terrestrial habitats at the relevant spatial scale for low-dispersing organisms than other traditionally used discrete categories of land use/cover. Our results support this notion, with genetic differentiation in the two species mainly associated with environmental factors related to land and vegetation cover.

Our comparative study revealed contrasting patterns of genetic structure in the two species, with stronger, finer scale genetic differentiation in *Pleurodeles waltl* (see Figure 1). This difference can be associated with demographic (especially differences in dispersal rates) and life history traits, or divergent terrestrial habitat preferences. These factors are not mutually exclusive, and our resistance surfaces provide some keys to sort out their relative effects. Leaving aside the common effect of topography on both species, land cover-related categorical variables did not have an important role in shaping genetic structure. Some studies have previously reported negative effects of roads on gene flow in anurans (Richardson, 2012) and salamanders (Coster et al., 2015). Nevertheless, models including road density as a variable were not well supported in either species (Table 3). Instead, we found remarkable differences associated with the role of fine-scale patterns of vegetation cover, vegetation water content and their spatial heterogeneity in shaping patterns of regional genetic structure for the two species. These differences were better captured by fine-scale remotely sensed spectral indices (NDVI/NDMI) portraying land cover, vegetation and moisture in a continuous fashion, highlighting their value in landscape genetics studies.

For *Pelobates cultripes*, genetic differentiation increased with both high and low values of vegetation coverage (NDVI avg.), with medium values in this variable associated with reduced resistance to gene flow (Table 3). Patches with high resistance are typically related to urban/artificial areas and roads (low NDVI values) or high altitude forested areas (high values; Figure 3). Habitats with mid-range canopy cover have been reported as areas preferentially selected during dispersal in other species of anurans, like *Bufo boreas* (Bartelt, Peterson, & Klaver, 2004). In *P. waltl*, high or very high values in variables related to the spatial heterogeneity of vegetation cover (NDVI and NDVI std.-dev.), and the spatial heterogeneity in vegetation water content (NDMI std.-dev.), were associated with higher connectivity between populations. These values correspond to heterogeneous and moderately disturbed areas with different types of natural and/or semi-natural vegetation frequently linked to water surfaces or lines (Table 3 and Figure 3). Thus, our results suggest a positive role of structural heterogeneity in population connectivity for the two species, with habitat patches of Mediterranean scrubland and open oak woodlands ("*dehesas*") facilitating gene flow.

Fine-scale aspects of the landscape, described by remotely sensed data, strongly influenced gene flow in both species, as anticipated. However, different variables were more important in shaping

FIGURE 2 Transformations applied to each variable (included in the most frequently selected models) to generate resistance surfaces for species *Pelobates cultripes* (left-side) and *Pleurodeles waltl* (right-side). Each curve represents a different transform parametrization optimized for D_{EST} (black colour), F_{ST} (dark-grey) and G''_{ST} (light-grey). Original values are represented in the *x*-axis (including the full range of the variable) while transformed (resistance) values are shown in the *y*-axis and should be interpreted in relative fashion between variables. The dashed line shows the minimum resistance for the original values of each variable. The boxplot on top shows the distribution of the original untransformed variable (boxes are the 25%, 50% and 75% quartiles, and whiskers show the smallest/largest observation greater/less than or equal to lower hinge −/+ 1.5 times the interquartile range). NDVI and NDMI values have a 10^{-4} scale factor


P. cultripes/Genetic distance: Dest Fst Gst
(a) Elevation (metres)
Min. = 790.9
Transformed resistance values
Original values (elev)
(b) Slope (%)
Min. = 10.4
Original values (slope)
(c) NDVI avg.
Min. = 3,812.2
Original values (ndvi_avg × 0.0001)
P. waltl/Genetic distance: Dest Fst Gst
(d) Elevation (metres)
Min. = 482
(e) NDVI std.-dev.
Min. = 1797.6
Original values (ndvi_std × 0.0001)
(f) NDMI std.-dev.
Min. = 1,739.2
Original values (ndmi_std × 0.0001)

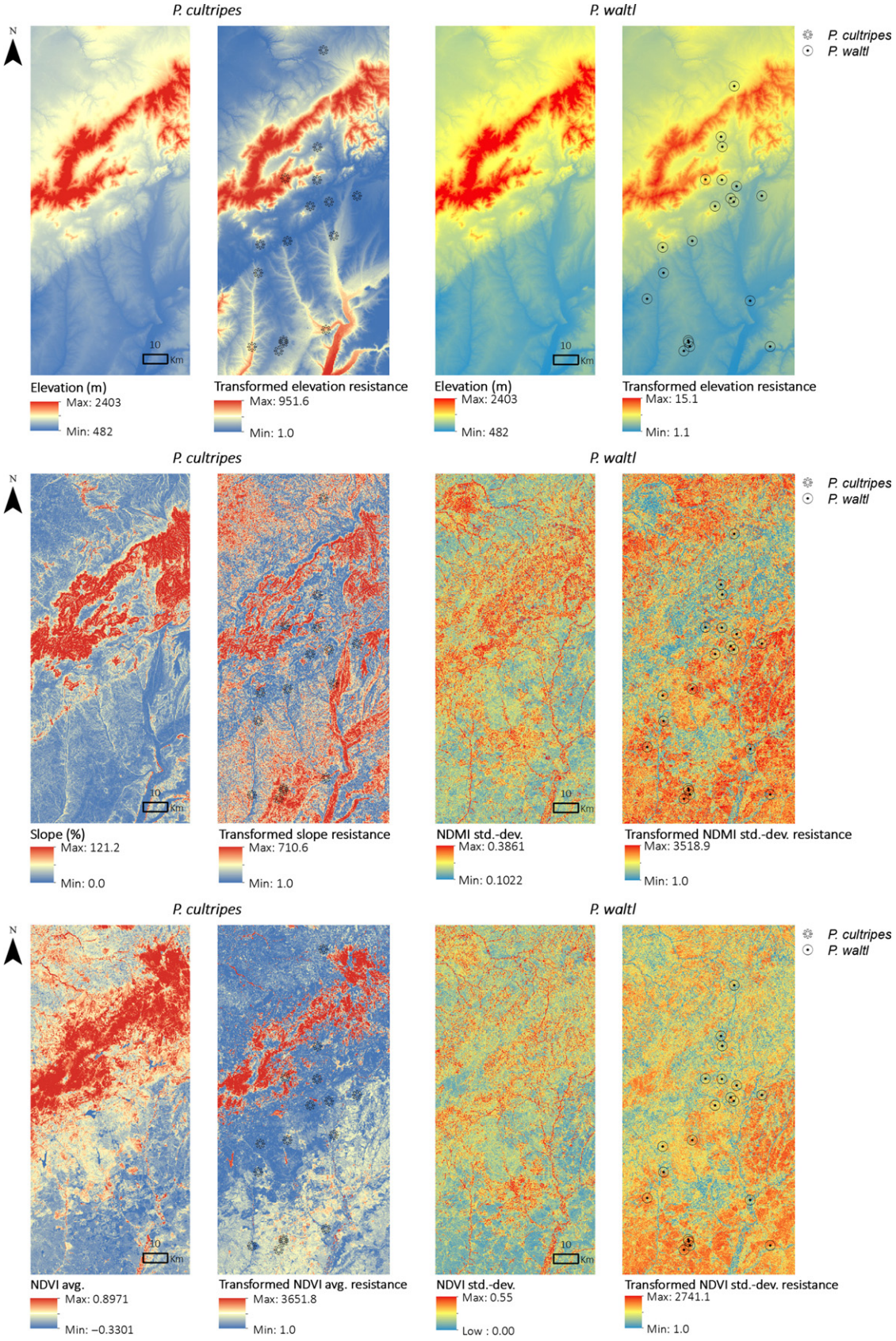


FIGURE 3 Optimized resistance surfaces for *Pelobates cultripes (left-side)* and *Pleurodeles waltl (right-side)* based on the most frequently selected variables and considering F_{ST} genetic distances. For each species, on the left are maps showing the original continuous values for each variable; on the right, the resistance surface generated through optimization. Points represent sampling localities [Colour figure can be viewed at wileyonlinelibrary.com]

patterns of gene flow between species. This is surprising in view of the extensive overlap in their geographic ranges, probably reflecting similar ecological requirements at the macro (bioclimatic) scale (Sillero, Brito, Skidmore, & Toxopeus, 2009). Regionally, vegetation amount played a key role in *P. cultripes*, whereas in *P. waltl*, the spatial heterogeneity in vegetation coverage and water content/water stress was more important. As NDMI is an indicator of water stress, its presence in models for *P. waltl* may indicate that this species is more sensitive to drought conditions. With potential alterations in hydrologic regimes derived from climate change (affecting wetlands and ponds through changes in hydroperiod), this has important consequences for connectivity and conservation assessments. Also, low-resistance areas (with importance for connectivity) are less common in the landscape for *P. waltl* than for *P. cultripes* (notice the difference in resistance curves in Figure 2: in *P. waltl*, low-resistance conditions are pushed to the right-side, close to extreme data values; while in *P. cultripes*, they are closer to the median). This observation, in conjunction with the inferred lower dispersal ability in *Pleurodeles*, probably makes this species more vulnerable to the effects of fragmentation, whereas *Pelobates* could be considered more of a generalist species, in the sense that it benefits from a higher availability of favourable areas for dispersal in the study area.

In addition to variables related with land and vegetation cover, our results also showed some effect of topography on regional patterns of genetic structure. Variables related to topography have been previously shown to explain genetic differentiation in a wide range of taxa (for examples in amphibians, see Funk et al., 2005; Funk et al., 2016; Giordano, Ridenhour, & Storfer, 2007; Igawa et al., 2013; Savage, Fremier, & Bradley Shaffer, 2010; Zancolli et al., 2014). In our study area, we also found that neither elevation nor slope favour gene flow in the two species studied (Table 3). Both are typical inhabitants of Mediterranean habitats at low and medium elevations, with upper altitudinal limits of up to 1,480 m in *P. waltl* and 1,770 m in *P. cultripes* (Cejudo, 1990; García-París, Montori, & Herrero, 2004; Montori, Llorente, Santos, & Carretero, 2002; Tejedo & Reques, 2002). Restrictions to population connectivity imposed by elevation and slope probably operate on deeper timescales than those related to land use/cover. Recent phylogeographic studies have shown that the Iberian Central System has acted as an historical barrier to gene flow in the two species, separating different evolutionary groups north and south of this mountain range (Gutiérrez-Rodríguez, Barbosa, & Martínez-Solano, 2017a,b). These groups would have experienced limited connectivity during the Last Glacial Maximum (21 ka), as glacial areas occupied lower altitudes around 1,350 m in the study area (Bullón, 2016). In contrast, vegetation dynamics in the Central System mountains have been constantly changing during the Holocene due to human activities (López-Sáez et al., 2014). In addition, large water surfaces and rivers are also associated with potential barrier effects in areas of low elevation and slope.

Demographic and life history traits, such as dispersal, philopatry, population effective size, generation time, clutch size and larval phenology, have been described as promoters of genetic differentiation in amphibians (Nowakowski, DeWoody, Fagan, Willoughby, & Donnelly, 2015; Richardson, 2012; Whiteley, McGarigal, & Schwartz, 2014). One of the most important biological traits affecting the genetic structure of populations is dispersal capacity, which is fundamental to maintain gene flow between populations. Amphibians generally present limited dispersal and significant differences have been observed between anurans and salamanders (Smith & Green, 2005). In the case of *P. cultripes* and *P. waltl*, there is no published information about dispersal distance or home range. Nonetheless, our analyses suggest low migration rates in both species (Tables S12 and S13). While these values may underestimate actual migration rates because of our sampling strategy (mostly larvae, which cannot be migrants) and other factors, like the potential presence of unsampled ponds that could act as stepping stones, low rates are consistent with our own field data. Over an 8-year period, we recorded 0.5% vs. 1.2% dispersal event rates involving distances over 250 m of marked adult individuals of *Pleurodeles* (six dispersal events per 1,172 recaptures) and *Pelobates* (16 dispersal events per 1,293 recaptures), respectively (Gutiérrez-Rodríguez, Sánchez-Montes, et al., 2017). The lower frequency of dispersal events in *P. waltl* is in agreement with the observed higher population genetic differentiation as compared to *Pelobates*. Other indirect evidence like higher frequency of road-kills in *P. cultripes* in areas where the two species co-occur (D'Amico, Román, de los Reyes, & Revilla, 2015) also suggests more dispersal capacity and propensity in *Pelobates*, in line with previous studies documenting higher migration potential in anurans compared to urodeles. Another life history trait linked to dispersal and population structure is breeding site philopatry, with the species with higher philopatry showing increased population genetic differentiation. Here, we inferred philopatry based on the comparison of inbreeding coefficients (F_{IS}), with results in agreement with dispersal estimates: mean values of F_{IS} in *P. waltl* (0.010) doubled those in *P. cultripes* (0.004), suggesting a more philopatric behaviour of the former species.

Our study represents a valuable contribution to the knowledge on the functioning and conservation of Mediterranean habitats, including the finding of a positive role of structural heterogeneity on gene flow in pond-breeding amphibians. More generally, it illustrates how remotely sensed continuous variables of vegetation cover and water content (e.g., NDVI, NDMI) have great potential to offer relevant insights about major drivers of population connectivity in landscape genetics studies. In our study area, variables associated with fine-scale structural habitat heterogeneity/complexity proved to have a major impact on regional patterns of genetic differentiation in two syntopic pond-breeding amphibians. This heterogeneity is a characteristic of Mediterranean landscapes at low/medium elevations, which include extensively managed areas together with natural and/or semi-natural habitats. While it remains to be seen whether our results are generalizable to other taxa, the differences observed in our comparison regarding two taxonomically and ecologically similar taxa highlight the conservation value of these areas and point to the need of considering species individually when identifying and designing corridors connecting local populations in Mediterranean habitats.

ACKNOWLEDGEMENTS

We thank Agentes Forestales de la Comunidad de Madrid and G. Astudillo, C. Grande, J.L. Gutiérrez, A. López, J.A. Lucas, L. Martín, B. Paños, R. Pereira, E. Recuero and G. Sánchez Montes for help in sampling and T. Cuartero and V. González (Laboratorio de Biogeografía Informática, MNCN-CSIC) for support with spatial data. JGR was supported by the Consejo Superior de Investigaciones Científicas of Spain (CSIC) and the European Social Fund (ESF) (JAE-pre PhD fellowship). JG was financially supported by FCT (Portuguese Foundation for Science and Technology) through PhD grant SFRH/BD/90112/2012 funded by POPH-QREN (2007–2013). EC was also supported by FCT through postdoctoral grant SFRH/BPD/109182/2015 funded by CSF (2014–2020). This research was funded by grant CGL2008-04271-C02-01/BOS and CGL2011-28300 (Ministerio de Ciencia e Innovación -MICINN-), Ministerio de Economía y Competitividad -MEC-, Spain, and FEDER) to IMS, who was supported by funding from the Spanish "Ramón y Cajal" (RYC-2007-01668) and "Severo Ochoa" (SEV-2012-0262) programmes. Authorizations for animal capture, marking and tissue sampling were provided by Consejería de Medio Ambiente y Ordenación del Territorio, Comunidad de Madrid and Consejería de Fomento y Medio Ambiente, Junta de Castilla y León (Spain).

DATA ACCESSIBILITY

The dryad archive (https://doi.org/10.5061/dryad.d3742) contains microsatellite genotypes of the two species and the spatial data used to develop connectivity analyses.

AUTHOR CONTRIBUTIONS

I.M.-S. and J.G.-R. designed the project and performed fieldwork. J.G.-R. conducted laboratory work and molecular analyses. J.G. and E.C. conducted spatial analyses. J.G.-R. wrote the manuscript, with input from all authors.

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How to cite this article: Gutiérrez-Rodríguez J, Gonçalves J, Civantos E, Martínez-Solano I. Comparative landscape genetics of pond-breeding amphibians in Mediterranean temporal wetlands: The positive role of structural heterogeneity in promoting gene flow. *Mol Ecol*. 2017;26:5407–5420. https://doi.org/10.1111/mec.14272