Molecular characterization of the contact zone between *Triturus pygmaeus* and *T. marmoratus* (Caudata: Salamandridae) in Central Spain and their taxonomic assessment

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Abstract: Two closely related parapatric taxa of the *Triturus marmoratus* complex, the marbled newt, *T. marmoratus*, and the pygmy newt, *T. pygmaeus*, inhabit adjacent areas in the central regions of the Iberian Peninsula. The geographic ranges of these taxa are separated by the mountains of the Sistema Central, except in at least two areas in which *T. marmoratus* is able to colonize the southern slopes of the mountains, the Sierras de Gata and Guadarrama. Contact between *T. marmoratus* and *T. pygmaeus* is presumed to take place in these two areas, since populations of both species are separated by a distance of 6 to 12 km. Both taxa can be unequivocally diagnosed in the contact zones based on their external morphology. Diagnostic traits include size, shape of the male dorsal crest, dorsal and ventral coloration patterns, vomerine teeth arrangement, and additional osteological traits. We conducted an enzyme restriction analysis of the cytochrome b mtDNA gene, using enzyme MVN-I on individuals from both sides of the contact zone. The restriction patterns obtained strictly correspond to the expectations derived from their morphological phenotypes. All individuals with a *T. marmoratus* morphological phenotype show the MV1 restriction pattern. All individuals from the contact zones with a *T. pygmaeus* phenotype show the MV2 restriction pattern. No signs of present or past hybridization among these taxa were detected in the areas studied. Lack of natural hybridization along 300 km of presumed contact zones in Spain, marked morphological differentiation without clinal variation and the strict correspondence between morphological phenotypes and mtDNA, support the specific status of the pygmy newt, *T. pygmaeus*.

Key Words: Caudata, Salamandridae, *Triturus*, Mitochondrial DNA, Evolution, Taxonomy, Spain.

Resumen: Caracterización molecular de la zona de contacto entre *Triturus pygmaeus* y *T. marmoratus* (Caudata: Salamandridae) en el centro de España y su asignación taxonómica.- Dos tritones estrechamente emparentados del complejo de *Triturus marmoratus*, el tritón jaspeado (*T. marmoratus*) y el tritón pigmeo (*T. pygmaeus*) poseen una distribución parapátrica en la Península Ibérica. Las distribuciones de estos dos taxones corren paralelas a lo largo del Sistema Central, excepto en la Sierra de Gata y en la Sierra de Guadarrama en donde *T. marmoratus* también coloniza parte de las laderas meridionales. En esas zonas las poblaciones de *T. marmoratus* y *T. pygmaeus* están separadas por una distancia mínima de 6 a 12 km, por lo que el contacto entre ellas es muy probable. Ambas especies se distinguen claramente por su morfología externa en las zonas de contacto. Entre los rasgos diagnósticos cabe destacar diferencias en la talla, la forma de la cresta dorsal de los machos, la coloración dorsal y ventral, la disposición de los dientes vomerianos y caracteres osteológicos adicionales. Los patrones de corte obtenidos en una región del citocromo b del ADN mitocondrial con la enzima de restricción MVN-I se corresponden estrictamente con los patrones morfológicos. Todos los individuos con morfología típica de *T. marmoratus* presentaron el patrón de restricción MV1, mientras que todos los individuos de las zonas de contacto con morfología de *T. pygmaeus* presentaron el patrón de restricción MV2. La ausencia de hibridación natural a lo largo de más de 300 km de zona de contacto potencial, la existencia de una marcada diferenciación morfológica no clinal y sobre todo la correspondencia estricta entre fenotipo morfológico y ADN mitocondrial apoyan el nivel específico del tritón pigmeo, *T. pygmaeus*.

Palabras clave: Caudata, Salamandridae, *Triturus*, ADN mitocondrial, Evolución, Taxonomía, España.

INTRODUCTION

The study of contact zones between closely related parapatric taxa offers the opportunity to evaluate the degree of hybridization and reproductive isolation that has evolved between them. The results derived from such studies are critical not only to our understanding of the processes underlying speciation (HARRISON, 1991; WAKE, 1997), but also to evaluate the taxonomic status of closely related taxa under the biological species concept (sensu MAYR, 1963).

The marbled, *Triturus marmoratus* (LATREILLE, 1800), and pygmy, *T. pygmaeus* (WOLTERSTORFF, 1905), newts in the Iberian Peninsula are an example of the importance of contact zone structure in assessing the taxonomic status of parapatric taxa. Traditionally the marbled and pygmy newts were considered subspecies of the widespread taxon *T. marmoratus*. The marbled newt, *T. marmoratus* (LATREILLE, 1800), is distributed throughout western France and northern Iberia, and the pygmy newt, *T. pygmaeus* (WOLTERSTORFF, 1905), is found in the southern half of the Iberian Peninsula. Previous studies on morphology, osteology, immunology and allozymes showed a higher degree of variation than expected among populations of a single species, and similar to that reached between other species of the *T. cristatus* group (ARNTZEN in DORDA AND ESTEBAN, 1986; BUSACK *et al.*, 1988; GARCÍA PARÍS *et al.*, 1993; and unpublished data). Based on these studies, the subspecific status of *T. marmoratus* and *T. pygmaeus* has been questioned, and their recognition as distinctive species suggested (GARCÍA PARÍS *et al.*, 1993). However, without data on the contact zone, the extent and degree of introgression at the border of their distributions is unknown, and therefore the taxonomic decission as to whether these taxa should be considered separate species has been delayed to this date. Our results support previous studies and suggest that the two taxa are differentiated sufficiently to be considered distinct species, so we treat both taxa as species in the following pages.

The geographical distribution of both species runs parallel with and along more than 300 km of the axis of the Sistema Central mountain range on the Iberian Peninsula (Figure 1). Generally, *T. marmoratus* does not cross this mountain chain, and is distributed primarily along the northern slopes, while *T. pygmaeus* inhabits only the southern slopes. Disruption of this pattern is known in two areas: the Sierra de Guadarrama (Province of Madrid) and the Sierra de Gata (Province of Cáceres), where *T. marmoratus* extends into the potential range of *T. pygmaeus*. Previous studies, based on color pattern and relative size, have not identified strict sympatry in the areas of expected contact, but the minimum distance between ponds inhabited by each form is 6 km in the Sierra de Gata and 20 km in the Sierra de Guadarrama (GARCÍA PARÍS *et al.*, 1993). These ponds are connected by favourable habitat, and contact between these taxa with sympatry or hybridization is expected.

Size, coloration pattern, and osteology of *T. marmoratus* and *T.pygmaeus* are distinctive in those contact zones and individual identification of adults is unequivocal. Distinctive coloration patterns are correlated with preliminary allozyme surveys in the Sierra de Guadarrama and the Sierra de Gata, which indicated very low levels of gene flow between the two taxa (Arntzen in DORDA AND ESTEBAN, 1986; and unpublished data). In this paper we analyze the congruence between morphological patterns and mtDNA in the two contact zones, in order to evaluate the degree of hybridization or introgression between marbled and pygmy newts. The exchange of nuclear markers between gene pools is not always correlated with mitochondrial DNA exchange (MORITZ *et al.*, 1992), and may provide additional information on the existence of past events of hybridization even if gene flow is now restricted. Mitochondrial DNA has also proven to be useful in detecting and assessing amounts of introgression in other hybrid zones involving *Triturus* (ARNTZEN & WALLIS, 1991; 1999).

The marbled and pygmy newts are protected under the Spanish and international laws. Although *T. marmoratus* is not endangered over most of its range, the status of *T. pygmaeus* is of special concern along the eastern and southern borders of its range where it is suffering a recent dramatic range reduction (ASTUDILLO *et al.*, 1997).

MATERIALS AND METHODS

Sampling design

One of our goals was to develop an effective marker to allow rapid, and non-destructive screening of a large number of individuals on either side of the contact zone. In order to find an appropriate mtDNA marker, we first sequenced a portion of the mitochondrial cytochrome b (cyt b) gene from tail tips of two individuals of *T.marmoratus* and *T. pygmaeus* located far from their contact zones, in Sanabria (Province of Zamora) and Fuentes de León (Province of Badajoz) respectively (Figure 1).

Based on the differences in sequences found between *T. marmoratus* and *T. pygmaeus* (Figure 2), we identified one restriction site which could effectively discriminate between the two taxa. Samples for the restriction analysis consisted of tail tips of 51 larvae and adults, collected along the southern slopes of the Sistema Central mountain range, including the contact zones. Localities and number of individuals screened are shown in Table 1.

Cyt b sequencing

Tail tips of adult newts were used to sequence a 385 bp long cyt b fragment, corresponding to codons 7 (part)-135 of the *Xenopus* cyt b gene (ROE *et al.*, 1985). Tail tip samples were collected from living individuals, and preserved in ethanol (70 to 90 %) up to six months prior to DNA extraction. DNA extraction was performed by boiling minute amounts (<5 mg) of tissue in a 5% (w/v) solution of Chelex (BioRad). PCR amplifications were done using the cyt b specific primers cyt-b2 (KOCHER *et al.*, 1989) and MVZ15 (MORITZ *et al.*, 1992). The annealing temperature for double-strand reactions was 55°C. Double-strand reactions ran for 35 cycles in a total volume of 12.5 µl, using 0.3 units of Taq polymerase (Cetus) in tubes containing 0.5 pmol of each primer, 0.75 mM dNTPs, and 1.5 mM MgCl2 in a pH 8.4 buffer with 50 mM KCl and 10mM Tris HCl (final concentrations). Aliquots of 5ml were run on 2 % low-melting agarose gels, from which plugs were taken and diluted 1:100 in 10 mM Tris-0.1 mM EDTA. Single-strand reactions were performed using asymmetric PCR (Gyllensten & Erlich, 1988) with 1:50 primer ratios and the same reaction profiles on 25 ml reactions. Appropriate size and purity of single-strand products was assessed by electrophoresis of 3 ml aliquots in 4% agarose gels. Dideoxy chain termination sequencing reactions (Sanger et al., 1977) were performed using U. S. Biochemicals Sequenase version 2.0 and S^{35}-labelled dATP. Extractions and double- and single-strand PCR reactions included negative controls to test for contamination of reagents with foreign DNA. Sequences were manually aligned to a corresponding region of *Salamandra* mtDNA (GARCÍA-PARÍS *et al.*, 1998). GenBank accession numbers for the sequences are AY046081-AY046082.

Restriction analysis

The use of the restriction enzyme MVN-I (Boehringer-Mannheim), which recognizes a 4 base pair nucleotide sequence (CGCG), provides an inexpensive but efficient discrimination of amplified cyt b products from *T. marmoratus* and *T. pygmaeus* in the contact zones.

Preservation of samples and DNA extraction followed the procedures given above for the sequencing analysis. Primers and conditions for PCR amplification were also the same. 5 ml of the PCR product were digested with MVNI (37°C for 4 hrs) and the digests

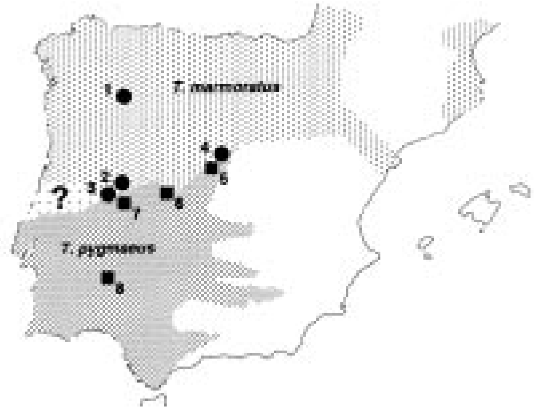


Figure 1.- Map of the Iberian Peninsula showing the geographic range of *Triturus marmoratus* and *T. pygmaeus* and sampling localities for mtDNA sequencing (seq) or restriction analysis. The localities (bold type) and number of specimens studied per locality are: *T. marmoratus*: (**1**) Sanabria (Zamora, Spain) (n=1, seq), (**2**) Valverde del Fresno (Cáceres, Spain) (n=14), (**3**) Cilleros (Cáceres, Spain) (n=1), (**4**) El Berrueco (Madrid, Spain) (n=10); *T. pygmaeus*: (**5**) Hoyo de Manzanares (Madrid, Spain) (n=5), (**6**) Losar de la Vera (Cáceres, Spain) (n=2), (**7**) Zarza La Mayor (Cáceres, Spain) (n=19), (**8**) Fuentes de León (Badajoz, Spain) (n=1, seq).

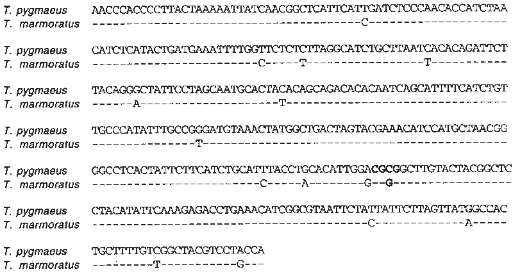
Figura 1.- Mapa de la Península Ibérica con la distribución geográfica de *Triturus marmoratus* y *T. pygmaeus*, las localidades de procedencia de los ejemplares utilizados para la obtención de secuencias (sec) para el análisis de restricción. Las localidades (en negrilla) y el número de ejemplares estudiados por localidad son: *T. marmoratus*: (**1**) Sanabria (Zamora) (n=1, sec), (**2**) Valverde del Fresno (Cáceres) (n=14), (**3**) Cilleros (Cáceres) (n=1), (**4**) El Berrueco (Madrid) (n=10); *T. pygmaeus*: (**5**) Hoyo de Manzanares (Madrid) (n=5), (**6**) Losar de la Vera (Cáceres) (n=2), (**7**) Zarza La Mayor (Cáceres) (n=19), (**8**) Fuentes de León (Badajoz) (n=1, sec).

were separated electrophoretically on horizontal 2% agarose gels alongside standard DNA markers (123 DNA ladder-Sigma).

RESULTS

Sequence divergence (KIMURA 2-parameter distance, KIMURA, 1980) between *T. marmoratus* and *T. pygmaeus* was 4.04 %. The 385 bp sequences included 15 variable positions. One of these sites was used successfully to discriminate between *T. marmoratus* and *T. pygmaeus* using restriction analysis (Figure 2).

The pattern obtained from enzymatic digestion of the amplified products of the cyt b gene was used to determine the mtDNA type of 51 individuals from both sides of the contact areas. The results of the restriction enzyme analysis are shown in Table 1. Two different restriction patterns were detected, one in which the enzyme did not cut the PCR amplified fragment of cyt b (MVN1-), designated here as MV1, and the other in which the enzyme cut the amplified fragment (MVN1+), designated as MV2. All individuals screened from the contact zone exhibited the mitochondrial haplotypes expected from their morphological phenotypes. All individuals with a *T. marmoratus* colouration pattern showed the MV1 restriction pattern and all individuals from the contact zone with a *T. pygmaeus* morphology showed the MV2



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T. pygmaeus     AACCCACCCCTTACTAAAAATTATCAACGGCTCATTCATTGATCTCCCAACACCATCTAA
T.  marmoratus  ---------------------------------------C--------------------

T. pygmaeus     CATCTCATACTGATGAAATTTTGGTTCTCTCTTAGGCATCTGCTTAATCACACAGATTCT
T.  marmoratus  ------------------------C-----T-----------------T-----------

T. pygmaeus     TACAGGGCTATTCCTAGCAATGCACTACACAGCAGACACACAATCAGCATTTTCATCTGT
T.  marmoratus  ------A--------------------T--------------------------------

T. pygmaeus     TGCCCATATTTGCCGGGATGTAAACTATGGCTGACTAGTACGAAACATCCATGCTAACGG
T.  marmoratus  ---------------T--------------------------------------------

T. pygmaeus     GGCCTCACTATTCTTCATCTGCATTTACCTGCACATTGGACGCGGCTTGTACTACGGCTC
T.  marmoratus  ------------------------C-----A--------G--G-----------------

T. pygmaeus     CTACATATTCAAAGAGACCTGAAACATCGGCGTAATTCTATTATTCTTAGTTATGGCCAC
T.  marmoratus  ----------------------------------------C-------------A-----

T. pygmaeus     TGCTTTTGTCGGCTACGTCCTACCA
T.  marmoratus  ---------T-----------G---
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Figure 2.- Sequences of 385 base pair length of the cytochrome b (mtDNA) corresponding to the haplotypes found in *T. marmoratus* (Sanabria, Zamora) and *T. pygmaeus* (Fuentes de León, Badajoz). Bold type indicate the portion of sequence where the enzyme MVNI breaks the chain.

Figura 2.- Sequencias de 385 pares de bases de longitud del citocromo b (ADNmt) correspondientes a los haplotipos encontrados en *T. marmoratus* (Sanabria, Zamora) y *T. pygmaeus*.(Fuentes de León, Badajoz). En negrilla se indica la porción de la secuencia donde la enzima MVNI corta la cadena.

Table 1: Restriction enzyme analysis. *MVN* patterns obtained, populations and number of individuals screaned.
Tabla 1: Análisis mediante enzimas de restricción. Patrones de corte con MVN-1, número de individuos y poblaciones examinadas.

Population	**N**	**cytb mtDNA**		**Phenotype**
		MVNI- MV1	**MVNI+ MV2**	
Eastern contact zone				
Hoyo de Manzanares	5	–	5	*T. pygmaeus*
El Berrueco	10	10	–	*T. marmoratus*
Western contact zone				
Losar de la Vera	2	–	2	*T. pygmaeus*
Zarza la Mayor	19	–	19	*T. pygmaeus*
Cilleros	1	1	–	*T. marmoratus*
Valverde del Fresno	14	14	–	*T, marmoratus*

restriction pattern. Therefore, we found no signs of past or present hybridization between *T. marmoratus* and *T. pygmaeus* in the contact zone.

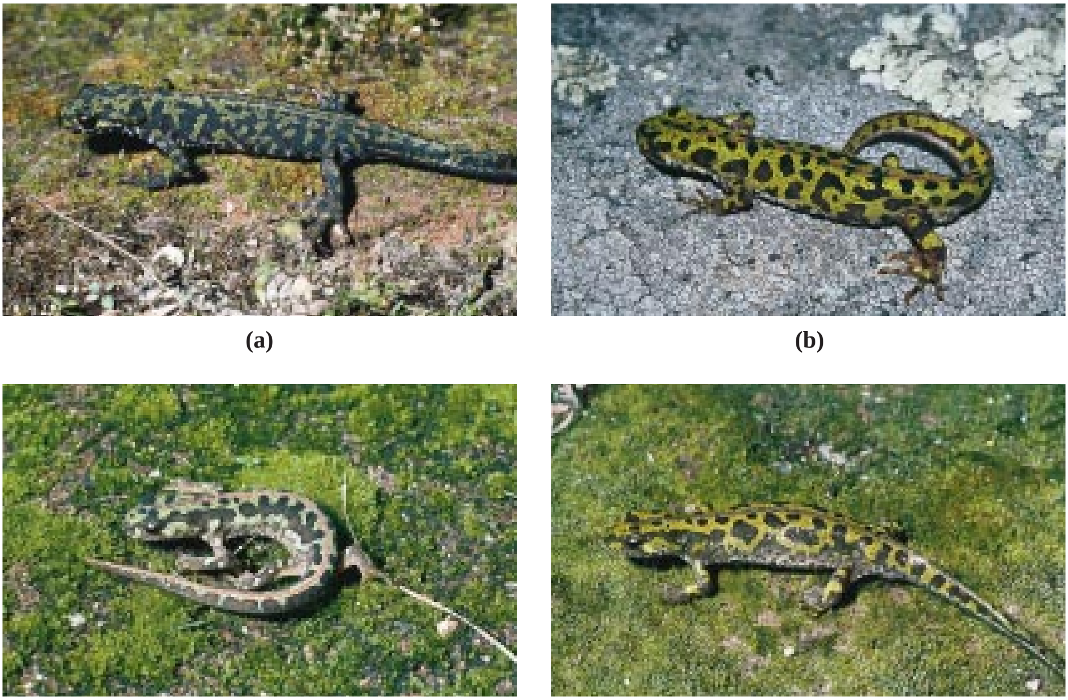
Molecular data gathered along the contact zone between *T. marmoratus* and *T. pygmaeus* favor the taxonomic recognition of both taxa at the species level, as discussed below. *Triturus pygmaeus* was originally described as a small form of *T. marmoratus* (Wolterstorff, 1905), long ignored, and subsequently redescribed as a subspecies (GARCÍA-PARÍS *et al.*, 1993). Both morphological descriptions were based on specimens from the southernmost regions of Spain in the Province of Cádiz. GARCÍA-PARÍS *et al.* (1993) indicated that size, dorsal coloration and the extent of ventral white dotting were variable within *T. pygmaeus*. Despite this variability, populations of *T. pygmaeus* located in or around the putative contact zones, are homogeneous morphologically, enabling indentificación of both taxa in the areas where they are geographically close. In the following paragraphs we redescribe the morphological diagnostic characters of *T. pygmaeus* adding notes on their variability, using the material from the Appendix of GARCÍA-PARÍS *et al.* (1993).

***Triturus (Triturus) pygmaeus* (Wolterstorff, 1905)**

Triton marmorata forma *pygmea* Wolterstorff, 1905: 260.
Triturus marmoratus pygmaeus (Wolterstorff): Mertens & Müller, 1928: 12.

Diagnosis and variation

The smallest species of the subgenus *Triturus*, with an average snout-vent length of 106.5 mm for males, and 116.5 mm for females. Snout-vent length can be used as a diagnostic character because there is little overlap between *T. marmoratus* and *T. pygmaeus* (GARCÍA-PARÍS *et al.*, 1993). The dorsal coloration pattern is variable within *T. pygmaeus*. The southernmost populations (Figure 3 a) show a marbled or reticulate pattern very similar to *T. marmoratus*, while the northern ones show a characteristic spotted pattern (Figure 3 b) formed by black to brown spots over a bright green background. The spotted pattern is basically constant in most the populations studied from Extremadura, Madrid, Castilla - León, Castilla - La Mancha, northern Andalucía (Sierra Morena), and Portugal. This pattern is less marked south to Sierra Morena where populations are transitional to



(a) (b)

(c) (d)

Figure 3.- Dorsal coloration variability in *T. pygmaeus*. A. Habitus of typical *T. pygmaeus* from Los Barrios, Province of Cádiz, showing the southern marbled pattern. B. Habitus of *T. pygmaeus* from Mérida, Province of Badajoz, with the characteristic northern spotted pattern (Photograph courtesy of I. Martínez-Solano). C. Dorsal pattern of a juvenile specimen from Gerena, Province of Sevilla, showing an intermediate pattern between northern and southern *T. pygmaeus*. D. Dorsal coloration of a female specimen from Zafarraya, Province of Granada, presenting an intermediate pattern between the spotted and the reticulated *T. pygmaeus* coloration patterns.
Figura 3.- Variabilidad en la coloración dorsal de *T. pygmaeus*. A. Aspecto de un ejemplar típico de *T. pygmaeus* de Los Barrios, Cádiz, con la coloración jaspeada característica de las poblaciones meridionales. B. Aspecto de un ejemplar de *T. pygmaeus* de Mérida, Badajoz, con el patrón moteado característico de las poblaciones septentrionales (Fotografía de I. Martínez-Solano). C. Coloración dorsal de un ejemplar juvenil de Gerena, Sevilla, con un patrón intermedio entre el típico jaspeado meridional y el moteado septentrional de *T. pygmaeus*. D .Coloración dorsal de una hembra de Zafarraya, Granada, que también presenta un patrón intermedio entre el típico jaspeado meridional y el moteado septentrional de *T. pygmaeus*.

the southern marbled pattern (Figure 3c, d). The spotted pattern is diagnostic of *T. pygmaeus* along the putative contact zones with *T. marmoratus* in central Spain. The marbled pattern of *T. marmoratus* becomes diffuse occasionally in males during the aquatic phase, sometimes resembling northern *T. pygmaeus*. The ventral coloration pattern is diagnostic for *T. pygmaeus*. It is typically creamy yellow marked with rounded large black spots and small white short flecks. Extensive variation exists on the shape and density of the white flecks, which occasionally may cover the entire ventral surface (Figure 4a, b).

The dorsal crest of aquatic males is relatively low, linear, and with very reduced or without any inflexion at the pelvic level. In contrast, *T. marmoratus* males show a high, crenulated crest with an evident inflexion in the pelvic region.

(a) **(b)**

Figure 4.- Ventral coloration variability in *T. pygmaeus*. A. Characteristic ventral coloration of *T. pygmaeus* (Hoyo de Manzanares, Province of Madrid). B. Variation involving shape and density of the white flecks, which almost cover the entire ventral surface (Los Barrios, Province of Cádiz).

Figura 4.- Variabilidad en la coloración ventral de *T. pygmaeus*. A. Coloración ventral característica de *T. pygmaeus* (Hoyo de Manzanares, Madrid). B. Variación en la coloración ventral que incluye un incremento en el tamaño y densidad de las manchas blancas, que cubren casi completamente toda la superficie ventral (Los Barrios, Cádiz).

Triturus pygmaeus and *T. marmoratus* differ in osteological characters, including proportions of limb and cranial bones, and in the degree of ossification of hyoid and ypsiloid cartilages (García-París, unpublished). A study of these characters, which are difficult to see in field studies, will be published elsewhere. The pattern of the paired two rows of vomerine teeth allows ready discrimination between *T. marmoratus* and northern *T. pygmaeus* along the putative contact zone. Northern *T. pygmaeus* from Madrid, Extremadura, and Castilla - La Mancha, show a characteristic inverted “Y” arrangement of the two rows of vomerine teeth (Figure 5 a). The rows run in parallel for approximately the first third of their length, then diverge posteriorly, forming a marked angle. The rows of vomerine teeth in *T. marmoratus* and southern *T. pygmaeus* show a “V” pattern (Figure 5 b), in which the rows diverge progressively without forming an internal angle.

Geographic range

Triturus pygmaeus is an endemic species of southern Spain and Portugal. The geographic ranges of *T. marmoratus* and *T. pygmaeus* in Spain are separated by the mountains of the Sistema Central, with the exceptions of the basin of the Lozoya River in the Sierra de Guadarrama, province of Madrid, and the Sierra de Gata in the province of Cáceres, in which *T. marmoratus* is present along the southern slopes, and the Puerto de Malagón in Madrid where *T. pygmaeus* moves into the mountains. In Portugal the northern limit of *T. pygmaeus* is unknown, and the existence of contact zones is likely in the central part of the country. Recent deliberate introductions of *T. pygmaeus* in the geographic range of *T. marmoratus* and vice-versa, are known in Madrid region (unpublished); these may seriously affect future genetic studies if hybridization between both taxa is possible.

Taxonomic comments

The coloration pattern and some osteological features are indicative of the existence of two geographically separated groups of populations within *T. pygmaeus*: A northern group characterized by the spotted pattern with “Y” shaped rows of vomerine teeth, and a southern group with a marbled color pattern and “V”


(a) (b)

Figure 5.- Vomerine teeth arrangement in *T. pygmaeus*. A. Teeth series ranged forming an inverted "Y" pattern characteristic of northern *T. pygmaeus* (Villalba, Madrid). B. Inverted "V" pattern characteristic of southern *T. pygmaeus* (Los Barrios, Cádiz), shared with *T. marmoratus*.
Figura 5.- Disposición de los dientes vomerianos en *T. pygmaeus*. A. Patrón característico de las poblaciones septentrionales de *T. pygmaeus* (Villalba, Madrid), con las series de dientes dispuestas en "Y" invertida . B. Patrón característico de las poblaciones meridionales de *T. pygmaeus* (Los Barrios, Cádiz), con las series de dientes formando una "V" invertida; este patrón en "V" lo comparten con *T. marmoratus*.

shaped teeth rows. The morphological transition between northern and southern population groups is not abrupt. The northern spoted pattern changes progressively to the southern marbled one, probably representing a cline. However, sampling at the osteological level is not sufficient to evaluate the transition between the two morphs.

Conservation status

Number and density of populations as well as availability of breeding sites are extremely variable along the geographic range of *T. pygmaeus*. Most of the western regions, including central and southern Portugal, and Extremadura, western Castilla-La Mancha and western Andalucía in Spain, seem to maintain healthy and numerous populations. The number of populations decreases abruptly in the drier eastern regions with limestone substrates, where current distribution gaps may surpass 60 km (ASTUDILLO *et al.*, 1997). Recent local extinctions have been detected in Castilla-La Mancha, especially in Ciudad Real and Albacete. This province represents the most extreme case, where a single known population remains (M. PARÍS *et al.*, unpublished report). A similar situation occurs in the provinces of Granada, Málaga and Jaén (Andalucía), where very few populations, most of them threatened, are known. Causes for the decline of the species in the eastern regions might be sought in the depletion of underground aquifers by over-exploitation, which causes the disappearance of breeding sites associated with springs and small streams. The destruction of traditional cattle or sheep throughs, and irrigation and washing tanks as a consequence of changes in land uses is also causing the elimination of many potential breeding sites. Finally, chemical pollution by pesticides or fertilizers, is affecting populations in the uplands of Granada (Zafarraya), Ciudad Real, Córdoba and Jaén provinces, while a dramatic recent urban expansion along mid-elevation areas of the Sistema Central Mountains in Madrid (Sierra de Guadarrama) and Avila (Valle del Tiétar), coupled with exotic fish and crayfish introductions, have already destroyed a large number of populations. The species is thus facing a dramatic reduction along its eastern and northern ranges, which in itself is enough to warrant endangered status for this southern Iberian endemic and apply urgently local conservation measures, including its listing in the regional and national endangered species lists as the Comunidad of Castilla-La Mancha has already done.

DISCUSSION

Hybridization among members of the *Triturus* clade is common (VALLÉE, 1959; CRNOBRNJA *et al.*, 1989). The hybrid zone between *T. marmoratus* and *T. cristatus* in northern France has been known for nearly a century and moves according to landscape changes and the ecology of the species involved (SCHOORL & ZUIDERWIJK, 1981; ARNTZEN & WALLIS, 1991). The fact that *T. marmoratus* hybridizes with *T. cristatus*, a species well-differentiated at both genetic and morphological levels (MACGREGOR *et al.*, 1990), suggests that hybridization between *T. marmoratus* and its closest relative, *T. pygmaeus*,is to be expected. In fact, captive breeding experiments (Herrero, unpublished) indicate that the two taxa hybridize in captivity, and therefore intrinsic prezygotic mechanisms of genetic isolation do not appear to exist between them. Sequence divergence between *T. marmoratus* and *T. pygmaeus* (4.0 %) is small in comparison to that between *T. marmoratus* and *T. cristatus* (17.5 % to 21.4 %) for the same DNA region (unpublished). However, despite the evidence suggesting that these taxa can hybridize, we have detected no genetic evidence of natural hybridization in Central Spain.

Lack of hybridization between *T. marmoratus* and *T. pygmaeus* may be a consequence of recent contact coupled with differential habitat use. As a rule, *T. pygmaeus* inhabits open areas in central Spain, corresponding to potential forest of *Quercus ilex* (RIVAS MARTÍNEZ, 1982), within the Mesomediterranean and Supramediterranean regions, whereas *T. marmoratus* is found most frequently in forested areas within the Supramediterranean region, where the dominant oak is *Quercus pyrenaica* (GARCÍA-PARÍS *et al.*, 1993). Evidence that *T. marmoratus* has strict and specialized habitat requirements derives from the displacement undergone by this species in areas of northern France where it is syntopic with *T. cristatus* (ARNTZEN & WALLIS, 1991). Different ecological requirements and habitat preferences could explain why we have not found areas of strict sympatry between *T. marmoratus* and *T. pygmaeus*. However, while it seems clear that *T. cristatus* is more successful than *T. marmoratus* in areas of sympatry (SCHOORL & ZUIDERWIJK, 1981), no data are available for the similar situation with *T. pygmaeus*. Although habitat segregation may play an important role in preventing contact, *T. marmoratus* and *T. pygmaeus* use a broad range of reproductive sites, from ditches, cattle troughs and ponds to rivers and small reservoirs. Given that they have been observed breeding in ponds only 6 km apart from each other, without any evident barrier to dispersal and more or less continuous suitable habitat between them (GARCÍA-PARÍS *et al.*, 1993), differential habitat use alone is likely not enough in itself to explain lack of hybridization.

Another barrier to hybridization between *T. marmoratus* and *T. pygmaeus* could be represented by differences in courtship and reproductive periods. Studies in progress on the courtship of *T. pygmaeus* suggest that there are marked differences with respect to *T. marmoratus* (HIDALGO *et al.*, 2000). The breeding season of *T. pygmaeus* in southern Spain is usually between November and February (DÍAZ-PANIAGUA *et al.*, 1996) while, in northern Portugal, *T. marmoratus* breeds from February until May, or even later in the case of high altitude populations (CAETANO *et. al.*, 1985). *Triturus pygmaeus* attains sexual maturity earlier and at smaller sizes than *T. marmoratus* (CAETANO and CASTANET, 1993; DÍAZ-PANIAGUA *et al*., 1996).

Sequence divergence between *T. marmoratus* and *T. pygmaeus* is relatively low, 4.0 %. Using the estimate of 0.8 % cyt b divergence per million years proposed by Tan & Wake (1995) for the salamandrid genus *Taricha*, we suggest that the split between *T. marmoratus* and *T. pygmaeus* happened about 3.2 My ago. This age is more recent to that proposed for the split among the species of the *T. cristatus* group (Kalezic & Hedgecock, 1980; Macgregor & Sessions, 1986). The hypothesis of a Pliocene split between *T. marmoratus* and *T. pygmaeus* along the Guadalquivir River Basin, which has been proved to be an important barrier for amphibian dispersal during the Miocene and Pliocene periods (LÓPEZ MARTÍNEZ, 1989; ARNTZEN & GARCÍA-PARÍS, 1995; GARCÍA-PARÍS *et al.*, 1998; GARCÍA-PARÍS & JOCKUSCH, 1999), would require local extinction of *T. marmoratus* fom the land included between the Guadalquivir River and its present range, and the recent colonization of that geographic region by *T. pygmaeus*.

A combination of recent arrival for *T. pygmaeus* and specific habitat requirements for *T. marmoratus* may be responsible for the lack of microsympatry or hybridization observed. Testing this hypothesis would require extensive sampling in the westernmost area of contact between these taxa: the slopes of the Serra da Estrela and the coastal region adjacent to it in central Portugal. These areas, where the presence of marbled newts is widely documented (CRESPO & OLIVEIRA, 1989), do not present any geographic barrier against free north-south movements, and habitat structure is less sharply defined than in continental central Spain, due to the increase in humidity and reduced seasonal contrasts provided by the proximity to the sea.

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