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

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CHAPTER 7

MOLECULAR IDENTIFICATION OF MARBLED NEWTS AND A JUSTIFICATION OF SPECIES STATUS FOR *TRITURUS MARMORATUS* AND *T. PYGMAEUS*

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Abstract

The marbled newts *Triturus marmoratus* and *T. pygmaeus* are common and well diagnosable species occurring in central Portugal, but difficult to survey in large and deep ponds. Conversely, embryos of both species are easy to locate but morphologically indistinguishable. We studied a panel of nuclear genetic loci by starch gel electrophoresis (the enzymes PEP-A, PEP-B and PEP-D) and isoelectric focusing techniques (the enzyme LDH-2, post-embryonic stages only) that together yield a species-specific signature (Cohen's kappa = 1.00). On a locus by locus basis the scores for correct classification range from kappa 0.12 to kappa 0.97. The method allows the reliable, fast and cheap identification of both species across life-stages, with a better behaviour and performance than mtDNA sequencing (i.e. bar-coding) and nuclear DNA microsatellite profiling. The observed distribution of *T. marmoratus* and *T. pygmaeus* over 25 aquatic breeding sites in the Caldas da Rainha area in western Portugal is parapatric, with no mixed populations and no F_1 interspecific hybrids. This demonstrates that *T. marmoratus* and *T. pygmaeus* are genetically isolated, even when populations are within the 'dispersal distance per generation' range of one another. We consider the data adequate for supporting the species status of *T. marmoratus* and *T. pygmaeus* under the Biological Species Concept.

Keywords: allozymes; Amphibia, principal coordinate analysis

Introduction

Newts are characterized by long annual periods (2-6 months) of pond breeding and dip-netting provides a mostly efficient and unbiased sampling technique for adults (Plethodon, ARNTZEN, 2002a; 2002b). This allows the reliable surveying over large areas for the purpose of, for example, the improved understanding of distribution patterns and the assessment of conservation status. In Portugal, we noted the frequent occurrence of newts in 'albercas'. These albercas are deep (> 3 m) and sizable (diameter 3-8 m), mostly circular stone structures for agricultural and household water supply [for typical examples of 'albercas', see Malkmus (1982; 2004: Figure 61). Albercas are exceedingly difficult to investigate. The dip-netting approach fails, because at the first sweep the adult newts dive beyond reach and hide in the crevices of the stone walls.

The presence of adults however may be revealed by the easy to spot eggs that are individually attached to the leaves of submerged, floating and overhanging vegetation (MIAUD, 1993). For the eggs to be useful in surveying, a method is required for species identification.

Aims of the present paper are, first, to develop an efficient and reliable method for marbled newts (*Triturus marmoratus* and *T. pygmaeus*) egg identification. Since the eggs of both species are morphologically indistinguishable these will be molecular genetic tools. On a technical note, most eggs in the field will be fertilized and should be referred to as embryos, but for convenience we will use the terms interchangeably. Secondly, we apply and test the new method to qualify the distribution of both species in central Portugal over and across a contact zone between them. Thirdly, we test the hypothesis of Garcia-Paris *et al*. (2001) that *Triturus marmoratus* and *T. pygmaeus* are full species under the Biological Species Concept.

Material and methods

Research was carried out in an area of *c.* 4000 km^2 around Caldas da Rainha to the north of Lisboa, Portugal, where *T. marmoratus* and *T. pygmaeus* have both been observed in a pilot study (JWA & E. Froufe, unpublished data). Ponds and other potential newt breeding sites were located by motorized field searches, assisted by military topographical maps and with help and information by local inhabitants. On

the spot we checked the submerged, floating and marginal vegetation for the presence of marbled newt eggs, which are clearly distinguishable from that of other amphibians, by size, structure and the way they are deposited.

The eggs of the sympatric small bodied newt *Triturus boscai* (placed in the genus Lissotriton by GARCÍA-PARÍS *et al.*, 2004; and in the genus Lophinus by LITVINCHUK *et al.*, 2005) are readily distinguished from marbled newt eggs on account of their small size, bipolar pigmentation and the round (as opposed to ovoid) shape of the jelly capsule around them. Eggs were collected from the vegetation all over the accessible parts of the water body and placed in Eppendorf vials. Occasionally, adult and larval marbled newts were caught by dip-netting. The adults were identified as *T. marmoratus* or *T. pygmaeus* on the basis of size, colour and colour-pattern, whereas larvae remained unidentified. Tail tips were removed and placed under buffer in individual Eppendorf vials. All vials were placed in liquid nitrogen for transportation to the laboratory and then stored in an -80º C freezer for future electrophoresis.

In the laboratory, the tail tips and entire embryos were homogenized in an aliquot amount of ice cold buffer (100 mM Tris, 1 mM EDTA, 0.05 mM NADP, adjusted to pH=7.0 with HCl) and centrifuged for 15 minutes at 13,000 rpm at 4 ºC. The supernatant was treated with dithiotreitol (120 μM DTT) for 1 hour at 37 ºC prior to electrophoresis and staining on starch gels for three peptidases (PEP-A, PEP-B and PEP-D) and on acrylamide gels with isoelectric focussing for the enzyme Lactate dehydrogenase (LDH-2), following standard protocols (e.g., PINHO *et al.*, 2003). Electromorphs were interpreted as alleles at the corresponding genetic locus. We used the program Genepop (RAYMOND and ROUSSET, 1995) to test for population genetic differentiation by Fisher's exact test, to calculate expected heterozygosity (H_e) and to test for departure from Hardy-Weinberg equilibrium (HW) under standard Bonferroni correction.

Principal Coordinate Analysis (PCA) was performed on a binary data set with alleles as characters and presence (1) or absence (0) of alleles as character states. Character states were assumed to be independent, although in reality limited to a maximum of two scores of 1 per locus. Homozygotes were not distinguished from heterozygotes (i.e., they were represented by a single score of 1). The subroutine SIMQUAL of the program NTSYS 1.7 (ROHLF, 1992) was used to compare the enzyme profiles and to calculate a matrix with pairwise similarity coefficients. We

choose the Jaccard similarity coefficient because it ignores joint absences. The subroutine DCENTER was used to transform the similarity matrix into scalar product form, after which it was factored using the subroutine EIGEN.

Results

The presence of marbled newts was confirmed in 25 aquatic sites, from which we sampled 101 eggs and embryos, 41 larvae and 84 adults. In 14 sites we failed to catch any adults, either because of timing (adults had left the water) or because the site had inaccessible parts. Tissue samples from adults and larvae were scored for four loci (with few exceptions) and embryos were scored for the three peptidase loci. The observed number of alleles was four at *Pep-A*, three at *Pep-B*, eight at *Pep-D* and three at the *Ldh-2* locus. The allele frequencies are presented in Table I. Observed genetic heterozygosity averaged at 0.20±0.11. A significant departure from Hardy-Weinberg equilibrium was observed for *Pep-D* in the sample from Valado dos Frades (population 4, $P<0.05$). No significant genetic differentiation was found between cohorts in any population. *Pep-A* showed significant population differentiation within *T. marmoratus* and *Pep-B* and *Pep-D* showed significant population differentiation within *T. pygmaeus* ($P<0.001$ in all three cases).

The PCA scores fell in two non-overlapping groups with values <-0.22 and >0.11, that we term the 'M'-group and the 'P'-group, respectively. Seven adult *T. marmoratus* had a genetic make-up that placed them in the M-group and 77 adult *T. pygmaeus* had a genetic make-up that placed them in the P-group. Common alleles with diagnostic properties are *Pep-D*^{d} and *Pep-D*^{f} and, to a lesser extent, *Pep-A*^{a} and *Pep-A*^{d}. Individuals heterozygous for the *Pep-D* diagnostic alleles were observed in the populations from Alqueidão (population 3), São Bartolomeu dos Galegos (5), Genrinhas (15), Santa Susana (16) and Fonte da Pena da Couvinha (23) (n=1 in all five cases). Less common and rare alleles associated with either group are *Pep-A*^{b}, *Pep-A*^{e}, *Pep-D*^{a}, *Pep-D*^{b} and *Ldh-2*^{f} in the M-group and *Pep-D*^{e}, *Pep-D*^{g}, *Pep-D*^{h}, *Ldh-2*^{c} in the P-group. Alleles shared between the groups are *Pep-B*^{bde}, *Pep-D*^{c} and *Ldh-2*^{b} (Table I). Correct classification on a locus-by-locus basis is very good for *Pep-D* (k=0.97) and *Pep-A* (k=0.92), moderate for *Ldh-2* (k=0.56) and poor for *Pep-B* (k=0.12), in the terminology of Altman (1991).

Table I.
Allele frequencies over four loci in Marbled newts from the Caldas da Rainha area, Portugal.
Values in parentheses are the electrophoretic mobilities relative to the most common allele that is shown as 100.
Six alleles present elsewhere in Portugal were not encountered in the Caldas da Rainha area.
Analysis of the results indicate the existence of two separate genetic units that correspond to *T. pygmaeus* (17 populations) and *T. marmoratus* (eight populations) respectively (details see text)..
Panels at the bottom present heterozygosity on the assumption of Hardy-Weinberg equilibrium and average score at the first PCA axis (details see text), with and without the locus Ldh-2.

		Triturus pygmaeus																	T. marmoratus							
Population	Total	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Sample size	226	13	19	12	22	6	8	8	9	8	1	7	10	8	2	7	3	4	9	8	10	21	10	7	4	10
adults	84	0	19	12	22	0	5	0	0	1	1	7	10	0	0	0	0	0	4	0	0	1	0	2	0	0
larvae	41	5	0	0	0	6	0	0	0	0	0	0	0	0	0	0	3	0	5	0	1	20	0	1	0	0
embryos	101	8	0	0	0	0	3	8	9	7	0	0	0	8	2	7	0	4	0	8	9	0	10	4	4	10
Locus and alelle																										
Pep-A																										
a	(122).																		0,56	0,83	0,85	0,91	0,95	1,00	1,00	1,00
b	(107).																						0,05			
d	(100).	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	0,17	0,15	0,05				
e	(78).																					0,05				
Pep-B																										
b	(113).		0,13	0,29	0,07	0,08	0,19						0,15				0,17		0,11	0,06		0,07				
d	(100).	0,38	0,76	0,46	0,66	0,92	0,75	0,81	1,00	0,94	1,00	0,93	0,60	1,00	1,00	0,79	0,83	1,00	0,89	0,94	0,95	0,88	1,00	0,93	0,88	0,75
e	(88).	0,62	0,11	0,25	0,27		0,06	0,19		0,06		0,07	0,25			0,21					0,05	0,05		0,07	0,13	0,25
Pep-D																										
a	(125).																		0,06			0,14		0,07		
b	(121).																		0,06							
c	(117).								0,50										0,06							
d	(112).			0,04		0,08										0,07	0,17		0,78	1,00	1,00	0,86	1,00	0,93	1,00	1,00
e	(106).		0,11	0,04	0,18		0,25			0,50																
f	(100).	0,96	0,84	0,71	0,71	0,92	0,56	0,94	0,50	0,50	1,00	1,00	1,00	0,94	1,00	0,79	0,83	1,00	0,06							
g	(95).	0,04	0,05		0,11			0,06						0,06		0,14										
h	(88).			0,21			0,19																			
Ldh-2																										
b	(100).	1,00	1,00	0,96	1,00	1,00	1,00			1,00		1,00	1,00				0,83		0,56		1,00	0,45		1,00		
c	(72).			0,04																						
f	(36).																0,17		0,44			0,55				
Three loci																										
He		0,19	0,23	0,38	0,32	0,11	0,35	0,15	0,18	0,22	0,00	0,05	0,20	0,04	0,00	0,25	0,22	0,00	0,38	0,14	0,12	0,22	0,03	0,10	0,08	0,13
SE on He		0,15	0,12	0,20	0,16	0,06	0,18	0,10	0,18	0,16	0,00	0,05	0,20	0,04	0,00	0,13	0,11	0,00	0,09	0,09	0,08	0,02	0,03	0,05	0,08	0,13
Weighted average									0.214±0.132													0.163±0.063				
Four loci																										
He		0,05	0,09	0,25	0,19	0,08	0,25				0,00	0,04	0,15				0,25		0,39			0,26		0,13		
SE on He		0,05	0,06	0,15	0,13	0,05	0,15				0,00	0,04	0,15				0,08		0,11			0,07		0,13		
Weighted average									0.150±0.100													0.284±0.084				
Average score along first PCA axis																										
Three loci		0,34	0,32	0,28	0,29	0,28	0,28	0,32	0,25	0,25	0,33	0,32	0,33	0,33	0,33	0,28	0,23	0,33	-0,44	-0,60	-0,66	-0,68	-0,73	-0,72	-0,72	-0,69
Four loci		0,41	0,37	0,36	0,35	0,33	0,37				0,37	0,36	0,39				0,23		-0,36			-0,57		-0,51		

The distribution of the two groups was spatially structured to the extent that, first, all ponds yielded either M- or P-group individuals and not both and second, ponds in the centre of the study area had M-group individuals whereas ponds at the fringe had P-group individuals. The average distance to the nadir point of the study area was 12.8±4.3 km for M-ponds and 14.0±7.5 km for P-ponds. Note that aspects of the spatial distribution of *T. marmoratus* and *T. pygmaeus* in western Portugal will be dealt with separately (CHAPTER 9).

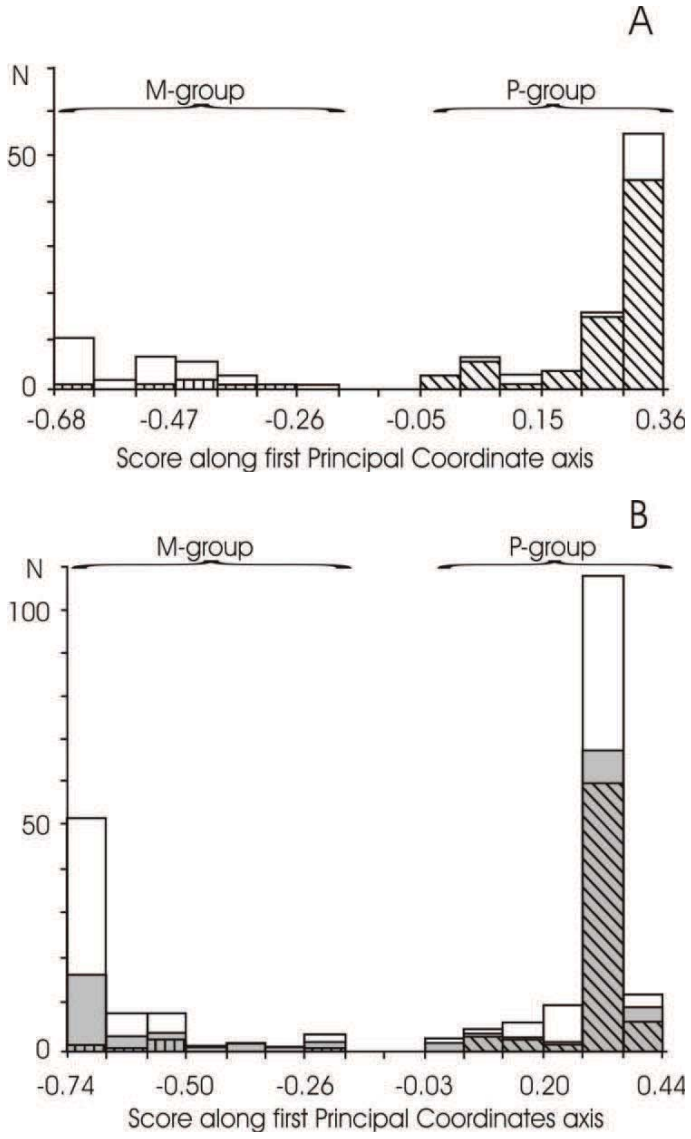

Figure 1. Histograms representing the scores along the first principal coordinate axis for enzyme genetic markers in marbled newts from the Caldas da Rainha area in western Portugal, with four enzyme loci studied in 39 larvae and 82 adults (A) and three enzyme loci studied for an additional 101 embryos, two larvae and two adults (B). Individuals in B also figuring in A are marked by grey shading. Adults identified from morphology are shown by horizontal hatching (*Triturus marmoratus*, *n*=6) and diagonal hatching (*T. pygmaeus*, *n*=76). Note that these fall into different, nonoverlapping M- and P- groups, respectively.

Discussion

We are interested in the distribution and ecology of amphibians from the Iberian Peninsula, with the particular aim to elucidate those environmental correlates that help to reconstruct, explain, predict and understand species ranges (e.g., TEIXEIRA *et al.*, 2001; ARNTZEN, 2006). This requires extensive surveying based upon reliable species identification. In Portugal and Spain, as in most other temperate regions, amphibians gather in ponds and streams for breeding which, by choosing the time and place of the fieldwork advantageously, facilitates the gathering of data. In practice, most surveys concentrate on offspring, because adult pond presence may be short, as in 'explosive breeders' (e.g. *Rana temporaria*), species that mate on land and only come to the water for offspring deposition (e.g. *Salamandra salamandra*), or species that are especially secretive (e.g. *Pelodytes punctatus*). As a rule, however, the earlier the life stage, the more problematic identification in the field proves to be. Classical keys for identification of eggs, spawn and larvae (HÉRON-ROYER and BAMBEKE, 1889; BOULENGER, 1891) have recently been upgraded (MIAUD and MURATET, 2004) and most modern field guides will include identification keys for adults, larvae and eggs (e.g., FERRAND DE ALMEIDA *et al.*, 2001; DUGUET and MELKI, 2003).

We set out to develop a molecular marker technique for the unambiguous species identification of marbled newt eggs that would allow all ponds to be investigated, including technically problematic ones such as albercas and lakes. In ponds with aquatic vegetation absent, rare or out of reach, collecting may be facilitated by the introduction of strings of thin plastic liner available for egg-deposition, cut from e. g. garbage bags. With the phenotypic identification of adult *T. marmoratus* and *T. pygmaeus* as a reference and acknowledging the equivalent allelic expression among embryos, larvae and adults, the observed 'M' and 'P' enzyme profiles can be equated with *T. marmoratus* and *T. pygmaeus*, respectively. The single case of departure from Hardy-Weinberg equilibrium, with a lower than expected number of heterozygotes, may well be attributed to a less than optimal resolution of PEP-D zymogram, perhaps caused by interference of the enzyme and the mucous components in larval tissue.

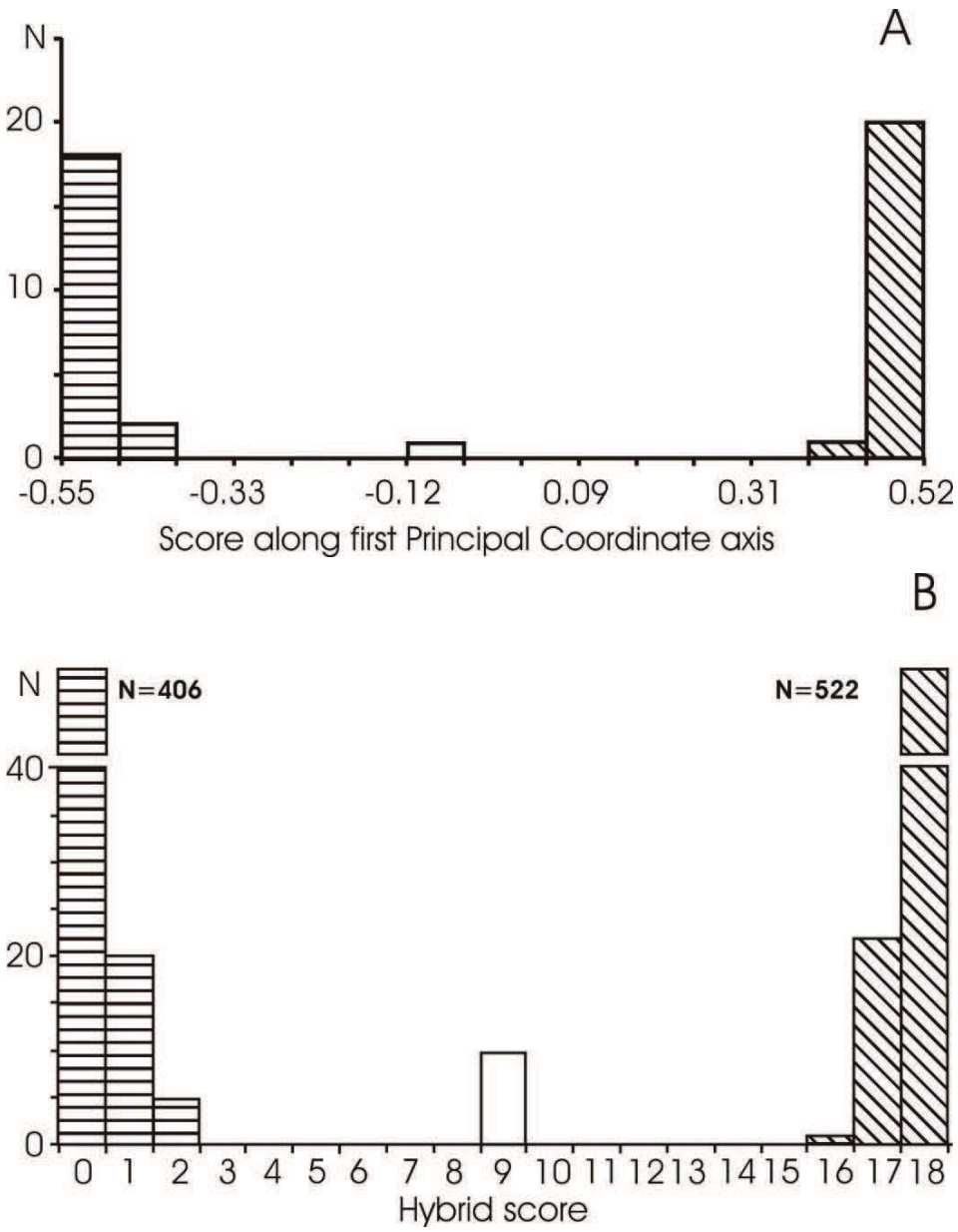

Figure 2. Histograms representing the scores along the first principal coordinate axis derived from 87 alleles in a panel of 30 variable enzyme genetic markers in the newts *Triturus helveticus* and *T. vulgaris* in Mayenne, western France (A: after ARNTZEN *et al.*, 1998) and hybrid index based on nine diagnostic enzyme genetic markers for the newts *T. cristatus* and *T. marmoratus* in the same area (B: after ARNTZEN and WALLIS, 1991).

The method of *T. marmoratus* versus *T. pygmaeus* identification through expressed proteins loci is lethal when applied to embryos. However, considering the high fecundity of marbled newts (ARNTZEN and HEDLUND, 1990) we presume that the effect will be negligible at the population level. Tissue sampling by clipping tail-tips has beenshown no ill effect in adult big-bodied newts (ARNTZEN *et al.*, 1999). The

effect of tail-tip sampling of larvae has not yet been studied. For applying our method of species identification in other parts of the Iberian Peninsula a note of warning is in place, since we observed geographic variation in the level of discrimination achieved by Pep-B and Pep-D (unpublished data). This reservation is in line with the observed population genetic differentiation in the Caldas da Rainha area for both species. An alternative technique for species identification would be the DNA sequencing of a mitochondrial gene such as COI, currently known as 'bar-coding' (HEBERT *et al.*, 2003; VENCES *et al.*, 2005). An important shortcoming of this marker is that it - in a phylogenetic sense - may not represent the species from which it is isolated correctly, due to hybridisation or incomplete lineage sorting, in combination with maternal inheritance and low effective population size compared to nuclear genetic markers. This phenomenon, that ultimately may involve the complete 'mtDNA-capture' by one species from the other, appears fairly frequent in salamanders. Discordance between the signature of mitochondrial and nuclear genetic markers has been found in various genera of plethodontid (e.g., Batrachoseps, WAKE and JOCKUSCH, 2000) and salamandrid salamanders (e.g., Salamandra, GARCÍA-PARÍS *et al.*, 2003; Taricha, KUCHTA and TAN, 2005; Chioglossa, SEQUEIRA *et al.*, 2005). Well-documented cases in big-bodied newts (genus *Triturus*) include i) the near-complete bias for *T. cristatus* mothered hybrids in *T. marmoratus* x *T. cristatus* interspecies hybrids in western France (ARNTZEN and WALLIS, 1991) and ii) the presence of mtDNA typical of *T. karelinii* in *T. carnifex* and *T. dobrogicus* over a large area of northern Serbia (WALLIS and ARNTZEN, 1989; ARNTZEN and WALLIS, 1999). An example among small bodied newts, genus *Triturus* (or *Lissotriton* or *Lophinus*) is the replacement across the entire Carpathian mountain range of the original *T. montandoni* mtDNA by that of *T. vulgaris* (BABIK *et al.*, 2005). Under the notion that flawed inferences from mtDNA may not be infrequent, its choice as a species marker was in this study *a priori* rejected. Nuclear microsatellite DNA markers have been successfully used to uncover genetic variation in *T. marmoratus* (JEHLE *et al.*, 2001; KRUPA *et al.*, 2002; JEHLE *et al.*, 2005) but in our experience, it is not easy to isolate and amplify nuclear DNA from freshly deposited embryos. This technique may require a larger number of copies of nuclear DNA than is available in this life stage, the one most frequently observed in the field and its application would involve raising the embryos in the laboratory.

The contact zone between *T. marmoratus* and *T. pygmaeus* runs over c. 600 km across central to western Iberia, from approximately Madrid in central Spain to north of Lisboa in Portugal. None of the 25 ponds around Caldas da Rainha had a mixed population and individuals with intermediate enzyme profiles were not found (Fig. 1). This suggests the absence in our sample of F_1-interspecies hybrids. On the other hand, the pattern of allozyme discrimination here revealed is flat U-shaped (Fig. 1), rather than more sharply ⎸_⎸- shaped as in the *T. helveticus* - *T. vulgaris* and *T. cristatus* - *T. marmoratus* situations (Fig. 2). The extent to which the shape of the curve represents incomplete diagnosticity of the enzyme genetic markers (as in *T. marmoratus* and *T. pygmaeus* and *T. helveticus* - *T. vulgaris*) versus gene flow has yet to be determined.

The minimum observed distance between *T. marmoratus* and *T. pygmaeus* populations in this study was 3.3 km. This contrasts to observations in Spain where the recorded minimum distance between the species was c. 26 km between Cilleros and Zarza la Mayor in western Spain and c. 6 km between Hoyo de Manzanares and Villalba in central Spain (GARCÍA-PARÍS *et al.*, 2001). With just three localities of *T. marmoratus* and eight localities of *T. pygmaeus* recorded in the province of Madrid (GARCÍA-PARÍS *et al.*, 1993) both species are locally rare and the contact zone between them has presumably deteriorated, effectively forming a residual contact zone, sensu Szymura (1993).

In the section of the *T. marmoratus* - *T. pygmaeus* distribution here considered, the contact zone between the species is firmly parapatric. Moreover, the data point to the complete or near-complete genetic isolation of the taxa, therewith supporting their specific status under the Biological Species Concept. The taxonomic change was first put forward by García-París *et al.* (2001) and implemented by e.g. Frost (2004); Montori *et al.* (2005). Our study differs from theirs in the following respects: i) a fully diagnostic panel of nuclear genetic markers, ii) utilization of the mitochondrial genetic marker rejected, iii) larger number of populations (25 versus 6), iv) small minimum inter-pond distances (3.3 km versus 6.0 km) and v) not part of a residual contact zone. All too frequent taxonomic and nomenclatorial change is proposed on the basis of a single type of data, including cases that rely on mtDNA data exclusively (e.g. Carlia, COUPER *et al.*, 2005; Salamandrina, MATTOCCIA *et al.*, 2005; Plethodon, MEAD *et al.*, 2005). This contrasts with studies that integrate evidence from two or more sources, such as morphology, allozymes, mtDNA and

nuclear DNA (e.g. Calotriton, CARRANZA and AMAT, 2005; Scaphiophryne, GLOS *et al.*, 2005; Salamandrina, NASCETTI *et al.*, 2005; Hyla,SALDUCCI *et al.*, 2005) .

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