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RESEARCH ARTICLE

Genetic management of an amphibian population after a chytridiomycosis outbreak

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Abstract An epidemic of the disease chytridiomycosis, caused by the pathogenic fungus *Batrachochytrium dendrobatidis*, induced a massive decline of populations of the common midwife toad (*Alytes obstetricans*) inhabiting the Peñalara Massif (Guadarrama National Park, Central Spain) in the years 1997–2001. The disease outbreak caused the disappearance of about 90 % of populations, leaving only eight remnant breeding populations. In response to the disease-induced population decline, a captive breeding program was started in 2008. Populations were kept separate to minimize possible outbreeding depression. Here, we examined indices of genetic diversity and population structure in these remnant populations to inform future reintroductions. Analysis of ten microsatellite loci showed strong genetic structure between breeding sites suggesting little genetic exchange and relatively low global genetic diversity. In accordance with the demographic bottleneck observed in the last years we found strong evidence for a reduction in genetic diversity. Our results suggest that the captive breeding program should mix animals from multiple sites from the Guadarrama Mountain Range, but avoid the genetically most divergent populations.

Keywords *Alytes obstetricans* · Fungal disease · Amphibian decline · Bottleneck · Reintroduction · Iberian Peninsula

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Introduction

Maintenance of genetic diversity in populations is a major focus in conservation biology (Frankham et al. 2002). Risk of extinction due to genetic factors can be pronounced in small and isolated populations (Hartl and Clark 1997). As such, continual gene flow through population connectivity is crucial, especially in species with small population sizes or limited dispersal abilities (Frankham 2005). For these reasons, one of the global priorities of International Union of Conservation of Nature (IUCN) is the conservation of genetic diversity in fragmented and small populations (McNeely et al. 1990).

Amphibians are good biological models for investigating the genetic effects of fragmentation events because they typically show high levels of genetic differentiation at fine scales (Andersen et al. 2004; Richardson 2012; Trumbo et al. 2013). This may be due to their high level of philopatry (Semlitsch 2008), their limited dispersal abilities (Allentoft and O'Brien 2010), or to frequent local extinction-recolonization dynamics (metapopulation system; Wade and Mc Cauley 1988).

It is widely recognized that the rate of loss of amphibians around the globe is increasing (Allentoft and O'Brien 2010; Collins 2010). Of the 6,600 described species, 43 % are currently threatened with extinction (Stuart et al. 2004).

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Pollution, climate change and emerging diseases are considered the major drivers behind the massive decline of amphibians (Collins 2010). The global emergence of diseases can lead to dramatic reductions in population size, especially when pathogen-induced mortality is additive (i.e., when pathogen-induced mortality is added to the normal mortality; Fisher et al. 2009; Tobler et al. 2012).

The chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) has affected amphibian populations on most continents, from tropical to temperate habitats with catastrophic consequences (Lips et al. 2006; Skerratt et al. 2007; Fisher et al. 2009). Chytridiomycosis is a non-typical emerging disease with a broad host range and heterogeneous impacts on host populations (Kilpatrick et al. 2010).The reasons for its sudden emergence as an amphibian pathogen remain under intensive study (Collins and Storfer 2003; Collins 2010; 2013). Although there is still no clear general agreement about the relationship between the presence of *Bd* and environmental variables (Walker et al. 2010), studies have shown that population level responses to infection can be explained by local environmental variables (Doddington et al. 2013).

The common midwife toad (*Alytes obstetricans*) is a widespread amphibian in central and western Europe with its southern range including the Iberian Peninsula. It is threatened over a great part of its distribution by the fragmentation and isolation of its populations (Bosch 2002) and more recently by the emergence of *Bd* (Bosch et al. 2001). Although recent studies have shown that populations can persist despite the enzootic presence of *Bd* under current environmental conditions (Tobler et al. 2012), *Alytes obstetricans* is one of the European amphibian species known to be highly susceptible to *Bd* (Bosch et al. 2001, Tobler and Schmidt 2010, Balaz et al. 2014). In Spain, the fungus has caused episodes of mass mortality in multiple species (*A. obstetricans*, *B. bufo*, *S. salamandra*), mainly in montane areas (Bosch et al. 2001; Walker et al. 2010).

Our study was conducted at the index site for *Bd* in Europe: the Peñalara Massif and its surroundings (Bosch et al. 2001). The Peñalara Massif (formerly the Peñalara Natural Park and currently the heart of the Guadarrama National Park) is an alpine area at about 2,000 m of elevation in central Spain. The area has been protected for the past 70 years and, in spite of the high number of visitors (>100,000 per year), conservation and restoration practices maintain its ecological health in good condition. More than half of all ponds in this area are permanent, whereas the rest are temporary ponds. The Peñalara Massif has 10 amphibian species, of which *A. obstetricans* used to be one of the most abundant. In spring, *A. obstetricans* males formed large choruses in several locations, and reproduction was known to occur throughout the massif in the past (J. Bosch, personal observations). Midwife toads have a small clutch size and a remarkable reproductive behavior, as males carry the eggs twined around their hind legs on land for about a month, from fertilization to hatching. After hatching, larval development takes place in water bodies until metamorphosis is completed and juveniles leave the ponds. In the summers of 1997 and 1998, thousands of dead post-metamorphic *A. obstetricans* were found around the ponds of the Peñalara Massif. This situation prompted an intense survey in 1999, which led to the discovery of *Bd* as the responsible agent of this decline (Bosch et al. 2001). Within 5 years, the fungus caused the extirpation of around 90 % of all populations, leaving only 8 breeding wild sites in the whole area. Although the species remains abundant in northern Spain, in Central Spain the situation is dramatic, especially in the Guadarrama Mountains Range. The neighboring populations are located more than 40–50 km away, are quite small and not genetically related (Bosch 2002).

As a consequence of this outbreak of chytridiomycosis, and to avoid the complete extinction of the *A. obstetricans* in the Peñalara Massif, in 2008 a captive-breeding facility was established by the local government (Madrid, Consejería de Medio Ambiente, Vivienda y Ordenación del Territorio), the Spanish Museum of Natural History (CSIC) and the Durrell Wildlife Conservation Trust. The purpose of the captive breeding program is to maintain captive populations representative of the remaining genetic diversity, and to be a source for reintroductions back into the park. Twenty one adults in total and some tadpoles were collected in 2008 from every remnant breeding site inside the Peñalara Massif and maintained in the facility in sterile conditions to avoid new *Bd* infection. At one site, Valdesquí (VQ), located outside the Peñalara Massif, individuals were collected in 1996 prior to the crash (60 tadpoles from a single temporary pond), and have been maintained in captivity since that time in a different location. Until the present study was concluded, all populations were kept separate to minimize possible outbreeding depression.

Here we evaluate the potential for captive stocks of *A. obstetricans* from the studied area to serve as a source of uninfected individuals for the reintroduction of this species into the Peñalara Massif. We used ten microsatellite markers to address three major questions: (1) are the populations from the Peñalara Massif and surrounding areas genetically differentiated or do they form a single panmictic unit? (2) can we detect signs of a genetic bottleneck in the extant populations of the *A. obstetricans* in the study area? (3) for future reintroductions, should we mix the present captive population before releasing them to the wild or should they be maintained separately?

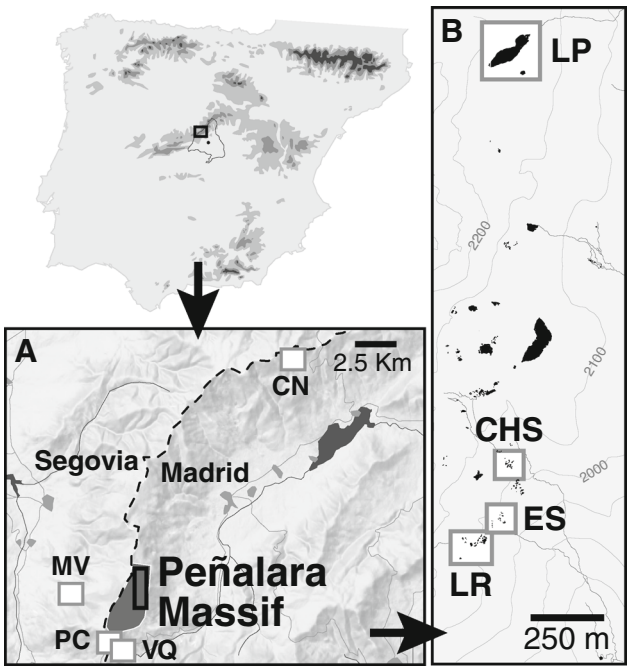

Fig. 1 Location of the Peñalara Massif (formerly Peñalara Natural Park) in the center of the Guadarrama Mountain Range (Central Spain) in dark shading. **a** Sampling sites outside the Peñalara Massif on the border between Segovia and Madrid: Circo del Nevero (CN), Montes de Valsaín (MV), Puerto de Cotos (PC) and Valdesqui (VQ). **b** Sampling sites inside the Peñalara Massif: Laguna de Pájaros (LP), Charcas del Salto (ES), Charcas Secas (CHS) and Charcas de la Rubia (LR)

Materials and methods

Sample collection

We analysed a total of 106 individuals from the eight extant demes in the area of the Peñalara Massif. Here, a deme includes all individuals that use the same breeding site for reproduction. We included four demes inside the Peñalara Massif: Laguna de Pájaros (LP), Charcas del Salto (ES), Charcas Secas (CHS) and Charcas de la Rubia (LR), and four populations outside the massif: Puerto de Cotos (PC), Montes de Valsaín (MV) on the north side of the Sierra de Guadarrama, Valdesquí (VQ) and Circo del Nevero (CN), the most remote population, located about 20 km from the Peñalara Massif (Fig. 1, Table 1). For all sites except VQ, samples were collected in 2009 in the field from larvae, or from individuals born in the wild but kept in the captive-breeding facility center. Samples from VQ were from individuals maintained in captivity since 1996. We collected tissue samples via tail clipping in the case of larvae and toe clips in the case of adults. All samples were stored in 70 % ethanol and maintained at −20 °C.

DNA extraction and PCR amplification

DNA was extracted using the QIAGEN DNeasy Tissue Extraction kit. We used 10 polymorphic microsatellites developed for *A. obstetricans* (Tobler et al. 2013) to characterize the genetic structure of the populations of Peñalara Park. Individual loci were PCR amplified in a final volume of 20 µl containing 1X PCR buffer [67 mM Tris–HCl pH 8.8, 16 mM $(NH_4)_2SO_4$, 0.01 % Tween-20], 2.5 mM $MgCl_2$, 0.01 % BSA (Roche Diagnostics), 0.25 µM dNTPs, 0.40 µM dye-labelled M13 primer, 0.25 µM reverse primer, 0.034 µM M13 tailed-forward primer, 0.5 U *Taq* DNA polymerase (Bioline) and 5 µl of genomic DNA. A sequence tail 5′-GTTTCT-3′ was added to the 5′ end of the reverse primer to improve adenylation and facilitate genotyping (Brownstein et al. 1996).

Samples were amplified in a 'touchdown' PCR in a BIO-RAD DNA Engine Peltier Thermal Cycler, with an

Table 1 Sample size (N), average number of alleles per locus (n_A), expected heterozygosity (H_e), observed heterozygosity (H_o), gene diversity (H), allelic richness (a_R), inbreeding coefficient (FIS) and number of private alleles per locality (P_A, number of loci) for ten microsatellite loci in eight populations of *Alytes obstetricans*

Population	N	n_A	H_e	H_o	H	a_R	FIS	P_A
LP	20	3.9	0.571	0.653	0.588	3.125	−0.114	3(2)
VQ	13	2.7	0.484	0.517	0.504	2.498	−0.024	0
PC	6	3.5	0.582	0.617	0.637	3.500	0.031*	1
ES	11	2.7	0.404	0.340	0.436	2.474	0.210**	0
CHS	8	2.7	0.485	0.529	0.517	2.607	−0.013	1
LR	17	4	0.485	0.430	0.581	3.303	0.165**	1
CN	20	3	0.415	0.395	0.426	2.610	0.073	1
MV	11	4.6	0.645	0.609	0.679	3.982	0.102**	6 (6)
Average					0.60	3.08	0.020	
SE					0.08	0.4	0.056	

Asterisks denote significant values: * $p < 0.05$, ** $p < 0.01$

initial 2 min of denaturation at 94 °C; 17 cycles at 92 °C for 30 s, annealing at 60–44 °C for 30 s (1 °C decrease in each cycle) and extension at 72 °C for 30 s; 25 cycles of 92 °C for 30 s, 44 °C for 30 s and 72 °C for 30 s with a final extension for 5 min at 72 °C. Amplified fragments were analyzed on an ABI 3130xl Genetic Analyser and scored using GeneMapper 4.0 (Applied Biosystems) and LIZ 500 size standard.

Population genetic differentiation and genetic diversity

Departure from Hardy–Weinberg equilibrium for each locus in each of the eight studied breeding sites was calculated using a test analogous to Fisher's exact test in GENEPOP 4.0 (Rousset 1997). Microsatellite diversity indices, average number of alleles per locus, observed heterozygosity (H_o) and expected heterozygosity (H_e) were computed from allele frequencies under the assumption of random mating using GENETIX 4.03 (Belkhir et al. 2001). Allelic richness and the population-inbreeding coefficient (F_{IS}) were calculated with FSTAT 2.9.3 (Goudet 2001).

Population structure was evaluated with the unbiased estimator of Wright's (1951) F_{ST} using GenAlex 6.2 (Peakall and Smouse 2006). Partitioning of genetic variance (AMOVA) among individuals and populations was calculated with the same software. Isolation by distance was assessed in IBDWS 3.16 (http://ibdws.sdsu.edu/~ibdws/) by testing the correlation between the logarithm of pairwise geographical distances (calculated from longitude and latitude data (http://www.chemical-ecology.net/java/lat-long.htm)) and Fst/(1–Fst) values.

We also examined population genetic structure using the model-based approach implemented in STRUCTURE 2.3.2 (Pritchard et al. 2000). Using a Bayesian approach, STRUCTURE estimates the number of genetic clusters without prior information on geographic clusters and assigns a posterior probability to each individual of belonging to each of K inferred clusters. We used the admixture model with correlated allele frequencies and the maximum number of the clusters was 11 (K = 1–11). For each K we ran 20 replicates of 1,000,000 iterations of Markov chain Monte Carlo with burn-in periods of 200,000 iterations. We applied the Evanno et al. (2005) method to estimate the most likely number of genetic clusters (ΔK).

Bottleneck test

To evaluate the evidence for recent bottleneck events, we used three different approaches. First, we assessed the effect of recent and severe population reduction using a one-tailed Wilcoxon signed rank test (10,000 iterations) to determine if observed heterozygosity was higher than expected under mutation-drift equilibrium for the observed number of alleles, as implemented in the software BOTTLENECK 1.2.02 (Piry et al. 1999). The Wilcoxon test is the most appropriate and powerful test when less than 20 loci are used (Piry et al. 1999). The distributions of expected heterozygosities were estimated under three models of microsatellite mutations: the two extreme models of the stepwise mutation model (SMM) and the infinite allele model (IAM), and the intermediate two-phase model (TPM) with the default settings of 30 % mutations under IAM and 70 % under SMM. Second, we used a mode shift test to detect distortion of the L-shape expected under equilibrium for the frequency distribution of allele classes for each of the eight populations (Luikart et al. 1998). In the third method, we calculated the statistical significance of the M statistic in each population using the *M*-ratio (*M*), as implemented in the software M_P_Val (Garza and Williamson 2001). In this test, M is the ratio between the number of alleles at a locus and the total range of allele sizes. M tends to be smaller in recently reduced populations than in equilibrium populations (Mc) because the range in allele size is expected to decrease less than the number of alleles after a reduction in population size (Garza and Williamson 2001). The critical values of M for different pre-bottleneck scenarios (Mc) were calculated using the software CRITICAL_M (Garza and Williamson 2001). We used the default parameters suggested by Garza and Williamson (2001); μ (microsatellite mutation rate) = 5.0×10^{-4}/locus/generation, Δ_g (average repetition frequency of multi-step mutation) = 3.5, and P_g (proportions of multi-step mutations) = 0.22 according to the suggestions of Peery et al. (2012) in order to avoid type I error. For the calculation of θ, which can vary depending on Ne ($\theta = 4Ne\mu$), we used Ne values of 50, 100, 500, and to 1,000 individuals per population, based on direct estimates of the individuals in the pond previous to the *Bd* mortality episode, using the maximal and the minimal number of counted individuals (J. Bosch, personal observations).

Results

Genetic diversity and population structure

We detected a total of 77 alleles in the 10 loci across all sampled demes of *A. obstetricans*, with individual loci ranging from 4 to 19 alleles. A few loci showed significant deviations from Hardy–Weinberg equilibrium after Bonferroni correction in two breeding sites: four loci in population LR (Aobs8, Aobs28, Aobs25 and Aobs17) and one locus in population MV (Aobs 8). Genetic diversity parameters appear in Table 1. Overall Fst value was 0.252 (SE = 0.023). Estimates of pairwise Fst ranged from

Table 2 Population differentiation among the midwife toad localities as measured by pairwise Fst (above diagonal), and geographic distances in Km between pairs of populations (below diagonal)

	LP	VQ	PC	ES	CHS	LR	CN
LP	–	**0.204**	**0.149**	**0.276**	**0.177**	**0.165**	**0.328**
VQ	4.88	–	**0.178**	**0.292**	**0.282**	**0.212**	**0.435**
PC	4.26	0.64	–	**0.197**	**0.180**	**0.187**	**0.282**
ES	1.60	3.60	2.75	–	**0.261**	**0.093**	**0.473**
CHS	1.38	3.54	2.95	0.21	–	**0.208**	**0.410**
LR	1.66	3.26	2.67	0.06	0.22	–	**0.447**
CN	16.86	21.43	20.90	18.17	17.99	18.26	–
MV	6.14	7.55	6.56	6.39	6.33	6.34	20.98

Bold numbers indicate values significantly different from zero ($\alpha < 0.05$)

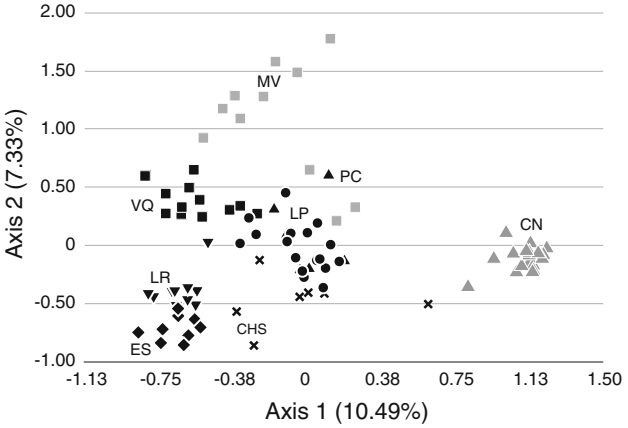

Fig. 2 Two-dimensional graph based on Factorial Component Analysis, illustrating the relationship between *A. obstetricans* individuals in the Guadarrama Mountains. The first two dimensions are shown with the percentage of variation described noted in parentheses. The different symbols correspond to the different study populations

0.093 to 0.447, and all pairs of breeding sites were significantly differentiated ($p < 0.05$) (Table 2). Four of the eight breeding sites showed significant positive F_{IS} values (PC, ES, LR, MV) indicating heterozygote deficits (Table 1).

The AMOVA analysis indicated that 61 % of the molecular variation occurred within breeding sites and 39 % between breeding sites. We found evidence for isolation by distance based on a significant Mantel test between genetic and geographic distances when all populations were considered ($R^2 = 0.3618$, $F_{1,27} = 14.739$, $p = 0.0007$), however significance was lost when we eliminated comparisons involving the most isolated and differentiated site CN ($R^2 = 0.0494$, $F_{1,20} = 0.9879$, $p = 0.3246$). The factorial correspondence analysis corroborated that the breeding site CN was the most distinct from all other breeding sites. This analysis also revealed high genetic differentiation of the individuals in the breeding site MV located on the opposite side of the mountain range (Fig. 2). When K was set to 2 during the clustering analysis performed with STRUCTURE, the CN deme was differentiated from the rest of the demes (data not shown). Applying the ΔK method of Evanno et al. (2005) we consistently obtained estimates of the highest likelihoods for the models with K = 6 across independent runs. Under K = 6, VQ, ES, CHS, and CN were all distinct clusters, while MV and PC were pooled in a single cluster (Fig. 4). LP was distinct from the remaining sites, but had evidence of some recent gene flow from VQ. LR was the most admixed site, being largely similar to ES but with some gene flow from CHS and PC/MV. The remaining three clusters identified by STRUCTURE corresponded to the other three breeding sites included in the analysis (LP, VQ and CHS), with some evidence for recent gene flow from VQ to LP (Fig. 4).

Bottleneck tests

Most breeding sites showed evidence of past population contraction, although no consistent evidence of population bottleneck was found across the three methods used. Results of the analysis with BOTTLENECK depended on the mutation model assumed: the infinite allele model (IAM) was significant for historical reduction in six of the eight populations, the two-phase mutational model (TPM) was only significant for LP and VQ, whereas the stepwise mutational model (SMM) did not show any significant results (Table 3). In addition, the mode shift test supported a population bottleneck in half of the analyzed populations (VQ, PC, CHS and CN). On the other hand, the third method, *M*-ratio was mostly consistent across populations, and indicated population contractions with values of $M < Mc$ for the assumed prebottleneck Ne values tested of 50 and 100. When these Ne values were larger (500 and 1,000) the results did not support a recent population contraction in ES, LR and CN (Table 3).

Discussion

Genetic structure and population contraction

Despite the short geographical distances between most of the ponds (a few kilometers) and the absence of apparent geographic barriers, dispersal between some of the studied breeding sites appeared to be limited. We found strong indication of isolation by distance when the farthest population CN was included in the analysis. Interestingly, that

Table 3 Results (P-values) of the three bottleneck tests. Results are based on ten microsatellite loci for eight populations of *Alytes obstetricans*

BOTTLENECK				Mode-shift	*M-ratio* (Mc)				
Pop	TPM	IAM	SMM		M	$\theta = 0.1$	$\theta = 0.2$	$\theta = 1$	$\theta = 2$
LP	**0.042**	**0.001**	0.347	–	0.644	**0.754**	**0.740**	**0.672**	**0.751**
VQ	**0.042**	**0.003**	0.080	+	0.508	**0.756**	**0.736**	**0.669**	**0.626**
PC	0.246	0.080	0.615	+	0.559	**0.753**	**0.737**	**0.661**	**0.599**
ES	0.179	0.064	0.673	–	0.642	**0.750**	**0.738**	**0.663**	0.618
CHS	0.138	**0.012**	0.278	+	0.461	**0.751**	**0.740**	**0.664**	**0.611**
LR	0.384	**0.012**	0.883	–	0.685	**0.752**	**0.742**	0.671	0.629
CN	0.191	**0.013**	0.472	+	0.688	**0.754**	**0.740**	0.671	**0.751**
MV	0.187	**0.012**	0.577	–	0.574	**0.750**	**0.738**	**0.663**	**0.618**

Analyses with BOTTLENECK used three models microsatellite mutation: two-phase mutation (TPM), infinite alleles (IAM) and stepwise mutation (SMM). For the Mode-shift test, modes are indicated by - for normal L-shaped and + for shifted mode. Observed *M-ratio* (M) and critical ratio (M_c) estimated for each of four values of pre-botleneck θ that correspond to a four values of Ne respectively (50, 100, 500, 1,000)

Bold numbers indicate values significantly different from zero ($\alpha < 0.05$) in the Bottleneck analysis and a population reduction in size when $M < M_c$ in *M-ratio* analysis

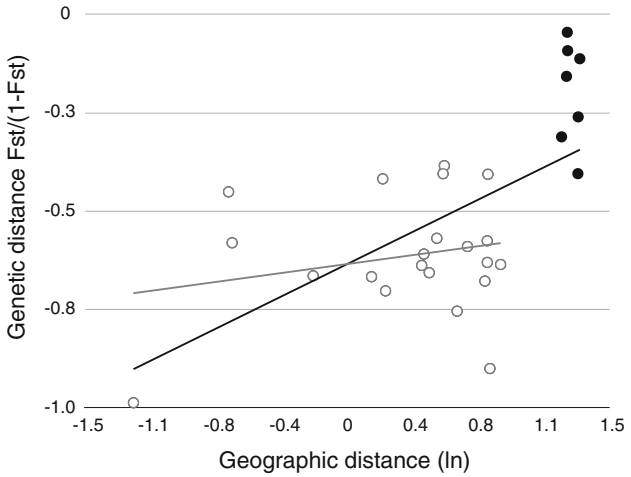

Fig. 3 Isolation by distance analyses representing the relationships between pairwise values of Fst/(1-Fst) and logarithm of geographical distance between sites. The linear curve represents the isolation by distance pattern observed ($p < 0.05$) including the population CN (*black dots*, *black line*) and without CN (*white dots*, *grey line*)

signature disappeared when this population was eliminated from the analysis (Fig. 3). Isolation by distance is an expected equilibrium pattern under limited dispersal and is commonly observed in amphibian metapopulations due to their poor dispersal abilities (Funk et al. 2005; Spear et al. 2005; Wang and Summers 2010). The absence of such a pattern within the focal area might be interpreted as an additional signal of recent local drift eroding the expected equilibrium pattern. Additionally, the Bayesian clustering analysis implemented in STRUCTURE supports the result of isolation by distance.

On the other hand, LR and LP showed some degree of admixture with other populations. Probabilities of population membership indicate the presence of some first generation immigrants and crosses between residents and immigrants, which may indicate sporadic dispersal events. A more striking case of genetic exchange is provided by the single cluster formed by the populations MV and PC, which are separated by 6.56 km (Fig. 4). These results are also supported by low F-statistic differentiation (see Table 2). One possible explanation could be the type of water bodies in this area. The small size, short hydroperiod and shortage of ponds in the basal areas of the mountains where these populations are located, might be inducing the dispersal of individuals in search of better breeding sites (Fig. 1).

In contrast, the general pattern of high genetic differentiation between nearby permanent ponds (as the case of PC and VQ) may indicate that these ponds are high quality habitat and thus individuals remain associated with the pond, that the nature of the surrounding terrestrial habitat limits migration, or, alternatively, that immigrants are less successful breeders than resident toads. Furthermore, small sample sizes could have biased our results and lead to the extreme genetic differentiation of ES and CHS despite their geographic proximity.

Population bottleneck analyses indicated reductions in genetic diversity in some populations under some mutation models and under the range of population sizes tested. In some populations, a bottleneck was inferred under IAM but not SMM, despite SMM being the more realistic mutation model when microsatellites are being used (Di Rienzo et al. 1994). The significance of the heterozygosity excess test is highly dependent on the assumed mutational model, so the results should be interpreted with caution. A small number of markers might not provide adequate resolution under the SMM model; however we used 10 microsatellite loci, which following Luikart and Cornuet (1998) is enough to

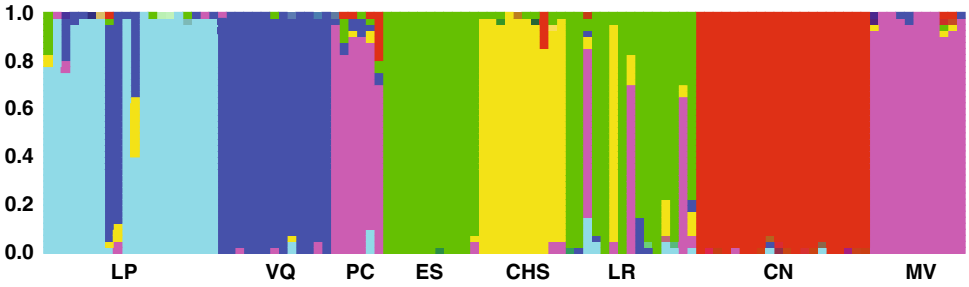

Fig. 4 Bayesian clustering analysis of *A. obstetricans* breeding sites in the Guadarrama Mountains using STRUCTURE. Each *vertical line* represents one individual, and color proportion shows individual membership coefficients. Colors represent different genetic clusters and letters show the breeding sites (see Fig. 1 for full names). The *y*-axis represents individual assignment probabilities

achieve sufficient statistical power. In contrast, the *M-ratio* approach detected a persistent bottleneck signature in all populations analyzed under all values of θ. Despite this finding, the interpretation of *M-ratio* results must be done with caution because we do not have specific information on either the mutation rate (μ) of our microsatellite set or on the average size of non-single step mutations (Δ_g), both of which can affect the *M-ratio* (Garza and Williamson 2001). Furthermore, when we varied the mutation rate (changing θ), the results were largely congruent with the heterozygosity excess test.

Although, the *M-ratio* and BOTTLENECK test measures different properties (in terms of duration and severity) of the bottleneck, the results may not be contradictory. Studies have shown that while *M-ratio* is more likely to identify a bottleneck that occurred in the distant past with severely reduced population size, BOTTLENECK will more frequently detect a weak or moderate bottleneck that occurred recently (Williamson-Natesan 2005). Thus, our results suggest that bottlenecks in the *A. obstetricans* populations occurred in the recent past and were fairly severe, which is consistent with the observed massive decline of the populations due to chytridiomycosis, but does not imply direct causation.

Conclusion and conservation implications

In the case of *A. obstetricans,* 90 % of the populations in the Peñalara Massif disappeared after the emergence of the disease chytridiomycosis (Bosch et al. 2001) and the entire population has remained below 100 individuals ever since. It has been argued that effective population sizes of <100 individuals can negatively affect the fitness and viability of populations (Lande 1998). Recently, congenital defects in the forelimbs and bones of the spine have been observed in captive individuals of midwife toads from Valdesquí (J. Bosch personal observation). These deformities may be early signs of inbreeding depression, a problem which has been identified as one of the most frequent causes of the failure of reintroduction and recovery programs (Spielman et al. 2004a; Frankham 2005). Especially when captive breeding is necessary, the appropriate selection of the source of founder individuals for future reintroductions may determine the success of the program. For these reasons, genetic studies prior to the management of threatened vertebrate populations have increased in number (Seddon et al. 2007), including for the conservation of endangered amphibians (Kraaijeveld-Smit et al. 2005; Vredenburg et al. 2007; Beauclerc et al. 2010). When populations are isolated and there are signs of inbreeding depression, genetic rescue can be achieved through induced migration (Moritz 1999). While this should be preferentially done with populations of the same genetic lineages in order to prevent inbreeding depression, in some cases the mixture of genetic lineages has been used when this first option is not feasible (Godoy et al. 2004; Beauclerc et al. 2010). Many authors have shown that by mixing lineages it is possible to obtain rapid population growth, an increase in heterozygosity, and a quick spread of new alleles (Madsen et al. 2004; Vilà et al. 2003; Johnson et al. 2010). This is especially important when populations are found in stressful environments, for example, when they must deal with a novel pathogen (Spielman et al. 2004b). On these grounds and for future reintroductions we recommend mixing populations using individuals of all remnant populations in the area as founders of the captive breeding program, with the possible exception of CN, a population that is most geographically separated and that showed evidence of lack of recent gene flow with Peñalara Massif. In contrast, as individuals from the higher elevations of the Peñalara Massif (LP, CHS, ES, LR) do share alleles with animals at lower elevations (PC, VQ, MV), we recommend treating all these populations as a single conservation unit. That strategy will help to recover the lost genetic diversity in the wake of the fungus disease and to maximize the short- and long-term viability of midwife toads in this area.

In summary, *A. obstetricans* in Guadarrama mountains showed evidence of a population bottleneck, limited

genetic diversity and strong genetic population structure among breeding sites. This translates into multiple genetic units with non-random mating between sites. Although low dispersal and geographical barriers can produce this pattern we suggest disease-induced population decline may also be a contributing factor. The genetic bottleneck observed and low genetic variability in these small and isolated populations indicates that they are at high probability of local extinction.

In addition, the high genetic subdivision among populations across very short geographic distance suggests low capacity for re-colonization of nearby ponds following local extinction events, and therefore, continued management should include artificial reintroductions.

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