Morphological and molecular characters to describe a marbled newt hybrid zone in the Iberian peninsula

Jan W. Arntzen^{1,2}

¹ *Naturalis Biodiversity Center, P.O. Box 9517, 2300 RA, Leiden, The Netherlands*

² *E-mail: pim.arntzen@naturalis.nl*

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Abstract

I document the contiguous distribution of the marbled newt species *Triturus marmoratus* and *T. pygmaeus* over the western part of the Iberian peninsula with a suite of morphological and molecular genetic data from altogether 141 populations. Morphological characters that identify the species are body size and the colour pattern character 'Links'. Links is the number of transversal connections following the green surface at the lateral sides of the newts' body. Large adults with few Links are *T. marmoratus* and small adults with many Links are *T. pygmaeus*. However, no morphological identification criterion is entirely adequate. Eight molecular genetic markers show markedly bimodal character state distributions that give rise to sharp species range descriptions, with *T. marmoratus* in the north of the Iberian peninsula and *T. pygmaeus* in the south and along most of the Atlantic coast of Portugal. I encountered ten genetically admixed populations that are all located at the *T. marmoratus* - *T. pygmaeus* species range interface, suggesting widespread but limited interspecific hybridization. A latitudinal transect across Portugal confirmed the narrow and steep transition from one to the other species for morphological and molecular characters alike. In central Portugal the position of the hybrid zone coincides with the river Tejo. However, the cline for mitochondrial DNA is relatively wide and shallow and its centre is positioned south of the river. In view of published data that reconstruct the northward advance of *T. pygmaeus* along the Portuguese coast at the expense of *T. marmoratus*, I propose that *T. marmoratus* had a wider range in central Portugal too, where it was eventually superseded by *T. pygmaeus*. I hypothesize that 'marmoratus' mtDNA haplotypes found south of the Tejo constitute a 'genetic footprint' left behind in *T. pygmaeus* by the receding species *T. marmoratus*.

Introduction

Hybrid zones figure prominently in evolutionary research. They are seen as 'windows on evolutionary process' (Harrison, 1990) and 'natural laboratories' (Hewitt, 1988). One particular asset of hybrid zones is the opportunity they provide to evaluate the performance of new, previously untested genetic combinations of differentiated genomes in nature. Possible outcomes range from the (near-)complete merger of previously allopatric lineages to a (near-)complete genetic isolation. Debated aspects are the modes by which post-zygotic isolation accumulates and how much gene flow can be tolerated before the speciation process goes in reverse. A good group to investigate these research questions would be monophyletic, species-rich, show contiguous species ranges where many closely as well as distantly related species engage in long hybrid zones. One group that qualifies is the European newt genus *Triturus*. The species group inner distribution ranges form a patchwork and most species are involved in intra-generic hybridization (Wielstra *et al.*, 2014). The analyses over nine hybrid zones support the idea that in *Triturus* post-zygotic hybrid incompatibilities accumulate gradually (Arntzen *et al.*, 2014). Results may, however, be dependent on local conditions. Moreover, for one species combination - that of the northern marbled newt *Triturus marmoratus* (Latreille, 1800) and the pygmy marbled newt *Triturus pygmaeus* (Wolterstorff, 1905) - the studied transect near Madrid, Spain may be allotopic and lack hybridization altogether. I here use morphological and molecular characters to describe the marbled newt species contact across Portugal and into Spain. I show that *T. marmoratus* and *T. pygmaeus* engage in limited but widespread hybridization, not unlike crested newts in the same genus at approximately the same level of divergence (Arntzen *et al.*, 2014). The approximately

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Figure 1. Ventral colouration of marbled newts with at the top row *Triturus marmoratus*, from left to right, male from Mayenne (western France), female from Mayenne, male from Barcelona (north-eastern Spain) and female from Barcelona. Bottom row - *T. pygmaeus* with male from Madrid (central Spain), female from Madrid, male from Cadiz (southern Spain) and female from Cadiz. Scale bars represent 10 mm. Colour morphology scores {Bc, WSs, WSn, BSs, BSn} given to the specimens are, in reading order {4,4,3,3,3}, {5,1,2,1,1}, {3,3,3,2,2}, {4,2,3,1,1}, {2,5,4,4,3}, {1,2,1,1,1}, {2,2,3,4,2} and {1,2,3,3,2}. Ambiguities arise for example because absent spots have no size, when the white or black spots obscure the background colouration, when white spots aggregate as to highlight dark spots, etc. Note that I use the word 'colour' even though a black and white image captures nearly all available information.

600 km long hybrid zone provides ample opportunities for further evolutionary research.

Material and methods

The morphological characters measured in marbled newts are SVL - snout-vent length up to and including the cloaca, HL - head length, Hw - head width, ILd - interlimb distance, FLl - fore limb length, TFl - third

finger length, HLl - hind limb length and FTl - fourth toe length. Measurements were taken in mm with a plastic ruler (SVl, ILd) and with Vernier calipers at 0.1 mm precision (the other characters). Ventral side colouration characters are Bc - belly colour, WSn and WSs - number and size of white spots, and BSn and BSs - number and size of black spots. These characters were scored at a 1-4 or 1-5 scale, from off-white to black (Bc), from absent or few to many (WSn and BSn) and from absent or tiny to large (WSs and BSs). To guide

Table 1. Studied populations of marbled newts (*Triturus marmoratus* and *T. pygmaeus*) in France, Spain and Portugal, with sample sizes and results of morphological analyses. Populations marked by a hash (#) have molecular data and are listed in Tables 2 and 3. Populations are labelled after the pond they were found, with pond numbers 2-436.

Pond cq. population number	Locality	Country	Longitude	Latitude	Sample size morphology	Mean discriminant score	Number of newts with toe abnormalities
2 #	Rio Maior	Portugal	-8.921	39.341	65	0.705	
3 #	Alqueidão	Portugal	-8.580	39.530	20	0.713	
4 #	Gavião 1	Portugal	-7.939	39.477	32	0.941	
5 #	Nisa 1	Portugal	-7.604	39.533	4	0.996	
6	Sobral do Campo	Portugal	-7.549	39.998	1	0.990	
7 #	Pedra do Altar	Portugal	-7.821	39.711	56	0.030	
9	São Domingos 1	Portugal	-8.174	39.603	22	0.060	
10	Montalvo	Portugal	-8.300	39.481	15	0.687	
11	Alfena	Portugal	-8.525	41.238	1	0.001	
12 #	Saide	Portugal	-8.329	40.449	49	0.018	
13	Vimeiro	Portugal	-9.019	39.468	1	0.631	
14	Martinchel 1	Portugal	-8.298	39.538	4	0.012	
15	Andreus 1	Portugal	-8.170	39.564	1	0.004	
16	Andreus 2	Portugal	-8.170	39.564	5	0.064	
19 #	Domingos da Vinha	Portugal	-7.947	39.523	30	0.322	
24	Gois	Portugal	-8.111	40.160	1	0.005	
25	Envendos	Portugal	-7.883	39.582	4	0.059	
26 #	Lagoa Seca	Portugal	-9.012	39.596	43	0.598	
28 #	Serra de St Antonio	Portugal	-8.731	39.516	7	0.437	
29 #	Mitra 1	Portugal	-8.001	38.535	23	0.992	
30 #	Mora	Portugal	-8.138	38.955	13	0.997	
31	Mindelo	Portugal	-8.727	41.317	2	0.482	
32 #	Gavião 2	Portugal	-7.932	39.459	22	0.974	
33	Cadafaz	Portugal	-7.943	39.470	6	0.980	
34	Martinchel 2	Portugal	-8.291	39.540	4	0.151	
38 #	Jublains	France	-0.472	48.253	40	0.052	
39 #	Dunes SW of Mira	Portugal	-8.785	40.389			
42 #	Casas de Ribeira	Portugal	-7.979	39.573	7	0.008	
46	dunes SW of Mira	Portugal	-8.807	40.324	4	0.987	
48	San Jose de Matos	Portugal	-7.845	39.566	2	0.958	
51 #	Gerês - Carris	Portugal	-8.046	41.815	71	0.024	
52	Zimbreirinha	Portugal	-7.873	39.536	3	0.966	
53	Vale Pedro Dias 2	Portugal	-7.878	39.533	9	0.151	
55 #	Areia 2	Portugal	-7.932	39.514	50	0.132	
56	Vila Flor	Portugal	-7.781	39.518	22	0.970	
57	Outeiro Cimeiro	Portugal	-7.901	39.496	28	0.042	
58 #	Areia 3	Portugal	-7.934	39.514	11	0.137	
60	Maxial	Portugal	-7.966	39.563	1	0.001	
62 #	Coimbra	Portugal	-8.404	40.203	10	0.040	
64 #	Degracias - Casas de S. Jorge	Portugal	-8.521	40.007			
65 #	Vila do Bispo - Sagres	Portugal	-8.904	37.072	31	0.964	
66 #	Nisa 2	Portugal	-7.593	39.550	17	0.962	
67 #	Rosmaninhal	Portugal	-7.091	39.732	40	0.976	
68	Monforte da Beira	Portugal	-7.300	39.738	1	0.841	
69 #	Soure	Portugal	-8.639	40.032			
70 #	Monte do Conde	Portugal	-7.250	40.167	12	0.015	
72 #	João Pires	Portugal	-7.154	40.106	17	0.024	

Table 1 continued

Pond cq. population number	Locality	Country	Longitude	Latitude	Sample size morphology	Mean discriminant score	Number of news with toe abnormalities
73	Medelim - Penha Garcia nr. Sidral	Portugal	-7.111	40.053	2	0.067	
76 #	São João do Deserto	Portugal	-7.198	40.144	5	0.005	
80 #	Barragem de Idanha-a-Nova	Portugal	-7.192	39.936	42	0.222	
81 #	Sra. do Almortão 1	Portugal	-7.206	39.907			
84 #	Zebreira	Portugal	-7.091	39.913	44	0.968	
87 #	Soverete	Portugal	-7.286	39.262	4	0.886	
88 #	Mourão	Portugal	-7.312	38.429	3	0.996	
90 #	Esperança	Portugal	-7.174	39.176	34	0.905	
91 #	Castelo Mendo	Portugal	-6.933	40.598	21	0.060	
92 #	Mogadouro	Portugal	-6.740	41.332	25	0.011	
93 #	Granja	Portugal	-7.262	38.318	11	0.986	
94 #	Mitra 2	Portugal	-8.001	38.535	22	0.978	
95 #	Aldeia Santa Margarida	Portugal	-7.274	40.050	21	0.082	
96 #	Ponte Maceira, Negreira	Spain	-8.703	42.901	10	0.010	
97 #	Calvão	Portugal	-8.705	40.460	26	0.847	1
98 #	Quiaios	Portugal	-8.816	40.237	20	0.890	
99 #	Praia de Vagueira	Portugal	-8.740	40.569			
100 #	Madeirã	Portugal	-8.101	39.937	24	0.000	
102 #	Nespereira de Baixo - Borallal	Portugal	-8.350	40.757	21	0.007	
103 #	Mezio	Portugal	-7.885	40.979	28	0.002	
104 #	Velada	Portugal	-7.701	39.580	22	0.965	
105 #	Fratel - Carepa	Portugal	-7.761	39.630			
106 #	Carepa	Portugal	-7.773	39.626			
107 #	Vila Velha de Rodão	Portugal	-7.667	39.668	40	0.899	
108 #	Vale de Figueira	Portugal	-7.747	39.681	31	0.005	
109 #	Vilar de Boi	Portugal	-7.736	39.672			
110 #	Zebreira West	Portugal	-7.112	39.839			
114 #	Torreira	Portugal	-8.710	40.764			
116 #	Porto - Botanical Garden	Portugal	-8.642	41.153			
119 #	Salir de Matos	Portugal	-9.083	39.427	1	0.000	
121 #	Fonte da Pena da Couvinha	Portugal	-9.019	39.477	2	0.001	
122 #	Punta Moreiras	Spain	-8.876	42.497	8	0.040	
127 #	Mosteiros de Alcanede	Portugal	-8.838	39.426	6	0.523	
134 #	Aceitunas	Spain	-6.317	40.152	8	1.000	
135 #	Santa Cruz de Paniagua	Spain	-6.335	40.201	7	0.937	2
136 #	El Bronco	Spain	-6.333	40.218	21	0.991	1
138 #	Pedro Muñoz 2	Spain	-6.343	40.275			
139 #	Pedro Muñoz 3	Spain	-6.340	40.257			
141 #	Puerto Nuevo	Spain	-6.536	40.361			
145 #	Alcuescar	Spain	-6.242	39.184	25	0.925	1
146 #	Rexaldia 1	Portugal	-8.548	39.572	2	0.951	
147 #	Rexaldia 2	Portugal	-8.549	39.571	10	0.960	1
148 #	Assertiz - Fungalvaz	Portugal	-8.515	39.598	20	0.868	
150	Covão da Fonte	Portugal	-8.802	39.538	2	1.000	
151	Carrascal - Évora de Alcobaça 1	Portugal	-8.961	39.531	2	0.804	
152	Molianos	Portugal	-8.920	39.507	1	0.185	
153	Covas	Portugal	-8.802	39.533	16	0.559	

Table 1 continued

Pond cq. population number	Locality	Country	Longitude	Latitude	Sample size morphology	Mean discriminant score	Number of news with toe abnormalities
155	Casais Monizes	Portugal	-8.896	39.449	11	0.459	
157	Carrascal - Évora de Alcobaça 2	Portugal	-8.960	39.532	2	0.723	
160	Juncal 2	Portugal	-8.916	39.597	7	0.266	
165	Andam	Portugal	-8.897	39.619	1	0.734	
177	Carrascal 2	Portugal	-7.878	39.547	1	0.010	
186	Zimbreira	Portugal	-7.849	39.576	1	0.000	1
196 #	Serradilla del Llano	Spain	-6.319	40.482	14	0.080	3
202	Mação 1	Portugal	-7.976	39.513	2	0.259	
203 #	Mação - Belver	Portugal	-7.980	39.538	1	0.001	
205 #	Tomar	Portugal	-8.418	39.602	46	0.841	6
206	Cem Soldos	Portugal	-8.458	39.585	1	0.594	
208 #	Sintra 1	Portugal	-9.418	38.793			
209 #	Sintra 2	Portugal	-9.388	38.788	15	0.660	2
216	Olas	Portugal	-8.339	39.665	2	0.000	
217 #	Nelas, Ponte Nove	Portugal	-7.836	40.486	21	0.014	1
218 #	Casar de Palomera 1	Spain	-6.241	40.278	4	0.010	
219 #	Casar de Palomera 2	Spain	-6.251	40.272	3	0.110	
221	Marchagaz	Spain	-6.270	40.270	1	0.002	
222 #	Palomero	Spain	-6.250	40.273	11	0.997	
224 #	Santa Catarina	Portugal	-8.357	39.651	4	0.075	
225	Casais	Portugal	-8.383	39.654	1	0.002	
243	Praia de Vagueira 2	Portugal	-8.740	40.568	10	0.714	
244	Albergaria dos Doze	Portugal	-8.612	39.797	2	0.064	
245	Montalvao - Spanish border	Portugal	-7.531	39.609	17	0.973	
247	Castelo de Vide railway line 1	Portugal	-7.479	39.444	13	0.995	
248	Beira	Portugal	-7.360	39.451	2	0.871	
250	São Domingos 2	Portugal	-8.171	39.602	11	0.101	
257	Carreiras	Portugal	-7.457	39.371	2	1.000	
271	Doñana	Spain	-6.394	36.853	31	0.949	
273 #	Archidona_Loja	Spain	-4.287	37.130			
300	Olhos de Agua	Portugal	-7.383	39.371	16	0.974	
307	Castelo de Vide railway line 2	Portugal	-7.444	39.455	10	0.954	
336 #	Confolens	France	0.667	46.017			
337 #	El Berrueco	Spain	-3.883	40.900			
338 #	Puerto de Galiz	Spain	-5.450	36.683			
339 #	Rochechouart	France	0.833	45.817			
340 #	Venta del Charco	Spain	-4.267	38.200			
341 #	Zafarraya	Spain	-4.133	36.967			
354	Marinha Grande	Portugal	-8.924	39.750	1	0.005	
436	Umbria near Monchique	Portugal	-8.506	37.335	1	0.996	
438	Faro University 1	Portugal	-7.979	37.050	2	1.000	
453	Picarras	Portugal	-8.563	38.680	2	0.990	
454	Fuenlabrada de los Montes	Spain	-4.956	39.129	2	1.000	
458	Los Baños del Robledillo	Spain	-4.358	39.496	1	0.987	
459	Buenasbodas nr. Belvis de la Jara	Spain	-4.890	39.681	3	0.974	
461	Los Yebenes - estacion de Urda	Spain	-3.821	39.418	20	0.992	
463	Villanueva de San Juan - Moron	Spain	-5.289	37.060	1	0.966	

the reader of what the colour classifications are like see Figure 1. Note that the image in black and white captures nearly all ventral ‘colour’ information. Since these characters are continuous, borderline cases are not infrequent. Dorso-lateral colour pattern characters are ‘Links’ and ‘Toplinks’. Links is the number of uninterrupted transversal connections following the green coloured surface at the lateral side of the body. Links were counted over both body sides independently,

over the section of the body covered by ILd. Toplinks is the number of dark spots that reach up to the mid-dorsal line and make it at least half way down the side and are also counted over both sides of the body. Illustrations of these characters are in Figure 2.

The morphometric data were ln-transformed. In order to analyze predominantly shape and not size, I calculated the standardized residuals of the regression of ‘ln character’ on lnSVL. Males and females were

Figure 2. Line drawings of the dorso-lateral sides of adult marbled newts. The dominant colours green and brown are shown by light and dark shading, respectively. On the left are *Triturus marmoratus* from Gerês in northern Portugal (A-C, locality 51) and Casas de Ribeira at the northern side of the contact zone (D-F, locality 42). On the right are *T. pygmaeus* from Gavião at the southern side of the contact zone (G, H and J, locality 32) and from Vale de Bispo - Sagres in southern Portugal (K-M, locality 65). The imagery illustrates how Links (numbers) and Toplinks (T) are counted. Links are the transversal connection following the green surface at the lateral side of the newts' body and are indicated by numbers (1-1, 2-2, etc.). Tick marks (^) to the left and right indicate genuine Links, but these are not counted because they fall (partly) outside the area covered by the measure interlimb distance. Toplinks are the dark spots that touch the mid-dorsal line and reach at least halfway down the newt's lateral side. Ambiguities in counting Links include the often vague colour transition from the lateral to the ventral side. This did not preclude recognizing links, such as Link 3 in specimen E. In specimen K Links 8 and 9 could also have been scored as a single one, because the Link paths share a short section in the middle. In counting Toplinks ambiguities may also arise, such as in specimen A where for the first Toplink the connection over the side is thin and in specimen J where it is arbitrary if the second Toplink reaches the mid-dorsal line. Reticulated (or 'wavy') colour patterns such as in specimen E and to a lesser extent in specimen D were only observed in the zone of contact and are tentatively associated with interspecific hybridization. Also 'colour islands' (I) such as in specimen J were found exclusively in and around the contact zone.

analyzed separately because of a documented sexual size dimorphism (García-París *et al.*, 1993). Altogether data were gathered for 1690 adult individuals in one French, 17 Spanish and 101 Portuguese populations (average $N=14.2$, range 1–71). The populations studied are listed in Table 1. However, colouration character states were defined during the project and are missing for 143 individuals (8.5 %). Occasionally, Links or Toplinks could not be counted due to albinism ($N=1$, population 51), melanism ($N=9$, populations 122, 151, 157, 165 and 206), or because of an aberrant, highly fragmented colouration pattern ($N=2$, population 307). The green dorso-lateral colour is overall darker and harder in *T. marmoratus* than the softer, olive shades of green that are prevalent in *T. pygmaeus*, but I was unable to score this character consistently. The tuberculate versus smooth skins and thick versus thin arms and legs for respectively *T. marmoratus* and *T. pygmaeus* were difficult to describe and also remained unrecorded. Finally, notes were taken on morphological abnormalities of fingers and toes, because these may be indicative of hybridization, as is the case of *T. marmoratus* and *T. cristatus* (Laurenti, 1768) in central France (Vallée, 1959; Arntzen and Wallis, 1991). To obtain an indication of measurement error 21 marbled newts were measured by myself in duplicate and by two volunteer students. To estimate measuring error the untransformed data were analyzed by principal component analysis, in SPSS v. 20 (IBM SPSS, 2016).

To be able to evaluate the diagnostic properties of the morphological data I also gathered data for enzyme genetic markers with proven performance for species identification, following Espregueira Themudo and Arntzen (2007a). The four marker loci that differentiated *T. marmoratus* from *T. pygmaeus* are *PepA* with reported κ values (Cohen's kappa, Cohen, 1960) $\kappa=0.92$, *PepB* with $\kappa=0.97$, *PepD* with $\kappa=0.12$ and *Ldh2* with $\kappa=0.56$. A total of 896 individuals was studied for ten Spanish and 46 Portuguese populations (average $N=16.0$, range 1–41). Tissues investigated were tail tips taken from adults. Missing data amounted to 3.4%, with no more than one locus with missing data for any individual. The individual genotypes are in Online Supplementary Information SI. DNA sequences of the nuclear genes β -fibrinogen intron 7 (FGBIT7, or *BF*), calreticulin intron 3 (CALRIT3, or *CALR*), platelet-derived growth factor receptor α -intron 11 (PDGFRAIT11, or *PDG*) and the mitochondrial gene NADH dehydrogenase subunit 4 (*ND4*) were taken from the literature (Espregueira Themudo *et al.*, 2012). A total of 1325 sequences were downloaded from Genbank and aligned with Clustal Ω

Figure 3. Bivariate scatter plots of molecular genetic classifications obtained with Structure (Q_p -scores along the horizontal axis) and NewHybrids (P -values along the vertical axis). Low and high Q_p -scores refer to *Triturus marmoratus* and *T. pygmaeus*, respectively. Note that 35 (3.9 %) individuals and one population (1.8 %) score as genetically admixed ($0.2 < Q_p < 0.8$). Top panel - results for individuals ($N=894$). Four individuals with relatively high hybridity ($P > 0.2$) are indicated by their population number. Bottom panel - results averaged for populations ($N=56$). Four populations for which individual newts are shown in Figure 2 are indicated by their numbers. Population 19 classifies as admixed; see also Table 2.

(Sievers and Higgins, 2014). Median joining haplotype networks were constructed for each sequenced marker with PopArt (Bandelt *et al.*, 1999; Leigh and Bryant, 2015). The internal node was located that maximized the separation between *T. marmoratus* and *T. pygmaeus* individuals from non-admixed populations. The fit of the haplotype groups to the species was $\kappa=0.87$ for *BF*, $\kappa=0.96$ for *CALR*, $\kappa=0.66$ for *PDG* and $\kappa=0.93$ for *ND4*. A total of 79 populations have sequence data available.

Species differentiation, population diversity and admixture were explored with the Bayesian clustering algorithm Structure (Pritchard *et al.*, 2000) with a predetermined $K=2$ number of groups, reflecting the species to be analyzed and the known diagnostic properties of the marker loci. The data were also analyzed with NewHybrids (Anderson and Thompson, 2002) under default settings, to identify possible cases of hybridization. The classes recognized were *T. marmoratus*, *T. pygmaeus* and a hybrid class composed of pooled F_1 's, F_2 's and backcross hybrid individuals. Structure Q -values in the 0.2 - 0.8 range and NewHybrid P_{NH} values > 0.5 were interpreted as

Figure 4. Western part of the Iberian Peninsula in quadrangular projection. Coloured symbols refer to genetically identified populations, with *Triturus marmoratus* shown by open green circles, *T. pygmaeus* by solid red circles and admixed populations by an X. Green and red diamond symbols refer to published data by (García-París *et al.* 2001; Arntzen *et al.* 2001). Small black dots are populations with morphological data only. Projected lines summarize the results of the discriminant analysis of morphological data, with the probability for classification as *Triturus marmoratus*, $P_m = P_p = 0.5$ shown by a solid line, $P_p = 0.4$ and 0.6 shown by thin lines and $P_p = 0.2$ and 0.8 by interrupted lines, with *T. marmoratus* to the north and *T. pygmaeus* to the south and southwest. The transect for the analysis of geographical clines is indicated by the box with coordinates 7.8 – 8.2 western longitude and 37.0 – 42.0 northern latitude.

evidence for recent (NewHybrids) or possibly more ancient (Structure) hybridization and interspecific gene flow. The basic population genetic analyses for Hardy-Weinberg and linkage (dis)equilibrium were carried out with GenePop (Raymond and Rousset, 1995) under population-wide Bonferroni correction.

The morphological data were analyzed by univariate and stepwise discriminant analysis in SPSS with Structure individual allocations to *T. marmoratus* ($Q_p < 0.2$), *T. pygmaeus* ($Q_p > 0.8$) or unknown. The ‘unknowns’ were either genetically admixed with Q -scores in the 0.2 – 0.8 range, or had no genetic data available. The unknowns were classified *a posteriori* from their morphology discriminant scores as *T. marmoratus* ($P_{DAP} < 0.2$), *T. pygmaeus* ($P_{DAP} > 0.8$) or intermediate ($0.2 < P_{DAP} < 0.8$). The fit of the

morphological to the genetic species identification was assessed with the AUC (area under the curve) statistic in SPSS. Ellipses summarizing variation around the mean in bivariate plots were produced with Mypstat 12 software (Mypstat, 2007). Spatial contour maps were also made with Mypstat, under default settings and with population data weighted for sample size.

Geographical clines were calculated for populations in a south to north transect across Portugal, encompassed by the coordinates 37.0 – 42.0 N and 7.8 – 8.2 W. I performed maximum likelihood geographical cline analyses with the R package HZAR (Derryberry *et al.*, 2014). Clines were fitted on probabilities of species assignment for morphological data from discriminant analysis (P_{DAP} , 31 populations), on Structure scores for the enzyme loci (Q_p , 14 populations) and on the

frequencies of species indicative haplotype groups for sequence data (F_p , eight populations) with values running from zero (*T. marmoratus*) to unity (*T. pygmaeus*). Clines were also fitted for the best performing morphological characters individually (SVI and Links, 27-29 populations, see below). The shape of each cline is described as a sigmoidal curve with a centre (maximum slope) and two tails (one in each side) modelled as an exponential decay, following Szymura and Barton (1986). Five different models were tested, varying on the presence/absence of left and right tails and their symmetry/asymmetry. Additionally, minimum ($P_{\min}$) and maximum ($P_{\max}$) character values were used to scale cline models in three different ways: with empirical estimates (using lower and higher observed values in the data), with best-fit values, and with no scaling. The combination of cline shapes and scaling thus produces a total of 15 different models for each cline, plus a null model (see for instance Prada and Hellberg, 2014). The Metropolis-Hasting algorithm in HZAR was run for the default value of 100,000 steps with a randomly selected seed and a burn-in of 10%. Given the uneven sample sizes it is important to note that HZAR cline estimates are made with sample size taken into account. After each run, I recorded the corrected Akaike Information Criterion score for each model within each cline and chose the one with the lowest score to infer cline width, cline centre and other cline shape parameters.

Results

Deviations from Hardy-Weinberg equilibrium with deficiencies of heterozygotes were found for the loci *PepB* and *PepD* in population 56. Significant linkage disequilibrium was not observed. With the Bayesian clustering algorithm Structure 393 individuals were classified as *T. marmoratus* ($Q_p < 0.2$), 466 were classified as *T. pygmaeus* ($Q_p > 0.8$) and 35 were admixed. At the population level 27 were *T. marmoratus*, 28 were *T. pygmaeus* and one was admixed (population 19, Table 2, Figure 3). With NewHybrids individuals and populations were allocated to the non-hybrid class (either *T. marmoratus* or *T. pygmaeus*), though four individuals (and no populations) showed a weak signal with P_{NH} values in the 0.2 - 0.5 range. The DNA sequence data allocated 34 populations to *T. marmoratus*, 36 to *T. pygmaeus* and nine were admixed (Table 3). The resulting species distribution is strongly partitioned with *T. marmoratus*

Table 2. Structure Q -scores derived from four enzyme loci (*LDH2*, *PEPA*, *PEPB* and *PEPD*) along with sample sizes and classification results, with m - *Triturus marmoratus* (27 populations with $Q_p < 0.20$), p - *T. pygmaeus* (28 populations with $Q_p > 0.8$) and x - admixed (one populations with intermediate Q_p). For locality information see Table 1; for individual genotypes see Online Supplementary Information S1; for Q -scores along with NewHybrid results see Figure 3. A .kml file for visualization with e.g. Google Earth is provided as Online Supplementary Information S2.

Population	Structure Q -score	Sample size	Species classification
2	0.958	20	p
3	0.949	19	p
4	0.935	22	p
5	0.989	17	p
7	0.026	36	m
12	0.038	10	m
19	0.215	14	x
26	0.977	22	p
29	0.988	23	p
30	0.977	7	p
32	0.885	10	p
42	0.036	7	m
51	0.038	25	m
55	0.083	10	m
58	0.054	7	m
62	0.032	5	m
65	0.989	10	p
67	0.942	34	p
70	0.017	7	m
72	0.055	10	m
76	0.015	5	m
80	0.037	38	m
84	0.925	41	p
87	0.990	4	p
88	0.991	3	p
90	0.978	27	p
91	0.081	21	m
92	0.014	25	m
93	0.988	11	p
95	0.016	21	m
96	0.013	10	m
97	0.970	26	p
98	0.973	20	p
100	0.108	24	m
102	0.031	21	m
103	0.033	28	m
104	0.963	22	p
107	0.840	40	p
108	0.053	31	m
119	0.008	1	m
121	0.014	2	m
122	0.017	8	m
127	0.980	6	p
134	0.960	8	p
135	0.981	7	p
136	0.982	21	p
145	0.978	24	p
146	0.974	2	p
147	0.982	10	p
148	0.978	20	p
196	0.073	14	m
203	0.030	1	m
217	0.024	21	m
218	0.012	4	m
219	0.151	3	m
222	0.983	11	p

Table 3. Frequency of species-indicative haplotypes for three nuclear genes (*BF*, *CALR* and *PDG*) and the mitochondrial *ND4* gene, along with sample sizes in brackets and classification results, with m – *Triturus marmoratus* (34 populations with $\bar{F}_p < 0.20$), p – *T. pygmaeus* (36 populations with $\bar{F}_p > 0.8$) and x – admixed (nine populations with $\bar{F}_p$ intermediate). For locality information see Table 1. A .kml file for visualization with e.g. Google Earth is provided as Online Supplementary Information S3.

Population	BF (N)	CALR (N)	PDG (N)	ND4 (N)	Classification
2	1.00 (5)	1.00 (5)	0.70 (5)	1.00 (6)	p
3	1.00 (1)	1.00 (4)	0.50 (5)	1.00 (5)	p
4	0.33 (3)	1.00 (5)	1.00 (6)	0.50 (8)	x
7				0.00 (4)	m
12	0.50 (2)	0.25 (2)	0.00 (2)	0.00 (5)	m
19				0.00 (5)	m
26		1.00 (3)		1.00 (5)	p
28	1.00 (5)	1.00 (5)	0.50 (5)	1.00 (5)	p
30	1.00 (4)	1.00 (5)	1.00 (4)	0.75 (4)	p
32	0.00 (4)			0.00 (5)	m
38		0.00 (4)			m
39				1.00 (4)	p
42	0.10 (5)	0.13 (4)	0.38 (4)	0.00 (5)	m
51	0.50 (4)	0.00 (5)	0.00 (7)	0.20 (5)	m
55	0.25 (4)	0.20 (5)	0.50 (5)	0.40 (5)	x
58				0.60 (5)	x
62				0.00 (3)	m
64				0.00 (5)	m
65	0.67 (3)	1.00 (7)	0.90 (5)	1.00 (5)	p
66	1.00 (1)	1.00 (1)	1.00 (1)	0.75 (4)	p
67	1.00 (5)	1.00 (5)	0.60 (5)	1.00 (5)	p
69				0.00 (10)	m
70				0.00 (3)	m
72				0.00 (3)	m
76	0.00 (5)	0.13 (4)	0.00 (5)	0.00 (5)	m
80	0.20 (5)	0.20 (5)	0.00 (5)	0.00 (4)	m
81	1.00 (4)	1.00 (5)	0.50 (9)	0.80 (5)	x
84	1.00 (2)	1.00 (3)	0.75 (4)	0.50 (4)	x
87				1.00 (3)	p
88	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (2)	p
90	1.00 (7)	1.00 (9)	1.00 (10)	0.63 (8)	p
91	0.33 (3)	0.00 (3)	0.00 (5)	0.00 (5)	m
92	0.30 (5)	0.00 (5)	0.00 (5)	0.00 (5)	m
93	1.00 (5)	1.00 (4)	1.00 (4)	1.00 (5)	p
94	1.00 (5)	1.00 (5)	1.00 (5)	1.00 (5)	p
95				0.00 (5)	m
96	0.38 (4)	0.00 (5)	0.00 (4)	0.00 (5)	m
97				1.00 (3)	p
98				1.00 (4)	p
99	1.00 (10)	1.00 (10)	0.30 (10)	1.00 (10)	p
100				0.00 (5)	m
102	0.10 (5)	0.00 (5)	0.00 (5)	0.00 (5)	m
103	0.00 (1)	0.00 (4)	0.00 (5)	0.00 (5)	m
104	1.00 (5)	1.00 (5)	0.75 (2)	1.00 (5)	p
105	0.00 (5)	0.00 (5)	0.20 (5)	0.00 (5)	m
106	0.20 (5)	0.00 (5)	0.60 (5)	0.00 (5)	x

Table 3 continued

Population	BF (N)	CALR (N)	PDG (N)	ND4 (N)	Classification
107	0.67 (3)	0.33 (3)	0.75 (4)	0.25 (4)	x
108				0.00 (5)	m
109	0.00 (5)	0.10 (5)	0.30 (5)	0.00 (5)	m
110	1.00 (5)	1.00 (5)	0.80 (5)	0.80 (5)	p
114	0.00 (2)	0.17 (3)	0.00 (3)	0.00 (3)	m
116	0.30 (5)	0.00 (5)	0.00 (5)	0.00 (5)	m
127	1.00 (4)	1.00 (4)	0.50 (4)		p
134	1.00 (4)	1.00 (4)	0.75 (4)	1.00 (4)	p
135	1.00 (3)	1.00 (4)	0.50 (4)	1.00 (5)	p
136	1.00 (5)	1.00 (5)	0.70 (5)	1.00 (5)	p
138	0.00 (2)	1.00 (1)	0.50 (1)	0.00 (3)	x
139	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (4)	p
141	0.00 (2)	0.00 (3)	0.00 (1)	0.00 (4)	m
145	1.00 (4)	1.00 (5)	1.00 (5)	1.00 (5)	p
146	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)	p
147	1.00 (2)	1.00 (3)	0.50 (4)	1.00 (4)	p
148	1.00 (5)	1.00 (5)	0.90 (5)	1.00 (5)	p
196	0.00 (5)	0.00 (5)	0.00 (5)	0.00 (5)	m
205	1.00 (5)	1.00 (5)	0.70 (5)	1.00 (5)	p
208	1.00 (4)	1.00 (3)	0.50 (1)	1.00 (2)	p
209	1.00 (4)	0.90 (5)	0.40 (5)	1.00 (4)	p
217	0.00 (5)	0.00 (5)	0.00 (5)	0.00 (5)	m
218	0.00 (4)	0.00 (4)	0.00 (4)	0.25 (4)	m
219	1.00 (1)	0.00 (1)	0.00 (1)	0.00 (1)	x
222	1.00 (5)	1.00 (5)	0.70 (5)	0.80 (5)	p
224	0.00 (3)	0.00 (4)	0.25 (4)	0.25 (4)	m
273	1.00 (4)		1.00 (4)	1.00 (3)	p
336		0.00 (1)	0.00 (1)		m
337		0.00 (3)	0.00 (4)		m
338	1.00 (4)		1.00 (5)		p
339	1.00 (1)	0.00 (3)	0.00 (3)		m
340	1.00 (3)		1.00 (3)	1.00 (3)	p
341			1.00 (3)	1.00 (3)	p

in the north and *T. pygmaeus* in the south of the Iberian peninsula. The transition between the species is sharp. The admixed populations are located at the species contact area in central Portugal (N=6), eastern Portugal (N=2) and western Spain (N=2, Figure 4; for details see Online Supplementary Information S2 and S3). Notable is the northerly distribution of *T. pygmaeus* along the Atlantic coast and the isolated occurrence of *T. marmoratus* directly north of the Lisbon peninsula. Because these results closely follow earlier reconstructions (e.g. Arntzen and Espregueira Themudo, 2008) and link up to the species borders recorded for Spain (García-París *et al.*, 1993; 2001; Pleguezuelos *et al.*, 2002) the results set the scene for

an evaluation of the performance of morphological data for species identification.

The replicated morphology measures were analyzed by principal component (PC) analysis. The first and second axes explained 28.1 % and 18.8 % of the total explained variance, respectively. The analysis revealed correspondence among three data sets out of four (Figure 5), suggesting that one observer measured in a different manner than the others. The outlying data shifted along the second PC-axis that has particularly high loadings for digit lengths (TFl and FTl) and the characters describing the extent of the black ventral colouration. The mean coefficient of variation (V) per character for the three

Table 4. Performance of morphological characters for *Triturus marmoratus* – *T. pygmaeus* species identification in stepwise discriminant analysis and univariate *t*-tests. Model fit is expressed by the ‘area under the curve’ (AUC) statistic. For character descriptions and abbreviations see text. Also shown are species characteristics and the coefficient of variation obtained from replicated measurements (see Figure 5).

Character	Unit,	Order of selection #		Model fit		Species with higher value \$		Mean coefficient of variation (% V)
	transformation &	Males	Females	Males	Females	Males	Females	
Size variable								
SVI	mm, ln	1	2	0.931	0.891	m	m	0.98
Shape variables								
HI	mm, lnstres			0.532	0.501			7.4
Hw	mm, lnstres	3	4	0.708	0.639	m	m	2.5
ILd	mm, lnstres	8	5	0.500	0.628		p	3.4
FLI	mm, lnstres		6	0.687	0.742	m	m	2.7
TFI	mm, lnstres	4		0.687	0.589	m	m	6.6
HLI	mm, lnstres			0.640	0.648	m	m	3.1
FTI	mm, lnstres			0.600	0.503	m		9.2
Ventral colouration characteristics								
Bc	Scale 1-5	5	3	0.728	0.719	m	m	16.9
WSn	Scale 1-5	9		0.556	0.520	m		12.4
WSs	Scale 1-4			0.645	0.605	m	m	26.9
BSn	Scale 1-5	6	8	0.505	0.514		p	15.6
BSs	Scale 1-4	7	9	0.648	0.515	m		11.4
Dorso-lateral colouration characteristics								
Links	Count	2	1	0.914	0.935	p	p	6.4
Toplinks	Count		7	0.704	0.693	m	m	24.3
Characters selected in stepwise discriminant analysis				0.990	0.981			
SVI and Links				0.976	0.966			

& lnstres - standardized residuals of the regression of ln ‘character’ on ln SVI

in stepwise discriminant analysis

\$ significant in univariate *t*-test; m - *T. marmoratus*, p - *T. pygmaeus*

coinciding data sets is low for the character SVI ($V < 0.01$), moderate for Hw, ILd, FLI and HLI ($0.01 < V < 0.04$), substantial for HI, TFI, FTI and Links ($0.04 < V < 0.10$) and large for the six remaining colouration characteristics ($V > 0.10$, Table 4).

The morphometric data show that *T. marmoratus* is the larger species (Table 4). It has a wider head and longer extremities than *T. pygmaeus*. Interlimb distance is relatively larger in *T. pygmaeus* than in *T. marmoratus*, but the difference is only statistically significant for females. Scores for the ventral colouration characters are generally higher for *T. marmoratus* than for *T. pygmaeus*, pointing to darker and lighter colourations, respectively. Scores for Links are higher in *T. pygmaeus*

and scores for Toplinks are higher in *T. marmoratus*. SVI and Links are the prime characters selected in the discriminant analysis. These characters stand out for efficient species discrimination to the extent that model fit on the basis of these two characters ($0.97 < \text{AUC} < 0.98$) is only marginally less than for the set of nine characters selected by discriminant analysis ($0.98 < \text{AUC} < 0.99$, Table 4). In two populations out of 59, species frequencies from enzyme genetic and morphological characters are significantly different (*G*-test for independence, $P < 0.05$ for population 3, and $P < 0.001$ for population 80). These populations are located at or close to (< 20 km) the centre of the hybrid zone (see Table 1 and Figure 4).

Second PC-axis

Figure 5. Analysis of replicated morphological measurement on 21 marbled newts (seven males and 14 females from population 91) by principal component analysis. Ellipses indicate one standard deviation of the mean. The results for ‘student 2’ differ from the others along the second PC-axis and mainly concern the measurements of digits (TFI, FTI) and the scores on the black ventral colouration (BSn, BSs).

The morphological identification criteria were also applied to populations for which enzyme data were unavailable. The species distributions on the basis of external morphology closely follows that of the molecules (Figure 4). However, in the contour plot of discriminant species probabilities, the transition zone is wide, in particular north of the Lisbon peninsula where the species distribution forms a mosaic. Along the coast the species transition zone reaches up to Aveiro. In central Portugal it follows the Tejo river and runs through the town Idanha-a-Nova into Spain where it follows the northern edge of Extremadura and, presumably, the southern edge of Castile and León, and into the province of Madrid where the species contact discontinues. Digital deformities I observed in 19 individuals in ten populations, located inside as well as outside the species transition zone (Table 1).

At the transect the *T. marmoratus* – *T. pygmaeus* species transition is sharp and coincides with the position of the Tejo river, for most characters studied (Table 5, Figures 6 and 7). With the pooled enzyme

genetic data set as a reference, the clines are congruent, with the exception of female SVI and *PDG* for which clines are marginally wider than the reference cline. The cline for mtDNA stands out on the basis of its width and southerly position. For the characters *lnSVI* and *Links* the clines revealed a prevalence of intermediate values at the very centre of the contact zone (Figure 6CDE). This raised the question if these morphologically intermediate populations were composed of genetically admixed individuals. Because the sample in the transect was small, I performed analyses with all data, with the *Q*-scores classification as above, and with admixture set as a wider class ($0.1 < Q < 0.9$). An ANOVA indicated significant differences for the three groups, with the genetically admixed group as morphologically intermediate (Table 6).

Discussion

I reconstructed the distribution of the hybridizing newt species *T. marmoratus* and *T. pygmaeus* over the western part of the Iberian Peninsula from morphological and molecular data. I report on ten admixed populations that all are located at the *T. marmoratus* – *T. pygmaeus* range interface. Yet, the molecular data show a strong bimodality with few admixed individuals, suggesting that interspecific hybridization is limited, possibly with strong selection against hybrids (Barton and Hewitt, 1985; Mallet, 2005). The level of hybridization is comparable to that in *Triturus* crested newts at the same level of divergence (Arntzen *et al.*, 2014). Some introgression appears to have taken place as shown by admixed individuals in e.g. populations 100, 104 and 107 (Figure 3). However, with few partially diagnostic markers ‘admixed’ genotypes may have arisen by chance recombination. This may be the case in population 100 that is located away from the species contact zone and figures as an outlier in cline analysis (Figure 6F). Morphologically intermediate individuals are more numerous than genetically admixed individuals, suggesting that the molecular markers do not reflect the full scope of *T. marmoratus* x *T. pygmaeus* hybridization. To document introgression and to elucidate hybrid zone structure at a fine scale, more genetic markers are required.

Morphological characters that stand out for species identification are body size (SVI) and the dorso-lateral

Table 5. Parameter estimates for the maximum-likelihood geographical clines for morphological data (top panel), nuclear genetic data (middle panel) and nuclear and mitochondrial sequence data (bottom panel) over the south to north transect shown in Figure 4. Cline position is relative to Tejo river at 8° W and width is 1/maximum slope. Confidence intervals are based upon the 2log-likelihood unit support limits. δ and τ are the shape parameters for the tails, with fitting at a mirror (M), at the right (R) or none (N). The parameters $P_{\min}$ and $P_{\max}$ are the estimated values at either end of the transect and are set to minimum and maximum values (typ models), fixed to their empirical values (fix models), or $P_{\min}$ and $P_{\max}$ are fitted (opt models). Clines are shown in Figures 6 and 7.

Character	Model	Position	Confidence interval		Width	Confidence interval		Mirror		Right		P		Cline shown in Figure
		(km) #	Min	Max	(km)	Min	Max	δ	τ	δ	τ	Min	Max	
Morphological data analysed with stepwise discriminant analysis - 31 populations														
All populations	OptR	0.73	-0.09	1.65	1.17	0.12	2.81			0.387	0.031	0.017	0.985	
Populations with genetic data	OptR	2.15	0.83	3.05	4.75	1.83	4.91			1.683	0.140	0.018	0.991	6A
Populations without genetic data	OptN	1.25	-1.07	1.91	0.23	0.00	2.73					0.119	0.979	6B
Morphology, single characters - 29 populations														
lnSVI, males	OptN	0.07	-24.47	5.03	16.54	0.28	109.92					4.028	4.263	6C
lnSVI, females	OptN	0.53	-11.64	6.00	14.65	5.60	86.51					4.055	4.299	6D
Links	OptN	1.68	-0.15	1.85	0.49	0.02	2.22					1.718	8.650	6E
Nuclear genetic data analysed with Structure - 14 populations														
Four loci	FixM	2.14	0.14	3.47	1.23	0.15	5.21	0.759	0.009			0.024	0.988	6F
PepA	TypM	1.24	0.25	3.42	1.80	0.20	8.63	0.727	0.003			0	1	6G
PepB	OptN	-1.17	-4.20	0.23	5.01	1.42	13.02					0.067	0.789	6H
PepD	OptN	0.77	-0.20	3.82	1.39	0.00	5.23					0.155	0.786	6J
Ldh2	FixN	26.72	-124	> 125	> 400	4.54	>> 400					0.079	0.869	6K
DNA sequence data, analyzed from species indicative haplotype frequencies - eight populations														
BF	OptN	-7.49	-57.62	2.67	18.63	0.002	106.71					0.180	0.997	7L
CALR	TypN	3.23	-0.49	7.29	7.53	2.91	34.15					0	1	7M
PDG	TypN	5.99	1.73	19.47	12.21	5.34	81.86					0	1	7N
ND4 mitochondrial	TypR	-19.28	-51.29	-5.54	78.86	26.24	182.44			51.88		0	1	7P

Relative to the Tejo river at 8.0° W. Note that the transect crosses the Tejo river at an angle, at positions varying from 39.446° to 39.543° N, amounting to a latitudinal difference of 10.8 km.

colour pattern (Links). Marbled newts with SVI > 63 (males), SVI > 65 (females) and Links ≤ 5 are likely to be *T. marmoratus*, whereas marbled newts smaller than that with higher link counts are likely to be *T. pygmaeus*. *Triturus pygmaeus* was described as a small sized ('pygmy') subspecies of *T. marmoratus* by Wolterstorff (1905) with Algeciras, Cadiz as type locality. Body size was considered a diagnostic character on the basis of material from the same region (García-París *et al.*, 1993). However, southern populations of *T. pygmaeus* may be of particular small body size (Díaz-Paniagua *et al.*, 1996). Body size may also be affected by local feeding conditions and other environmental variables. Moreover, newts show indeterminate growth (Arntzen, 2000) so that individual age is a confounding factor. The character Links has higher diagnostic power than body size and

is equally applicable to males and females. However, diagnostic power appears to break down in the very south of Spain where link counts for *T. pygmaeus* are close to zero (García-París *et al.*, 1993). Observer bias may be an issue in morphological species identification, but was not shown to affect SVI or Links. Counting links may be ambiguous if there are many, but with a threshold set at 5.5 this is not an identification issue. Marbled newts with aberrant colour patterns were found at the hybrid zone. In some ways similar 'reticulate' or 'wavy' colouration patterns have been observed in *Bombina bombina* (Linnaeus, 1761) x *B. variegata* (Linnaeus, 1758) reciprocal crosses (Héron-Royer, 1891; Michalowski and Madej, 1969). Given the observations of toe abnormalities well outside the hybrid zone it is unclear if these have to do with hybridization, as was

Table 6. Analysis of variance on morphological characters selected for species discrimination, applied to the groups *Triturus marmoratus* (1), admixed (2) and *T. pygmaeus* (3).

Genetically classified groups	Mean character values #				Significance in Tamhane's T2 post-hoc test			
	Sample size	SVI (mm)		Links number	Groups compared	SVI		
		Males	Females			Males	Females	Links
Intermediate group small								
1 - <i>T. marmoratus</i> ($Q_p < 0.2$)	393	69.1	71.8	1.49	12	NS	NS	***
2 - admixed ($0.2 < Q_p < 0.8$)	35	62.6	66.8	6.29	23	NS	NS	NS
3 - <i>T. pygmaeus</i> ($Q_p > 0.8$)	466	58.2	62.9	7.97	13	***	***	***
Analysis of variance, F-value		453.5 ***	194.6 ***	150.9 ***				
Intermediate group large								
1 - <i>T. marmoratus</i> ($Q_p < 0.1$)	361	68.8	71.8	1.43	12	***	**	***
2 - admixed ($0.1 < Q_p < 0.9$)	80	62.9	67.7	5.61	23	**	***	***
3 - <i>T. pygmaeus</i> ($Q_p > 0.9$)	449	58.3	62.8	7.90	13	***	***	***
Analysis of variance, F-value		409.4 ***	171.3 ***	144.6 ***				

NS - not significant, ** - $P < 0.01$ and *** - $P < 0.001$
To reduce the skewness of the character state distributions to marginally significant levels the data were transformed following the Box Cox procedure

inferred for *T. cristatus* x *T. marmoratus* hybrids in France (Arntzen and Wallis, 1991).
The clines reconstructed for the transect in central Portugal are largely coincident (located at the same place) and congruent (of the same shape). Hybrid zones are frequently located in areas where populations are sparse and dispersal is impeded (i.e., ‘density troughs’, Barton and Hewitt, 1985), as may be the case here by the Tejo river. However, the course of the river is not exactly perpendicular to the transect and a more detailed survey over two spatial dimensions is in order to evaluate hybrid zone position as determined by the environment. The different profile of the mtDNA cline (Figure 7P) versus all other, nuclear markers is remarkable. With the knowledge that *T. pygmaeus* has been enlarging its range at the expense of *T. marmoratus* in the nearby coastal area of Portugal (Espregueira Themudo and Arntzen, 2007b) the most straightforward explanation is that *T. marmoratus* had a wider range in central Portugal too, was superseded by *T. pygmaeus*, but not without introgressive hybridization taking place (i.e. hybrid zone movement; Buggs, 2007). Seen like this, the southern mtDNA constitutes a ‘genetic footprint’ left behind by the receding species *T. marmoratus*.

Modelling has shown that even limited hybridization can result in substantial introgression, when one species is competitively displacing the other (Currat *et al*, 2008). This genetic footprint is reflected in the cline by an increased width and a displaced, southern position. Mitochondrial DNA is more than nuclear loci prone to chance introgression due to its small effective population size and it is more prone to detection because it stays intact, due to its non-recombining nature (Toews and Brelsford, 2012). The hypothesis of hybrid zone movement can be tested by surveying a larger number of nDNA markers and search for further ‘genomic footprints of hybrid zone movement’ (Wielstra *et al.*, 2017).
Marbled newt populations are infrequent in the Madrid area (García-París *et al.*, 1989; Martínez-Solano, 2006). The two populations studied by Arntzen *et al.* (2014) are ca. 20 km apart, with no evidence for hybridization. It may here concern a residual hybrid zone with past but no present opportunities for hybridization and gene flow. Both species appear to suffer from regressing ranges in the east of the Iberian peninsula, as suggested by the isolated occurrences around Barcelona (*T. marmoratus*) and in Albacete province (*T. pygmaeus*). The southern fringe of

Figure 6. Geographical clines for morphological (left, panels A-E) and nuclear genetic data (right, F-H, K). Solid dots represent populations and the grey areas the 95% credibility interval. The parameters for formal cline descriptions are in Table 5. Note that the character transitions are mostly steep and located at the Tejo river. Clines are mostly coincident and congruent. The combination of characters (panels A, B and F) outperforms single characters (C-E and G, H, J, K) for species classification purposes. Morphological data - discriminant analysis (cf. Table 4) separates the species adequately, for individuals with species identity known (panel A), or unknown from genetic data (B). Note that in B the credibility interval is wider than in A and the P_{min} and P_{max} values are less differentiated. The character Links (panel E) outperforms SVI (C and D) in species diagnostics. In panels C and D note the preponderance of populations with intermediate SVI at the center of the transect, further analyzed in Table 6. Nuclear genetic data - the loci *PepA* and *PepB* outperform *Ldh2*, as they do in western Portugal (Espregueira Themudo and Arntzen, 2007). The locus *PepD* has a performance at par with *PepA* and *PepB* and accordingly discriminates species better in central than in western Portugal (see α values reported in Espregueira Themudo and Arntzen, 2007a). Population 100 is an outlier for the molecular data (panel F, see also Figure 3).

Figure 7. Geographical clines for DNA sequence data at three nuclear loci (*BF*, *CALC*, *PDG*) loci and the mitochondrial marker *ND4*. Haplotypes were pooled into groups that are indicative of the species *Triturus marmoratus* versus *T. pygmaeus*. Solid dots represent populations and the grey areas the 95% credibility interval. The parameters for formal cline descriptions are in Table 3. Note that the character transitions are steep and located at the Tejo river. Clines are coincident and congruent to those in Figure 6, except for the *ND4* mitochondrial marker.

Castile and León remains to be studied. In the north of Extremadura the species contact is located at the lower altitudes of the Sierra de los Ángeles (south of Sierra de Gata), with *T. pygmaeus* at lower and *T. marmoratus* at higher altitudes. No obvious ecological correlate for hybrid zone position was found in eastern Portugal. I suggested that *T. marmoratus* and *T. pygmaeus* engage in a tension hybrid zone (*sensu* Barton and Hewitt, 1985) that locally follows the minimum spanning distance between fixed points at either side, these flanking positions being the Sierra de Gata in the east and the Tejo river in the west (Arntzen and Espregueira Themudo, 2008). The Tejo river as an approximate species border is followed over a length of at least 20 km. In the west of Portugal *T. marmoratus* and *T. pygmaeus* show more of a mosaic distribution, with no evidence for recent hybridization in the present data. The new morphological and molecular data confirm the presence of an isolated occurrence of *T. marmoratus* at the north of the Lisbon peninsula and show that the *T. pygmaeus* range follows the coastal zone up to Aveiro and the river Ave in the north, with local climate and soil type affecting the mutual species distribution (Espregueira Themudo and Arntzen, 2007ab; Arntzen and Espregueira Themudo, 2008). The diversity in environmental correlates to the joint species' border illustrates the intricate and complicated relationship of hybrid zone position and the environment, the more so

if the hybrid zone is, or has been moving northwards. Other congeneric Iberian amphibians and reptiles with to a large extent contiguous range borders show similar - but not coinciding - latitudinal bi- or tripartitions (eg. *Alytes* Wagler, 1829 - midwife toads; *Blanus* Wagler, 1830 - worm lizards) (Carretero *et al.*, 2014). Several tripartitions along a longitudinal axis have recently come to light (*Pelodytes* Bonaparte, 1838 - parsley frogs; *Psammmodromus* Fitzinger, 1826 - sand lizards). Yet other contiguous congenics show a distribution mosaic with no particular axis dominating the distribution pattern (*Podaris* Wagler, 1830 - wall lizards). Given the observed disparity, spatio-temporal correlations between groups are unlikely to exist and evolutionary scenarios for the Iberian herpetofauna will be unique and taxon specific.

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References

- Anderson EC, Thompson EA. 2002. A model-based method for identifying species hybrids using multilocus genetic data. *Genetics* 160: 1217–1229.
- Arntzen JW. 2000. A growth curve for the newt *Triturus cristatus*. *Journal of Herpetology* 34: 227–232.
- Arntzen JW, Espregueira Themudo G. 2008. Environmental parameters that determine species geographical range limits as a matter of time and space. *Journal of Biogeography* 35: 1177–1186.
- Arntzen JW, Wallis GP. 1991. Restricted gene flow in a moving hybrid zone of the newts *Triturus cristatus* and *T. marmoratus* in western France. *Evolution* 45: 805–826.
- Arntzen JW, Wielstra B, Wallis GP. 2014. The modality of nine *Triturus* newt hybrid zones assessed with nuclear, mitochondrial and morphological data. *Biological Journal of the Linnean Society* 113: 604–622.
- Bandelt H, Forster P, Röhl A. 1999. Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* 16: 37–48.
- Barton NH, Hewitt GM. 1985. Analysis of hybrid zones. *Annual Review of Ecology and Systematics* 16: 113–148.
- Buggs RJA. 2007. Empirical study of hybrid zone movement. *Heredity* 99: 301–312.
- Carretero MA, Martínez-Solano I, Ayllón E, Llorente G (2014) *Lista patrón de los anfibios y reptiles de España (Actualizada a diciembre de 2014)*. Asociación Herpetológica Española, Barcelona, Spain.
- Cohen J. 1960. A coefficient of agreement for nominal scales. *Educational and Psychological Measurement* 20: 37–46.
- Curran M, Ruedi M, Petit RJ, Excoffier L. 2008. The hidden side of invasions: massive introgression by local genes. *Evolution* 62: 1908–1920.
- Derryberry EP, Derryberry GE, Maley JM, Brumfield RT. 2014. HZAR: hybrid zone analysis using an R software package. *Molecular Ecology Resources* 14: 652–663.
- Díaz-Paniagua C, Mateo JA, Andreu AC. 1996. Age and size structure of populations of small marbled newts (*Triturus marmoratus pygmaeus*) from Donana National Park (SW Spain). A case of dwarfism among dwarfs. *Journal of Zoology* 239: 83–92.
- Espregueira Themudo G, Arntzen JW. 2007a. Molecular identification of marbled newts and a justification of species status for *Triturus marmoratus* and *T. pygmaeus*. *Herpetological Journal* 17: 24–30.
- Espregueira Themudo G, Arntzen JW. 2007b. Newts under siege: range expansion of *Triturus pygmaeus* isolates populations of its sister species. *Diversity and Distributions* 13: 580–586.
- Espregueira Themudo G, Nieman AM, Arntzen JW. 2012. Is dispersal guided by the environment? A comparison of interspecific gene flow estimates among differentiated regions of a newt hybrid zone. *Molecular Ecology* 21: 5324–5335.
- García-París M, Arano B, Herrero P. 2001. Molecular characterization of the contact zone between *Triturus pygmaeus* and *T. marmoratus* (Caudata: Salamandridae) in Central Spain and their taxonomic assessment. *Revista Española de Herpetología* 15: 115–126.
- García-París M, Herrero P, Martín C, Dorda J, Esteban M, Arano B. 1993. Morphological characterization, cytogenetic analysis, and geographical distribution of the Pygmy marbled newt *Triturus marmoratus pygmaeus* (Wolterstorff, 1905) (Caudata: Salamandridae). *Contributions to Zoology* 63: 3–14.
- García-París M, Martín C, Dorda J, Esteban M. 1989. *Los anfibios y reptiles de Madrid*. Ministerio de Agricultura, Pesca y Alimentación, Madrid, Spain.
- Harrison RG. 1990. Hybrid zones: windows on evolutionary process. *Oxford Surveys in Evolutionary Biology* 7: 69–128.
- Héron-Royer [LF]. 1891. Nouveaux faits d'hybridation observés chez les batraciens anoures. *Memoires Société Zoologique France* 4: 75–85.
- Hewitt GM. 1988. Hybrid zones – natural laboratories for evolutionary studies. *Trends in Ecology and Evolution* 3: 158–167.
- IBM SPSS. 2016. *Statistical Package for the Social Sciences*. SPSS Inc., Chicago, USA.
- Leigh JW, Bryant D. 2015. PopArt: full-feature software for haplotype network construction. *Methods in Ecology and Evolution* 6: 1110–1116.
- Mallet J. 2005. Hybridization as an invasion of the genome. *Trends in Ecology and Evolution* 20: 229–237.
- Martínez-Solano I. 2006. Atlas de distribución y estado de conservación de los anfibios de la comunidad de Madrid. *Graellsia* 62: 253–291.
- Michalowski J, Madej Z. 1969. Studies on the relationship of *Bombina bombina* (Linnaeus) and *Bombina variegata* (Linnaeus) III. Taxonomic characters in both species from laboratory and in interspecific hybrids. *Acta Zoologica Cracoviensis* 14: 173–200.
- Mystat. 2007. *Mystat 12*. Systat Software, San Jose, CA, USA.
- Petit RJ, Excoffier L. 2009. Gene flow and species delimitation. *Trends in Ecology and Evolution* 24: 386–393.
- Pleguezuelos JM, Márquez R, Lizana M. 2002. *Atlas y libro rojo de los anfibios y reptiles de España. Second Edition*. Organismo Autónomo de Parques Nacionales, Madrid, Spain.
- Prada C, Hellberg ME. 2014. Strong natural selection on juveniles maintains a narrow adult hybrid zone in a broadcast spawner. *American Naturalist* 184: 702–713.
- Pritchard JK, Stephens M, Donnelly P. 2000. Inference of population structure using multilocus genotype data. *Genetics* 155: 945–959.
- Raymond M, Rousset F. 1995. GenePop (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity* 86: 248–249.
- Sievers F, Higgins DG. 2014. Clustal omega. *Current Protocols in Bioinformatics* 12: 3–13.
- Szymura JM, Barton NH. 1986. Genetic analysis of a hybrid zone between the fire-bellied toads, *Bombina bombina* and *B. variegata*, near Cracow in southern Poland. *Evolution* 40: 1141–1159.
- Toews DP, Brelsford A. 2012. The biogeography of mitochondrial and nuclear discordance in animals. *Molecular Ecology* 21: 3907–3930.
- Vallée L. 1959. Recherches sur *Triturus blasii* de l'Isle, hybride naturel de *Triturus cristatus* Laur. x *Triturus marmoratus* Latr. *Bulletin Société Zoologique France* 31: 1–95.
- Wielstra B, Burke T, Butlin RK, Arntzen JW. 2017. A signature of dynamic biogeography: enclaves indicate past species

- replacement. *Proceedings of the Royal Society B* 284: 20172014.
- Wielstra B, Sillero N, Vörös J, Arntzen JW. 2014. The distribution of the crested and marbled newt species (Amphibia: Salamandridae: *Triturus*) – an addition to the New Atlas of Amphibians and Reptiles of Europe. *Amphibia-Reptilia* 35: 376-381.
- Wolterstorff W. 1905. Zwergformen der paläarktischen Urodelen. *Comptes Rendus Séances 6 Congrès International de Zoologie, Berne* 1904: 258-263.

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Online supplementary material

S1. Allele frequencies at four nuclear loci over 56 populations of *Triturus marmoratus* and *T. pygmaeus* in France, Spain and Portugal. Data are in GenePop format.

S2. Marbled newt populations identified to the species with enzyme genetic data in .kml format, for visualization with e.g. Google Earth. Populations are identified as: large green dots - *Triturus marmoratus*, large red dots - *T. pygmaeus* and large grey dots - admixed. Data are as in Table 2.

S3. Marbled newt populations identified to the species with DNA sequence data in .kml format, for visualization with e.g. Google Earth. Populations are identifies as: small green dots - *Triturus marmoratus*, small red dots - *T. pygmaeus* and small grey dots - admixed. Data are as in Table 3.