Newts in time and space: the evolutionary history of Triturus newts at different temporal and spatial scales

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CHAPTER 9

NEWTS UNDER SIEGE: RANGE EXPANSION OF *TRITURUS PYGMAEUS* ISOLATES POPULATIONS OF ITS SISTER SPECIES

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Contents

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Abstract

The newt species *Triturus marmoratus* and *T. pygmaeus* are both present in central Portugal where they have parapatric distributions. We used four genetic markers to determine which species was present in 31 populations. In the centre of the study area we found a *T. marmoratus* enclave. Despite small inter-population distances, hybridization is locally rare. We built several models to try to explain this distribution using environmental data. The best model, chosen by Akaike's Information Criterion, relates the presence of *T. marmoratus* with the temperature in July, the relief of the landscape and a higher use of the land for orchards. The current distribution can best be explained by *T. pygmaeus* expanding north and replacing *T. marmoratus*, the latter only persisting where ecological conditions are relatively favourable.

Keywords: Akaike's Information Criterion, Allozymes, Amphibia, Enclaves, Mosaic Hybrid Zones, Parapatry

Introduction

Secondary contact between closely related species often generates spectacular events and provides insight into the evolutionary process. Hybrid zones formed this way are considered 'natural laboratories' or 'windows on evolutionary processes' (e.g. HEWITT, 1988 in Anartia butterflies; HARRISON, 1990). Evolution is not a static process and to understand it, we must look into dynamic systems like these.

The dynamics of species ranges are not independent of ecological conditions, competition with sister species, or dispersal capabilities. Present day distributions are echoes from past events. One particular event that provides clues to the relative movement of species is the formation of enclaves. Enclaves are here defined as populations of one species *completely* surrounded by populations of closely related species and genetically isolated from other populations of the same species (ARNTZEN, 1978; in geographical terms, they are simultaneously exclaves and enclaves). This is reminiscent of the 'internal parapatry' concept of Key (1981) that, however, does not deal with disjunct distributions.

Mosaic hybrid zones are bimodal hybrid zones with few hybrids and predominantly parental genotypes present. The contact between the species is more strongly shaped by ecological constraints than by genetic interactions. In a recent review, Jiggins & Mallet (2000) go one step further and suggest that ecology contributes more to speciation than genetic incompatibility. Well known mosaic hybrid zones are for example those in *Gryllus* crickets (RAND and HARRISON, 1989), *Chorthippus* grasshoppers (BRIDLE *et al.*, 2001), and *Mytilus* mussels (BIERNE *et al.*, 2003). Examples of mosaic hybrid zones in salamanders are *Triturus cristatus* and *T. marmoratus* in western France (ARNTZEN and WALLIS, 1991) and *Plethodon cinereus* and *P. shenandoah* in the Appalachian Mountains of North America (JAEGER, 1970; JAEGER, 1971; see also SITES *et al.*, 2004). Although they present patches of populations of one species distributed among patches of the other, these are not all necessarily enclaves because dispersal among patches may be frequent.

To the best of our knowledge the only enclaves recorded in the literature are those for *Bombina* toads in central Europe (ARNTZEN, 1978) and *Triturus* newts in western France and the northern Balkans (ARNTZEN and WALLIS, 1991; ARNTZEN and WALLIS, 1999). Perhaps enclaves are more likely to arise in organisms with structured populations and low dispersal capability than in organisms that disperse well. Amphibian populations in particular are well-delimited because of their dependence on water for reproduction and they have low individual mobility. Once formed, enclaves will take some time to dissolve, or be stable or disappear by reconnection to the main distribution.

The two species of marbled newts living in the Iberian Peninsula, *Triturus marmoratus* (Latreille, 1800) and *T. pygmaeus* (Wolterstorff, 1908) have a parapatric distribution. Some reports, however, indicated the presence of *T. marmoratus* where only *T. pygmaeus* was expected, near Caldas da Rainha and a spatial-environmental model for the two species suggests that the local conditions may indeed be favourable to *T. marmoratus* (see the southernmost record in Fig. 1c in ARNTZEN, 2006)). This would indicate an area of sympatry or a mosaic distribution. A mosaic distribution would point to differential ecological requirements with patches where the environmental conditions are more suitable for one species than for the other.

An intuitive explanation from fieldwork across the Iberian Peninsula is that *T. pygmaeus* thrives in ephemeral ponds with a fluctuating reproductive output and that *T. marmoratus* thrives in smaller, more permanent water bodies (e.g., springs) with regular but low annual recruitment reaching metamorphosis (J. W. Arntzen, unpublished). To confirm the record at Caldas da Rainha and to learn more about the events that lead to this occurrence and its extent, we conducted a detailed study. Adult marbled newts have clearly distinguishable morphologies while embryos and larvae are difficult to identify. Conversely, embryos and larvae are easy to find at the aftermath of the reproductive season while adults may be elusive. To facilitate a fast, detailed and reliable surveying we employed genetic markers for species identification (CHAPTER 7).

In the present paper, we look into the spatial structure of the distribution of the two species of marbled newts and determine if and what ecological constraints are shaping it. We also analyze the presence of hybrids in our sample.

Material and Methods

Sampling

In April 2003, we searched around Caldas da Rainha, in central Portugal, for water bodies containing marbled newts. The region is characterized by flat dunes on the coast and an undulating agricultural land, with an abundance of orchards in the centre. We located and sampled 31 populations of *Triturus marmoratus* and *T. pygmaeus*, over 1600 km^2. Breeding female newts obtain one or several spermatophores from which eggs are internally fertilized and then deposited one by one, mostly on aquatic vegetation. The process takes place over a ca. two month period.

To increase random collection and to reduce pseudoreplication we screened the entire accessible area of each pond and collected no more than one egg per leaf or two eggs per plant. In marbled newts adult population size can be very small, especially in small water bodies such as springs (SCHOORL and ZUIDERWIJK, 1980; JEHLE *et al.*, 2001; 2005) and this explains why sample size is small on some occasions (N<5 in four populations). Adults and larvae were captured by dip-netting. Tail tips were collected from adults and larvae were sacrificed. All samples were immediately stored in liquid nitrogen and later transferred to -80º C until the day they were analysed.

Genetic Data

All the tissue samples (from n≥10 individuals for most locales) were analyzed for four allozyme loci: peptidase A (*Pep-A*), peptidase B (*Pep-B*), peptidase D (*Pep-D*) and lactate dehydrogenase (*Ldh-2*; this locus is not yet expressed in embryos), that yield a species specific enzyme profile using standard starch gel electrophoresis and isoelectric focusing. The genetic signature is consistent with morphological identification of adult marbled newts (CHAPTER 7).

With the program ARLEQUIN (Version 3.1; EXCOFFIER *et al.*, 2005), we tested for departures from Hardy-Weinberg expectations and linkage disequilibrium. We used the program FSTAT (v. 2.9.3.2; GOUDET, 1995) to calculate F-statistics and, to detect population sub-structuring, we analysed the results with STRUCTURE 2.1 (PRITCHARD *et al.*, 2000). Using a Markov chain Monte Carlo (MCMC) algorithm, STRUCTURE assigns individuals to a population, or jointly to two or more populations, if their genotypes indicate that they are admixed. This is done assuming a model with K populations (where K may be unknown), where each is characterized by a set of alleles for each locus. Individuals are assigned to populations as to maximize Hardy-Weinberg and linkage equilibrium. We choose for the 'admixture model' because neighbouring populations may interbreed and varied K from 1 to 5 with 10 000 generations as the length of burn-in period and 100 000 MCMC generations after burn-in. Convergence occurred before 10 000 in test runs. The value of λ was inferred by the program. We accepted K as indicating the true number of genetic partitions when the difference in likelihood between two consecutive values of K was maximal.

We investigated the existence of hybrids in our sample by performing a Bayesian model-based clustering as implemented in the software NEWHYBRIDS (ANDERSON and THOMPSON, 2002). This computes the posterior probability that each individual belongs to each of six predetermined classes (pure species A, pure species B, F_1-hybrid, F_2-hybrid, backcross to species A and backcross to species B).

Ecological Data

For the spatial environmental analysis in a Geographical Information System (GIS), we selected 21 ecological parameters following Teixeira *et al.* (2001) and an additional explanatory variable (land surface occupied by orchards, arcsin transformed percentages – ORCH) that appeared locally informative. We used ORCH as a proxy to one or more unidentified variables that - possibly more directly than ORCH - would help to explain the newt distribution. The advantage of the parameter ORCH is that it manifests itself from field observations and that blanket data are readily available (INE, 1999).

For all variables, information was available in digital format for Portugal (DGA, 1995). A vegetation map (normalised difference vegetation index or NDVI) was obtained courtesy of the Royal Dutch Meteorological Institute (KNMI). An altitude map was taken from the internet (http://edcwww.cr.usgs.gov/doc/edchome/ datasets/edcdata.html) and used to produce a relief map by a set of filter operations (ITC, 1997). Maps on the mean January and July temperature were digitalised from the Portuguese climate atlas (SCN, 1974).

A hierarchical clustering based on Spearman's correlation coefficient was used to evaluate the level at which ecological information appeared redundant.

One variable was selected arbitrarily out of a set of variables that correlated at Spearman's $r_s > 0.8$. Sixteen variables were retained: acidity of the soil (ACID), altitude , chlorates content of subterranean water (CHLO), frost days (FROD), frost months , hardness of subterranean water (HARD), humidity of the air (HUMI), insolation (INSO), lithology (LITH), vegetation index (NDVI), orchard land coverage , mean annual precipitation (PRET), relief (RELI), the sulphate content of subterranean water (SULP), mean annual temperature (TEMP) and mean July temperature (TJUL). To increase the comparability of their effects, all continuous variables were standardized to an average of zero and a standard deviation of one. The variables were introduced into the GIS analytical software as raster layers with 1 km spatial resolution. Values for 10*10 km UTM grids were obtained by averaging the data (modal values for the categorical variable LITH).

Model selection

We used an information-theoretic model selection approach for the statistical analysis of the data (BURNHAM and ANDERSON, 2002). Using our field knowledge, we built several *a priori* models that would explain the current distribution of the two species. By contrasting the presence of one species against the other, we circumvented the inclusion of false absence data. To understand the effect of the variable ORCH, we used our available data on fruit growing in the region as dependent variable, and applied a Stepwise Multiple Regression (SMR) with the same environmental data (all but ORCH) as explanatory variables, using the software SPSS v14 (SPSS, Inc., 2005). We then substituted ORCH by the model derived from this analysis in our *a priori* models, and added them to the list as *a posteriori* models. In a second step, we used the small-sample Akaike's Information Criterion (AICc; BURNHAM and ANDERSON, 2002) to rank the models and chose the best one. AICc is defined as:

$$AIC_c = -2\ln L + 2K + \frac{2K(K+1)}{n-K-1}$$

where ln L is the natural logarithm of the likelihood function, K is the number of parameters from the model, and n is the sample size. Akaike's Information Criterion prevents overfitting the model, by taking the number of parameters into consideration. To fit the models to the data and obtain the log-likelihoods of the models, we used the logistic regression procedure available in SPSS.

Results

We analyzed 398 individuals (101 adults, 50 larvae and 247 embryos; see allele frequencies in supplementary material – Table S1).

F-statistics show a high degree of population substructuring (Fst) in *Pep-A* (0.84) and *Pep-D* (0.66), intermediate in *Ldh-2* (0.41) and low in *Pep-B* (0.14). The exact test on Hardy-Weinberg expectations showed that population 4 presents a significant departure at one locus (*Pep-D*) under Bonferroni correction. Linkage disequilibrium test are significant at $P<0.05$ in two *T. marmoratus* populations that are neighbouring *T. pygmaeus* populations (population 23 and 31; see Figure 1 and Table S2 in supplementary information).

The model-based clustering method implemented in STRUCTURE 2.1 showed that the most likely number of partitions for the present data is K=2 (Table 1; for details see table S2 in supplementary material). The partitions correspond to *T. marmoratus* and *T. pygmaeus*. Since the likelihood continues to increase with K, we looked into the population structure when K=3, to examine the possibility that additional sub-structuring existed. The only result was that one of the clusters split into two equal parts. Fst values are consistent with the relative diagnostic power of the *loci* (high for *Pep-A* and *Pep-D*, intermediate for *Ldh-2* and low for *Pep-B*). Accurate species identification is independent of the number of loci studied (Figure 1).

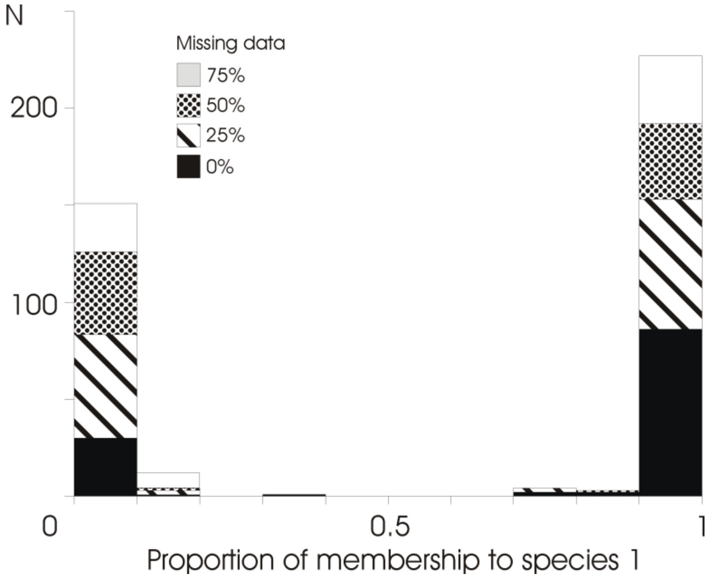

Figure 1 Proportion of membership of individuals to species 1 (= *Triturus pygmaeus*) using the software Structure 2.1 (Pritchard *et al*., 2000). Data are separated out for samples with 0, 25, 50 and 75% missing data to show that, in practical terms, discriminatory power is independent of the *loci* used.

K	logL	SD
1	-1737,7	5,3
2	-961,9	0,6
3	-887,5	1,4
4	-849,4	30,7
5	-802,2	6,9

Table 1 – Log-likelihood (logL) and standard deviation (SD) of the number of partitions (K) in our data set. K varied from 1 to 5. The increase in likelihood is not significant other than at K=2.

The spatial distribution of the two groups indicates the existence of a set of *T. marmoratus* populations surrounded by *T. pygmaeus* populations, forming an enclave in the centre of our study area (Figure 2; note that the isolated pocket is - in proper terms - an exclave of *T. marmoratus* and an enclave of *T. pygmaeus*). Only population 18 had individuals of both species.

The Bayesian-based assignment of individuals to hybrid classes using NEWHYBRIDS indicates that hybridization, backcrossing and introgression are locally rare Thirty out of 31 populations were classified as either *T. marmoratus* or *T. pygmaeus* and not both. Population 18 contained both species as well as one individual with about equal probability of being a pure *T. marmoratus* (p=0.53) as having mixed species parentage (p=0.47, being either a F2-hybrid (P=0.35) or a backcross hybrid in direction of *T. marmoratus* (p=0.12)). This mixed population (population in grey in figure 2) was excluded from the spatial-environmental analysis.

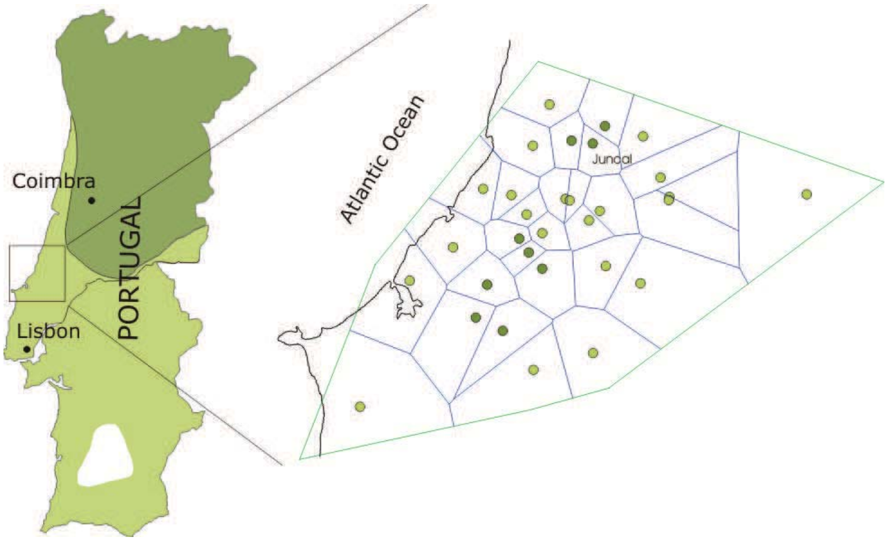

Figure 2 Distribution of marbled newts in Portugal with *Triturus marmoratus* (hatched) and *Triturus pygmaeus* (shaded). The box shows the research area around Caldas da Rainha (a). The detailed map (b) shows the localities with *T. marmoratus* (solid dots) and *T. pygmaeus* (open dots). See Table 1 for population details. Voronoi polygons are used to estimate the contiguous species distribution over the area. Note that the scale of extrapolation at the exterior of the study area is set at *c*. 6 km as to match the level of interpolation.

Table 2 List of *a priori* and *a posteriori* models used to determine the relationship between the distribution of *Triturus marmoratus* and *Triturus pygmaeus* in the Caldas da Rainha area, Portugal. The parameter values were obtained by logistic regression analysis of presence data in the area (by contrasting the presence of one species with the presence of the other). The log likelihood of the models (–2 ln *L*), number of parameters in each model (*K*), and the number of data points (*n*) were used to calculate the small-sample Akaike Information Criterion (AICc). The hypothesis behind each model is also listed. Lowest AICc value is highlighted in bold.

Model	-2 ln L	K	n	AICc	Hypothesis	Model equation												
						ORCH	RELI	TJUL	FROD	ALTI	PRET	HARD	HUMI	NDVI	TEMP	SULP	ACID	Constant
a priori																		
1	25.50	1	30	53.14	Extent of orchard plantations	2.85												-1.23
2	36.87	1	30	75.88	Relief		-1.04											-0.12
3	40.24	1	30	82.62	July temperature			-0.42										0.05
4	41.23	1	30	84.60	Number of frost days				-0.04									0.08
5	24.85	2	30	54.15	Combination of model 1 and model 2	2.57	-0.61											-1.24
6	24.02	3	30	54.96	Model 5 plus altitude	2.78	-0.33			-0.83								-1.49
7	24.74	3	30	56.40	Model 5 plus precipitation	2.50	-0.56				-0.22							-1.21
8	24.77	3	30	56.47	Combination of model 4 and model 5	2.62	-0.57		-0.18									-1.25
9	20.72	3	30	**48.36**	Combination of model 3 and model 5	5.10	-0.40	-2.10										-3.30
10	28.52	6	30	72.70	from Arntzen (unpublished)		-0.98	-0.44				0.16	-1.81	-0.87	-1.88			-0.68
a posteriori																		
11	28.73	5	30	69.97	SMR model					-0.92			-3.07		-2.88	0.79	-0.38	-0.72
12	27.06	6	30	69.76	Model 5 with ORCH replaced by SMR model		-1.06			-0.70			-3.18		-2.22	0.90	-0.75	-0.77
13	26.12	7	30	71.32	Model 7 with ORCH replaced by SMR model		-0.74			0.00	-1.63		-3.69		-2.70	0.24	-0.48	-0.90
14	26.80	7	30	72.70	Model 8 with ORCH replaced by SMR model		-1.14		0.42	-0.92			-3.19		-2.14	1.02	-0.84	-0.83
15	22.82	7	30	64.75	Model 9 with ORCH replaced by SMR model		-2.17	-2.82					-4.53		-2.07	2.24	-0.31	-2.01

SMR, stepwise multiple linear regression; ORCH, orchard land coverage; RELI, relief; TJUL, mean July temperature; FROD, frost days; ALTI, altitude; PRET, mean annual precipitation; HARD, hardness of subterranean water; HUMI, humidity of the air; NDVI, vegetation index; TEMP, mean annual temperature; SULP, sulphate content of subterranean water; ACID, acidity of the soil (details in Teixeira *et al*. 2001 and Arntzen, 2006).

GIS-model 9 (ORCH + TJUL + RELI; see table 3) showed the lowest AICc value. This model explains 50% of the total variance observed. The presence of *T. marmoratus* relates to the higher abundance of orchards, lower July temperature and smoother relief than for *T. pygmaeus*. In the subsequent analysis, five environmental variables: ALTI, ACID, HUMI, SULP and TEMP explained the variable ORCH. This model explained 69% of the variance of ORCH. When, however, ORCH was replaced by this set of variables, the models did not perform as well (Table 2).

Discussion

One of us (JWA) first described the existence of *T. marmoratus* in the research area through a sporadic observation in March 1998. Our results confirm the presence of *T. marmoratus* in six populations outside its documented range in a pocket near Caldas da Rainha. This pocket of *T. marmoratus* is fully surrounded by populations of *T. pygmaeus*. Considering that i) the distance of ca. 10 km that separates it from the main *T. marmoratus* distribution exceeds the dispersal capability of large bodied newts (ARNTZEN and WALLIS, 1991; THIESMEIER and KUPFER, 2000) and ii) that the species are locally strongly parapatric, we conclude that the pocket equals to an enclave.

The variable ORCH has a stronger effect than its fellow explanatory variables in models 5-9 (Table 3). It would be inappropriate though to extrapolate any model with ORCH over wider areas because in Portugal extensive fruit growing is particular to the Caldas da Rainha region.

In the habitat preference model, we assume that strongly preferred habitat is of high quality for the species (RAILSBACK *et al.*, 2003). So, if a model has a good fit, it is usually assumed that the species/habitat system is in equilibrium and that the species distribution will only change if the environment around also changes. This ignores, however, effects of life history and dispersal. Areas may be suitable but out-of-reach, due for example to unsuitable habitat in between realized and prospective ranges.

The current distribution of marbled newts in central Portugal is best explained by *T. pygmaeus* moving north from its previous range and superseding *T. marmoratus* that only persisted in areas with ecological conditions more suitable for the species. Because *T. marmoratus* and *T. pygmaeus* occasionally hybridize, this scenario can in principle be tested by looking for *T. marmoratus* 'genetic footprints' in areas that are hypothesized to have been inhabited by *T. marmoratus* in the past (ARNTZEN and WALLIS, 1991). Similarly, we predict the absence of *T. pygmaeus* 'genetic footprints' inside the enclave.

There are several accounts of moving hybrid zones in, for example, butterflies (MALLET *et al.*, 1990; BLUM, 2002; DASMAHAPATRA *et al.*, 2002), birds (PEARSON, 2000; ROHWER *et al.*, 2001), crayfish (PERRY *et al.*, 2001), lizards (HILLIS and SIMMONS, 1986) and amphibians (ARNTZEN, 1978; ARNTZEN and WALLIS, 1991); see also Barton & Hewitt (1985; 116-119). Most studies provide direct evidence of hybrid zone movement through the tracing of genetically interacting species distributions over time. Our study utilizes a single temporal window and provides compelling evidence for spatial change in a mosaic hybrid zone nevertheless, through the demonstration of an enclave.

The distance between *T. marmoratus* in the enclave and the main distribution is minimally 6 km (the distance between populations 13 and 15) and maximally 15 km (the distance between populations 22 and 23). Although we cannot pertinently exclude the presence of long distance dispersal, such a scenario is unlikely given the absence of *T. marmoratus* or genetically mixed individuals in populations 13 and 15. Similarly, we cannot exclude the possibility that a human introduction is responsible for the enclave. There is, however, no tradition of newt husbandry in Portugal, and a deliberate or accidental release is improbable.

To infer the direction of the movement in a hybrid zone, it is equally possible to follow a direct or an indirect strategy. Direct demonstrations employ two or more temporally separated observations, either on position (e.g. HILLIS and SIMMONS, 1986 in *Pholidobolus* lizards) or shape of the cline that separates in this case connects the hybridizing species (e.g. DASMAHAPATRA *et al.*, 2002 in *Anartia* butterflies). An indirect way is to look at disequilibrium measures. Cruzan (2005) showed in the wide *Piriqueta coaroliniana/viridis* (flowering plants from the family Turneracea) hybrid zone that *P. viridis* alleles were moving north. This was done by showing that the southern border of the hybrid zone presented relatively high levels of disequilibria, indicating recent gene flow from parental populations south of the hybrid zone. Interestingly, we observed significant levels of linkage disequilibrium in two *T. marmoratus* populations (populations 23 and 31) that are both within the dispersal range of *T. pygmaeus* populations. This strengthens the argument that *T. pygmaeus* advances into *T. marmoratus* territory, even though we were unable to demonstrate current *T. pygmaeus* presence.

As a further test to our explanation, we predict that the presence of alien *T. marmoratus* genes in *T. pygmaeus* exceeds that of the reverse condition, in a spatial pattern consistent with enclave formation.

Despite the range expansion of *T. pygmaeus* in its northern border, the situation in the south of Spain and Portugal is different. Due to desertification and an intensification of agricultural practices, *T. pygmaeus* is losing many breeding sites and has been classified as 'near threatened' (ARNTZEN *et al.*, 2006). This is consistent with recent evidence that suggests that climate warming will not only increase the northern range of species but also decrease the southern one (THOMAS *et al.*, 2006).

If *T. pygmaeus* continues its competitive advance, the *T. marmoratus* enclave would eventually disappear. As yet, we have no indication on the speed of the process. Monitoring the area would provide an additional test to the hypothesis of *T. pygmaeus* expansion and document the speed of the process. It is remarkable that *T. marmoratus* is not only losing out to its sister-species at the southern edge of its range, but also to the related species *T. cristatus* at its northern edge. The advance of *T. cristatus* at the expense of *T. marmoratus* was estimated to occur at a speed of ca. one km per year (ARNTZEN and WALLIS, 1991). If range replacement would proceed at this speed at either side of its range *T. marmoratus* would be squeezed out in ca. 500 years.

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Appendix S1. Allele frequencies in XLS format. Allele frequencies over four loci in marbled newts from the Caldas da Rainha area, Portugal. Analysis of the results indicates the existence of two separate genetic units that correspond to *T. pygmaeus* (22 populations) and *T. marmoratus* (nine populations). Pond numbering and alleles indication are as in Espregueira Themudo & Arntzen (2007). N is sample number; zero sample size refers to missing data.

	Triturus pygmaeus																						*Triturus marmoratus*								
Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	26	27	28	29	30	18	19	20	21	22	23	24	25	31
Adults	0	20	20	22	0	6	0	1	1	1	8	10	1	0	0	0	0	0	0	1	0	0	7	0	1	1	0	2	0	0	0
Larvae	5	0	0	0	6	0	0	0	0	0	0	0	1	0	0	3	0	0	0	0	0	0	9	0	1	23	0	2	0	0	0
Eggs	10	0	5	0	0	8	11	10	0	13	0	0	8	2	10	0	11	5	10	10	10	2	0	21	14	0	14	13	18	20	21
Total	15	20	25	22	6	14	11	11	1	14	8	10	10	2	10	3	11	5	10	11	10	2	16	21	16	24	14	17	18	20	21
Locus and alleles																															
Pep-A																															
N	*13*	*19*	*12*	*22*	*6*	*10*	*10*	*10*	*1*	*10*	*7*	*10*	*9*	*2*	*10*	*3*	*10*	*0*	*10*	*10*	*10*	*2*	*9*	*10*	*10*	*22*	*10*	*10*	*11*	*10*	*10*
a																							0,56	0,85	0,85	0,91	0,95	0,95	1,00	1,00	0,60
b																											0,05	0,05			
d	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00		1,00	1,00	1,00	1,00	0,44	0,15	0,15	0,05					0,40
e																										0,05					
Pep-B																															
N	*15*	*20*	*25*	*22*	*6*	*13*	*10*	*10*	*1*	*11*	*8*	*10*	*10*	*2*	*8*	*3*	*4*	*5*	*0*	*2*	*0*	*1*	*16*	*20*	*14*	*24*	*14*	*14*	*11*	*11*	*9*
b		0,13	0,24	0,07	0,08	0,23					0,06	0,15				0,17							0,09	0,03		0,06					0,06
d	0,37	0,78	0,54	0,66	0,92	0,69	0,80	1,00	1,00	0,95	0,88	0,60	0,95	1,00	0,81	0,83	1,00	0,70		1,00		1,00	0,84	0,95	0,96	0,90	1,00	0,96	0,91	0,77	0,89
e	0,63	0,10	0,22	0,27		0,08	0,20	0,00	0,00	0,05	0,06	0,25	0,05		0,19			0,30					0,06	0,03	0,04	0,04		0,04	0,09	0,23	0,06
Pep-D																															
N	*15*	*20*	*24*	*22*	*6*	*12*	*9*	*10*	*1*	*12*	*7*	*10*	*8*	*2*	*8*	*3*	*11*	*5*	*0*	*1*	*2*	*1*	*16*	*20*	*15*	*23*	*14*	*15*	*11*	*20*	*11*
f	0,97	0,85	0,69	0,70	0,92	0,71	0,94	0,50	1,00	0,33	1,00	1,00	0,94	1,00	0,81	0,83	1,00	0,90		1,00	1,00	1,00	0,13					0,03			
d			0,02		0,08										0,06	0,17							0,63	1,00	1,00	0,83	1,00	0,87	1,00	1,00	1,00
e		0,10	0,02	0,18		0,17				0,67																					
g	0,03	0,05		0,11			0,06						0,06		0,13			0,10													
c			0,02					0,50															0,03					0,03			
a																							0,09			0,17		0,07			
b																							0,13								
h			0,25			0,13																									
Ldh-2																															
N	*5*	*20*	*20*	*22*	*6*	*6*	*0*	*0*	*0*	*1*	*8*	*10*	*2*	*0*	*0*	*3*	*0*	*0*	*0*	*1*	*0*	*0*	*14*	*0*	*1*	*23*	*0*	*3*	*0*	*0*	*0*
c			0,03																												
b	1,00	1,00	0,98	1,00	1,00	1,00				1,00	1,00	1,00	1,00			0,83				1,00			0,46		0,50	0,43		0,67			
f																0,17							0,54		0,50	0,57					
e																												0,33			

Appendix S2

Clustering assignment of 31 sample sites in XLS format. Assignment of 31 populations of marbled newts (*Tritrus marmoratus* and *T. pygmaeus*) to two clusters in STRUCTURE 2.1 . Columns represent sample sites localities, Universal Transverse Mercator coordinates (UTM_X and UTM_Y), sample number (N) and the probability to belong to cluster 1 (that corresponds to *T. marmoratus*).

Code	Sample site	UTM_X (km)	UTM_Y (km)	N	Inferred cluster 1
1	Porto de Mós	514.0	4384.3	15	0.009
2	Rio Maior	507.3	4355.2	20	0.008
3	Alqueidão	536.0	4376.6	25	0.020
4	Valado dos Frades	499.2	4383.1	22	0.007
5	São Bartolomeu dos Galegos	476.0	4348.0	6	0.044
6	Mosteiro de Alcanene	513.7	4364.5	14	0.014
7	Foz do Arelho	482.6	4365.0	11	0.016
8	Casais dos Morgados	488.6	4369.5	11	0.015
9	Carrascal	503.7	4375.9	1	0.010
10	Molianos	506.8	4373.0	14	0.016
11	Covas	517.5	4376.1	8	0.020
12	Casais Monizes	509.0	4367.0	10	0.007
13	Carrascal II	503.8	4375.8	10	0.020
14	Cela	496.3	4376.6	2	0.011
15	Genrinhas	498.4	4373.9	10	0.037
16	Sta Susana	499.3	4353.0	3	0.119
17	Pataias Gare	501.5	4388.6	11	0.011
18	Famalicão da Nazaré	492.5	4377.3	5	0.018
19	Chão	516.4	4378.8	10	0.032
20	Covão da Fonte	517.6	4376.3	11	0.031
21	Molianos II	508.2	4374.4	10	0.029
22	Ribeira da Maceira	500.4	4371.4	2	0.027
23	Juncal	507.3	4383.4	16	0.778
24	Casal da Charneca	491.5	4360.0	21	0.984
25	Andam	508.9	4385.8	16	0.974
26	Salir de Matos	493.0	4364.4	24	0.987
27	Cós	504.4	4383.8	14	0.990
28	Fonte da Pena da Couvinha	497.5	4370.6	17	0.971
29	Vidais	495.2	4358.3	18	0.984
30	Casal da Coita	500.5	4366.5	20	0.986
31	Vimeiro	498.6	4368.8	21	0.934

Sample	Probability Inferred cluster 1
J1	0.035
J2	0.036
J3	0.226
J4	0.631
J5	0.870
J6	0.938
J7	0.941
J8	0.942
J9	0.955
J10	0.974
J11	0.974
J12	0.977
J13	0.987
J14	0.987
J15	0.987
J16	0.992