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A subspecies of marbled newt (*Triturus marmoratus*) in the Iberian Peninsula newly resolved from congruent nuclear and mitochondrial DNA data

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Abstract

The herpetofauna of the Iberian Peninsula is relatively well-researched, yet detailed studies, at least in part relying on molecular genetic data, continue to reveal taxa new to science, mostly species and subspecies. Newts of the genus *Triturus* are one such group with undiscovered yet taxonomically relevant variation, as shown by the recent description of new (sub)species of pygmy newts (*T. pygmaeus*, *T. rudolphi*). The marbled newt, *Triturus marmoratus*, shows an equally deep and geographically coherent spatial-genetic diversification. It is here shown that a northern and a southern group are characterized by different mitochondrial DNA profiles and are also differentiated in morphometry and colouration pattern. With no firm evidence for selection against intermediate genotypes, the southern group is described at the subspecies level, as *T. marmoratus harmannis* **ssp. nov.** The subspecies' contact zone is situated at ca. 41.5 northern latitude and stretches from the Atlantic coast near Porto, Portugal to the northeast of Madrid, Spain.

Keywords

colouration pattern – gene capture – haplotypes – mitochondrial DNA – morphometrics – pygmy newts – reciprocal monophyly – species status – *Triturus marmoratus harmannis* **ssp. nov.**

Introduction

Biological diversity is not homogeneously distributed across the planet and outstandingly rich areas are known as biodiversity hotspots (Myers et al., 2000). The Mediterranean basin is one of those 25–36 global biodiversity hotspots. Therein, the Iberian Peninsula contains more than 50% of the recognized European vertebrate and plant species and at ca. one-third of the total for plants and vertebrates, the endemism rate is also high (Médail & Quézel, 1997; Williams et al., 2000). Among amphibians and reptiles, the Iberian Peninsula is by now relatively well-researched and well-documented, probably contributing to the documented pattern. I here delve into the morphological and genetic variation of western Mediterranean large-bodied newts (genus *Triturus*). The data so far resolved taxonomically relevant variation in pygmy newts, leading to the recognition of the species *T. rudolphi* Arntzen, 2024, as different from *T. p. pygmaeus* (Wolterstorff, 1905) and the subspecies *T. p. lusitanicus* Arntzen, 2023. However, within marbled newts, *Triturus marmoratus* (Latreille, 1800), two groups with coherent geographical ranges are also apparent (Kazilas et al., 2024). It is here investigated if the documented nuclear genetic differentiation of *T. marmoratus* has parallels in morphometry, colouration pattern and mitochondrial DNA, to which the answer is affirmative. The data obtained so far suggest the presence of a wide transition area between the groups. With no evidence for selection against admixed genotypes, the new taxon is described at the subspecies level. Given that the type locality of *T. marmoratus* is situated at the very north of the species range ('Paris'; Mertens and Müller, 1928) it is the southern taxon that requires recognition, for which the formal description (as *Triturus marmoratus harmannis* ssp. nov.) is in the Appendix.

Materials and methods

Fieldwork and morphological data

Fieldwork was carried out in France, Portugal and Spain under licenses as appropriate (see acknowledgements). Newts were captured by dip-netting or with funnel traps and released on the spot immediately after processing. Adults were measured and studied for their dorso-lateral colour pattern (N_{links}). Small amounts of tissue for molecular genetic analyses were taken as larval or adult tail tips and stored on 95% ethanol. Alternatively, recently deposited eggs (embryos) were raised in 5l buckets until hatching.

Measurements made were snout-vent length from the tip of the snout up to and including the insertion of the hind leg, or the posterior side of the cloaca (SV1, SV2), head length and head width (HL, Hw), interlimb distance (ILD), fore- and hindlimb length (FL, HLL) and third finger and fourth toe length (TFL, FTL). Measurements were taken in mm with a plastic ruler or on plasticized graph paper for SV1 and ILD, and with plastic Vernier callipers at 0.1 mm precision for the other characters. Extremities were measured at the right side of the body. The measurement SV2 was only used for descriptive purposes. To facilitate data interpretation and for comparability with published data, body proportions were calculated from untransformed data as *character*/SV1. To further reduce the effect of overall size and to increase normality of the data, the standardized residuals of the regression of $\ln(\text{character})$ to $\ln(\text{SV1})$ were used in multivariate analysis.

N_{links} is counted as the number of uninterrupted connections over the green coloured surface, from the position of the crest in males or the mid-dorsal line in females to the belly, over the region in between the insertions of the fore- and hindlegs, with numbers summed for the left and right side of the body (Arntzen,

2018). Analyses of morphological data were restricted to four groups of marbled newts (*T. marmoratus*) defined by two sexes and two inferred subspecies. Statistical analyses were done with SPSS 26 (IBM SPSS, 2019).

Mitochondrial and nuclear genetic data

DNA was extracted from the preserved tissue samples under standard protocols with the Kingfisher™ (Thermo Scientific) and DNeasy extraction kits (Qiagen, Valencia, CA, USA). A fragment of the mitochondrially encoded NADH dehydrogenase 4 gene (ND4) was amplified and sequenced with established primers, also following standard protocols (Wielstra and Arntzen, 2011; Wielstra et al., 2013). Sequences were aligned with the web version of Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo>) under default settings and the result was exported in Nexus format. Sequences were trimmed to 600 bp to fit an earlier analysis and analysed by the minimum spanning network procedure (Bandelt et al., 1999) as supported in PopArt software (Leigh & Bryant, 2015) under default settings. This revealed the presence of six main haplotypes that each accommodate >2% of the data. A distance matrix was calculated in PAUP*4 (Swofford, 2003) for all individual sequences that were then allocated to the haplogroups H1–H6, *in casu* the representative main haplotype to which the uncorrected p-distances was smallest. For each population the dominant haplogroup (i.e., the one observed at highest frequency) was determined. It has been documented that the species border between marbled and pygmy newts is sharp, with limited hybridization and introgression (e.g., Arntzen, 2018). Yet, mtDNA haplotypes typical for one species were regularly found associated with the nuclear genome of the opposite species (Wielstra et al., 2013; Kazilas et al., 2024; present paper), but the analysis of interspecific cyto-nuclear discordances is left for a forthcoming article.

I reanalysed the nuclear gene capture data published by Kazilas et al. (2024), with the aims to estimate the geographical ranges of (four) taxa that were resolved as reciprocally monophyletic groups, and to scrutinize the intermediate status of several individuals that a Structure analysis had identified as mildly admixed. Analyses were done with H1est software (Fitzpatrick, 2012) and were restricted to markers with largely diagnostic properties for non-admixed reference samples. The panel contained 32 markers for within *T. marmoratus*, with a Cohen's kappa (κ) measure of (dis) association as $(1-\kappa) > 0.70$ and 33 markers for *T. pygmaeus* and *T. rudolfi* with $(1-\kappa) > 0.80$.

Blanket distribution maps composed of so-called Dirichlet cells (Matérn, 1979; Burroughs et al., 2015) were obtained by the spatial interpolation of taxon and haplogroup information with the 'NearestPoint' procedure in ILWIS 3.8.6 (ILWIS, 2019), with a maximum extrapolation of ca. 90 km. The map derived from the gene capture data was used to allocate *T. marmoratus* populations not studied by Kazilas et al. (2024) to either the nominotypical subspecies, the new subspecies, or to the class of intergrades. The latter group was excluded from the morphological data analysis on account of its sparse representation.

Results

The gene capture data resolved two species of pygmy newts (*T. rudolfi* and *T. pygmaeus*) as well as two spatially coherent groups of marbled newts (*T. marmoratus*) (fig. 1). One individual pygmy newt standing out in the triangular H1est plot is allocated to *T. rudolfi*, in line with its origin from Serra de Sintra at the south of the species range. Another individual genetically akin to *T. m. harmannis* ssp. nov. has a coastal position and yet another three akin to *T. m. marmoratus* have an inland geographical position.

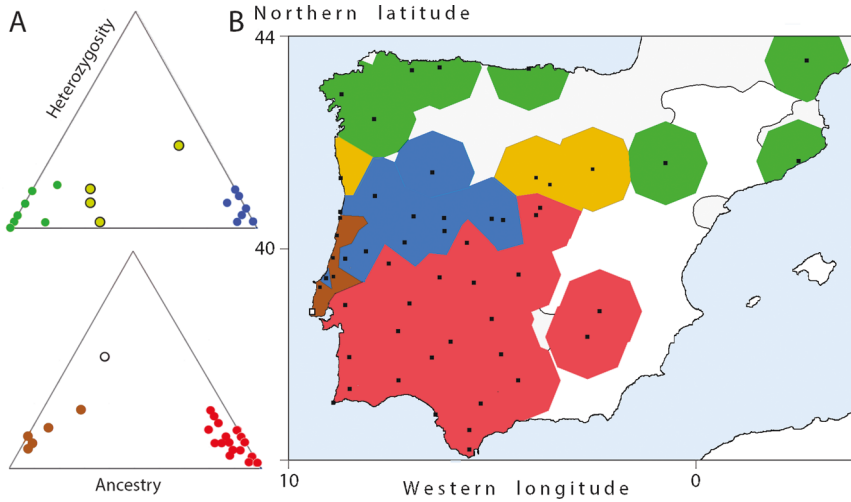


FIGURE 1 Classification and geographical distribution of European marbled and pygmy newts from a panel of 32–33 nuclear genetic markers (data from Kazilas et al., 2024). (A) Hiests plot with ancestry and heterozygosity for within marbled newts (top panel, with *Triturus m. marmoratus* left and *T. m. harmannis ssp. nov.* to the right) and for pygmy newts (bottom panel, with *T. rudolfi* to the left and *T. pygmaeus* to the right). (B) Investigated Iberian populations shown by black dots with surrounding areas coloured as in A. Areas shown in white fall outside the documented range of the *T. marmoratus* species group and areas in shown grey are distant from a sampled locality. The open square symbol in the Lisbon Peninsula corresponds to the open round symbol in A.

Of all adult marbled newts with N_{links} data, 167 individuals from 16 populations were allocated to *T. m. marmoratus* and 635 individuals from 36 populations were allocated to *T. m. harmannis ssp. nov.* Both subspecies show a wide range of values and have the same modal value ($N_{\text{links}} = 0$). However, character state distributions are different (Median Chi² test, d.f. = 1, Chi = 33.2, $P < 0.0001$), with on average higher values for *T. m. marmoratus* ($\tilde{N}_{\text{links}} = 3.0$) than for *T. m. harmannis ssp. nov.* ($\tilde{N}_{\text{links}} = 1.7$) (fig. 2A). The difference between the averages ($\Delta\tilde{N}_{\text{links}}$) is thus 1.3. For 28 well-sampled populations ($N \geq 10$) a full separation among subspecies is achieved, with a cut-off point at $\tilde{N}_{\text{links}} = 2.3$ (fig. 2B).

Of all populations with morphometric data, five populations were allocated to *T. m. marmoratus* (70 males and 80 females) and 46 were allocated to *T. m. harmannis ssp. nov.* (247

males and 338 females). Body size is smaller in *T. m. harmannis ssp. nov.* than in *T. m. marmoratus*. The relative sizes of extremities are shorter and head sizes larger in *T. m. harmannis ssp. nov.* than in *T. m. marmoratus* (table 1). A discriminant analysis of size corrected morphometric data retrieved a substantial differentiation across subspecies and sexes, more pertinent in females than in males (fig. 3). The first discriminant axis has high loadings (L) for the extremities (TFL, FLL, FTI and HLL, $0.48 < L < 0.82$) and not the other characters ($0.00 < L < 0.13$) whereas the second axis has the highest loadings for HI (L = 0.68) and Hw (L = 0.72).

A total of 3324 ND4 sequences was obtained for altogether 278 populations of *T. marmoratus*, *T. pygmaeus* and *T. rudolfi*, increasing the data for Iberian *Triturus* species by up to two orders of magnitude. Sequences were

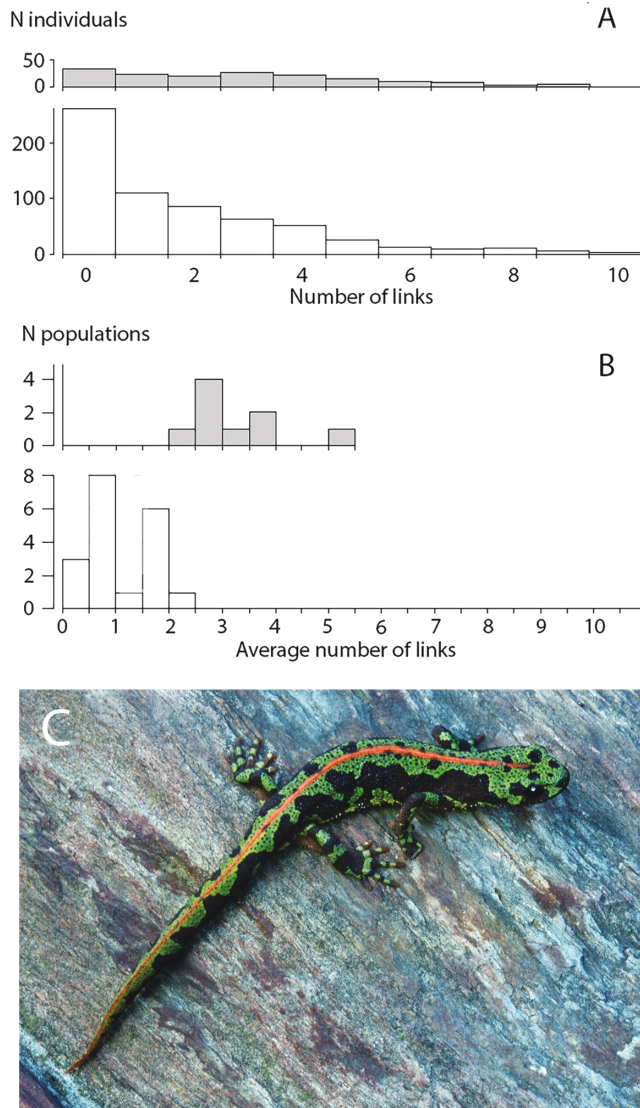


FIGURE 2 Histograms of N_{link} values observed for adults of *Triturus m. marmoratus* ($N = 167$, shaded bars) and *T. m. harmanniss ssp. nov.* ($N = 635$, open bars), with (A) all data and (B) averages for populations with a sample size ≥ 10 . (C) Juvenile *T. marmoratus* from the subspecies transition area (Poço do Inferno near Valongo, Portugal). The photo aims to illustrate the species, and to discuss the usefulness of the character N_{links} for subspecies identification (details see text).

dissimilar for pygmy versus marbled newts (fig. 4). *Triturus pygmaeus* and *T. rudolfi* fall in the same haplotype group (H6) that has a haplotype diversity $\pi = 0.00088$. *Triturus marmoratus* is characterized by five haplogroups (H1–H5), with a haplotype diversity ranging from $\pi = 0.00015$ for H5 to $\pi = 0.00269$ for

H3 (table 2). The haplogroups H1–H5 are not randomly distributed over *T. m. marmoratus* and *T. m. harmanniss ssp. nov.* (χ^2 test, $\chi = 729.23$, d.f. = 4, $P < 0.0001$). *Triturus m. marmoratus* has mostly the H1 and H2 haplogroups whereas *T. m. harmanniss ssp. nov.* carries mostly the H3–H5 haplogroups.

TABLE 1 Summary of morphometric data on males and females in two subspecies of *Triturus marmoratus*. Values to the left are averages of untransformed data in mm and to the right are the relative values obtained from division by snout-vent length (SVL). The panel to the right gives the results of statistical testing on SVL and relative sizes, with in parentheses the identity of the group with significantly higher values.

Subspecies	<i>marmoratus</i> (m)		<i>harmannis</i> ssp. nov. (h)		Mann-Whitney <i>U</i> -test
Males	N = 70 (5 populations)		N = 247 (38 populations)		
	As measured	Relative	As measured	Relative	
SVL	65.31		60.60		*** (m)
ILd	35.07	0.537	32.99	0.545	NS
FLl	26.66	0.409	25.14	0.416	NS
TFI	8.22	0.127	8.14	0.134	NS
HLl	27.04	0.415	25.70	0.425	NS
FTI	8.97	0.139	8.63	0.143	NS
Hw	13.17	0.202	12.84	0.212	*** (h)
HI	17.88	0.275	17.52	0.290	*** (h)
Females	N = 80 (5 populations)		N = 338 (38 populations)		
	As measured	Relative	As measured	Relative	
SVL	67.38		65.17		*** (m)
ILd	36.31	0.538	35.70	0.548	NS
FLl	25.89	0.385	23.94	0.368	*** (m)
TFI	7.71	0.114	7.11	0.109	* (m)
HLl	25.73	0.383	23.75	0.365	*** (m)
FTI	7.98	0.119	6.90	0.107	*** (m)
Hw	13.48	0.200	13.42	0.206	*** (h)
HI	18.01	0.267	17.84	0.274	* (h)

Significance levels are: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and NS $P > 0.05$.

The distribution of dominant haplogroups (fig. 5) is largely geographically coherent and coincides with subspecies ranges as estimated by the nuclear data (fig. 1). Five populations located in the coastal transition zone have H2 as the dominant haplogroup. For 64 *T. marmoratus* populations with an mtDNA sample size ≥ 10 the majority carried a single haplogroup (39 times, 61%). Co-occurring haplogroups were most often H3 with H4 (three times), H3 with H5 (two times) and H4 and H5 (18 times) whereas the co-occurrence of H1 and H2 was not observed.

Discussion

The study of nuclear genetic variation in western European *Triturus* species revealed two reciprocally monophyletic and geographically coherent groups of individuals of similar evolutionary age of ca. 2.5 Ma (Arntzen, 2024; Kazilas et al., 2024). One group is composed of the pygmy newts *T. rudolfi* and *T. pygmaeus* from the west and the south of the Iberian Peninsula (Arntzen, 2024; fig. 5). The other group is made up by marbled newts, *T. marmoratus*, from western-central Iberia and

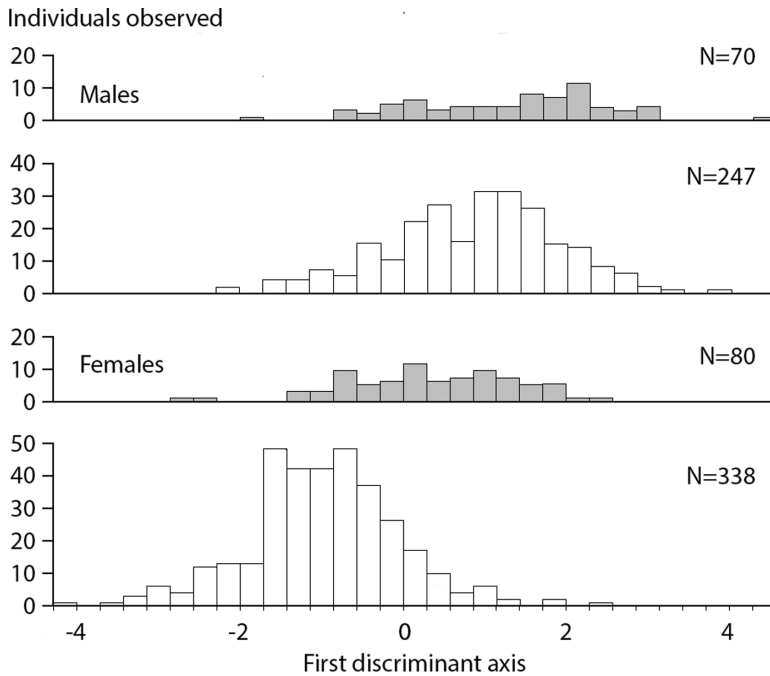


FIGURE 3 Histogram of scores along the first axis for the discriminant analysis of size corrected morphometric data for *Triturus marmoratus marmoratus* (shaded bars) and *T. m. harmannis ssp. nov.* (open bars). This first discriminant axis is most strongly correlated with extremity lengths and not the other characters. Highest loadings on the second axis are for head length and head width (results not shown).

from northern Iberia and France (fig. 1). It is here shown that these marbled newt groups also possess different mtDNA haplotype profiles and have dissimilar morphologies. These results justify the recognition of a new taxon (see Appendix).

Even though both taxon-pairs are about equally deeply differentiated, the contact zones are narrow in pygmy newts (Arntzen, 2024) and wide in marbled newts (present paper). This observation is in line with the loose negative correlation of genetic differentiation and hybridization potential found in a wide range of organisms, including the genus *Triturus* (Jiggins & Mallet, 2000; Arntzen et al., 2014). Compared to the survey over 15 hybrid zones in frogs and toads (Dufresnes

et al., 2021), the (sub)speciation events in Iberian *Triturus* are relatively recent, yet on par with the earlier events (also estimated at ca. 2.5 Ma) for the anuran subspecies *Discoglossus g. galganoi* Lanza, Nascetti, Capula and Bullini, 1986 and *D. g. jeanneae* Busack, 1986 that show a wide intergradation zone (ca. 140 km), and the species *Alytes obstetricans* (Laurenti, 1768) and *Alytes algavarii* Arntzen and García-París, 1995, that engage in a narrow hybrid zone (ca. 18 km). In pygmy newts the steep transition zone (ca. 35 km; Arntzen, 2024) suggests selection against hybrids and consequently *T. rudolfi* was described as a species. Conversely, in the absence of evidence for selection against admixed genotypes in what is taken to be a

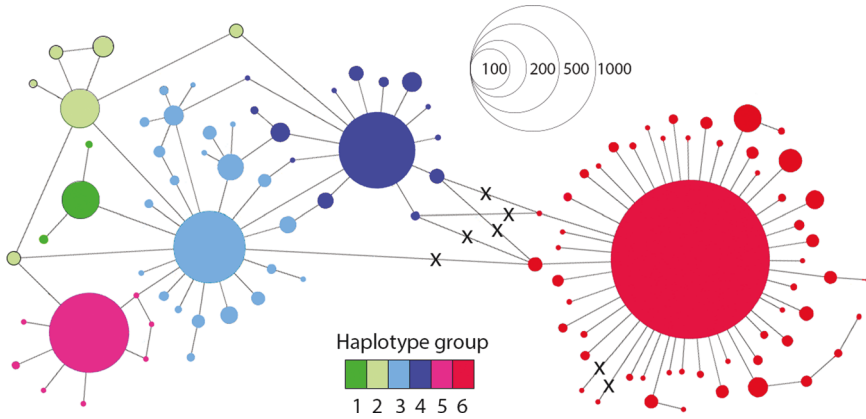


FIGURE 4 Minimum spanning network for mitochondrial ND4 sequences retrieved from pygmy newts (*Triturus rudolfi* and *T. pygmaeus*) with a single haplogroup in red (H6), and marbled newts (*T. marmoratus*) with five haplogroups shown as H1 – green, H2 – light green, H3 – light blue, H4 – blue and H5 – purple. Satellite haplotypes are allocated to the nearest main haplotype, based on minimum uncorrected p-distances (details see text). Size of the circles corresponds to sample size (see legend). Internal and external branches have lengths of one or two substitutions, except for those marked with an ‘x’ that have 5–7 substitutions.

wide transition zone, the southern taxon of the marbled newt is classified as a subspecies, namely *T. m. harmannis ssp. nov.*

The subspecies *T. m. marmoratus* and *T. m. harmannis ssp. nov.* are morphologically differentiated. The former taxon is of larger size than the latter one and has a relatively smaller

head. The subspecies differ in the length of the extremities, that are longer in the former than in the latter taxon, be it that the difference is more pronounced in females than in males. While a strong sexual dimorphism in limb lengths is already documented for the genus *Triturus* (Malmgren & Tholesson, 1999), it

TABLE 2 Mitochondrial DNA haplotype variation at the ND4 gene, its classification and numbers observed in marbled and pygmy newts (*Triturus marmoratus*, *T. rudolfi* and *T. pygmaeus*).

	N	Haplogroup					
		Populations	H1	H2	H3	H4	H5
Species, subspecies or area							
<i>T. m. marmoratus</i> (m)	10	79	27	1	6	0	8
Transition zone m-h	5	0	34	0	2	0	0
<i>T. m. harmannis ssp. nov.</i> (h)	90	1	66	163	347	318	93
<i>T. pygmaeus</i> (p) and <i>T. rudolfi</i> (r)	167	0	344	4	45	70	1644
Species hybrid populations m-p, m-r	6	0	0	5	14	29	24
Associated data							
Haplotype diversity ($\pi * 1000$)		0.26	2.69	2.02	0.86	0.15	0.88
Typical for (sub)species		m	m	h	h	h	p and r

is here shown that in *T. marmoratus* it aligns with subspecies differentiation (table 1, fig. 3).

Colour and colouration pattern of western European *Triturus* species are highly variable and the character \bar{N}_{links} captures just an aspect of the variation in marbled and pygmy newts (Arntzen, 2024). Moreover, the diagnostic power of \bar{N}_{links} to identify geographically adjacent (sub)species varies as seen from the difference in the average number of links ($\Delta\bar{N}_{\text{links}}$) that is 2.9 for *T. marmoratus* – *T. rudolfi*, 7.3 for *T. marmoratus* – *T. p. lusitanicus*, 4.5 for *T. rudolfi* – *T. p. lusitanicus* and 7.4 for *T. p. lusitanicus* – *T. p. pygmaeus* (Arntzen, 2018, 2023, 2024). Accordingly, the weakest signal ($\Delta\bar{N}_{\text{links}} = 1.3$) is for the *T. marmoratus* subspecies here considered. Confounding factors are sample size (fig. 2AB) and the possibly wide zone of subspecies intergradation (fig. 1B). Ontogeny has also been implicated (Arntzen, 2023), but photographic material for *T. pygmaeus lusitanicus* from Guadalupe, Central Spain, shows that juveniles possess a colouration pattern already like that of the adults (Sergé Bogaerts, pers. comm., 8 November 2023). The juvenile *T. marmoratus* here shown here as example (fig. 2C) has no transversal green bands at either side of the body ($N_{\text{links}} = 0$) and is from inside the subspecies' transition area and therewith remains of unknown subspecies affiliation. A remarkable feature for the group at large is that two southerly subspecies (*T. marmoratus harmannis* ssp. nov. and *T. p. pygmaeus*) are more striped (i.e., with lower N_{link} values) than both northerly conspecifics (*T. m. marmoratus* and *T. p. lusitanicus*).

The observed mitochondrial DNA variation is partitioned in five haplotype groups of which two associate with *T. m. marmoratus* (H1 and H2) and three with *T. m. harmannis* ssp. nov. (H3, H4 and H5), to which it may be noted that the haplogroups H1 and H2 are spatially partitioned whereas H3, H4 and H5 are

frequently found together, yet separate out when the *dominant* haplogroup is considered (table 2, fig. 5). Because the mitochondrial and nuclear DNA spatial distributions are largely concordant, i.e., with no widespread cyto-nuclear discordances for the subspecies, particular phenomena such as e.g., sex-dependent dispersal, adaptive introgression and hybrid zone movement (Ballard & Whitlock, 2004; Toews & Belsford, 2012) do not have to be invoked. The prime task at hand is the sequencing of the complete mtDNA molecule, to test the hypothesis that the H3, H4 and H5 haplogroups associated with the new subspecies are more closely related to one another than they are to H1 and H2.

Triturus from the Iberian Peninsula has now 'trituated' into five taxa with, from north to south *T. m. marmoratus*, *T. m. harmannis* ssp. nov., *T. rudolfi*, *T. p. lusitanicus* and *T. p. pygmaeus*. An overall similarity in spatial – temporal relationships within this monophyletic group and the continental European representatives of the genus *Alytes* can be discerned (García-París & Martínez-Solano, 2001; Ambu et al., 2023), in which the range of *T. m. harmannis* ssp. nov. coincides with that of *Alytes obstetricans* cf. *boscai* Lataste, 1879, as represented by the 'green lineage' of Maia-Carvalho et al. (2018). While the Douro River basin may explain the local phylogeography of *Alytes* midwife toads (Maia-Carvalho et al., 2018; Ambu et al., 2023) and was also implicated in the patterning of genetic variability in the golden-striped salamander, *Chioglossa lusitanica* Bocage, 1864 (Alexandrino et al., 2000), the correspondence is incomplete because *T. m. harmannis* ssp. nov. is found at either side of that river (Kalezic et al., 2023; fig. 1). Other recent additions to the amphibian checklist of western Iberia are *Chioglossa lusitanica longipes* Arntzen and Alexandrino, 2007 (Arntzen et al., 2007; Sequeira et al., 2022), *Lissotriton maltzani* (Boettger, 1879)

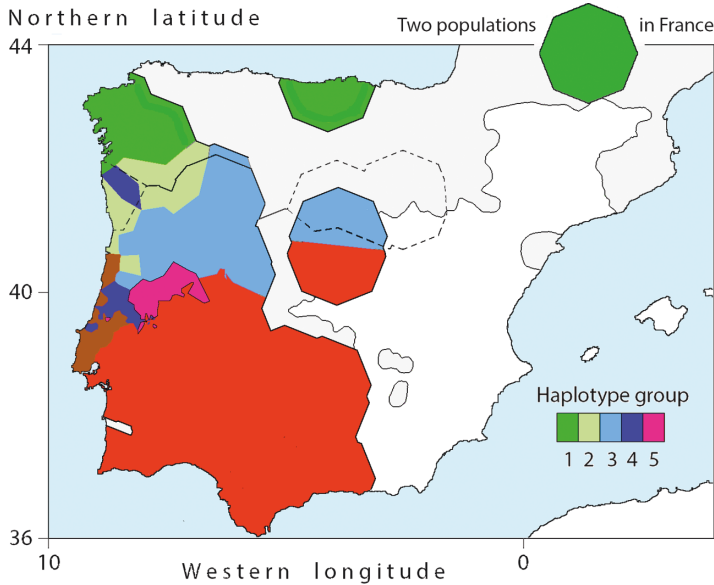


FIGURE 5 The Iberian Peninsula with the ranges of *Triturus pygmaeus* in light red and *T. rudolfi* in brown (Arntzen, 2023, 2024). Populations of the counterpart species *T. marmoratus* are coloured according to the dominant mitochondrial haplogroups with colours as in the legend and fig. 4. The solid or interrupted black line shows the northern range of *T. m. harmanniss ssp. nov.*, as determined by the green, yellow and blue sections in fig. 1. Note that haplogroups 1 and 2 are associated with *T. m. marmoratus* and that haplogroups 3, 4 and 5 are associated with *T. m. harmanniss ssp. nov.* Haplogroup 6 is associated with *T. pygmaeus* and *T. rudolfi* (for details see table 2). Areas shown in white fall outside the documented range of the *T. marmoratus* species group and areas shown in grey are distant from a sampled locality.

(Sequeira et al., 2020), *Pelodytes atlanticus* Díaz-Rodríguez, Gehara, Márquez, Vences, Gonçalves, Sequeira, Martínez-Solano and Tejedro, 2017 (Díaz-Rodríguez et al., 2017), *Rana parvipalmata* Seoane, 1885, and *R. p. asturiensis* Dufresnes, Ambu, Galán, Sequeira, Viesca, Choda, Álvarez, Alard, Suchan, Künzel, Martínez-Solano, Vences and Niecieza, 2023 (Dufresnes et al., 2020, 2023). With these discoveries, the pinnacle of amphibian diversity in the Iberian Peninsula accentuates towards the Atlantic region, partly outside the Mediterranean biodiversity hotspot. Several of the above cases are, however, in need of a more thorough characterization of contact zones. This also applies to *T. m. marmoratus*

– *T. m. harmanniss ssp. nov.* Because their intergradation zone stretches over 2/3 of the width of the Iberian Peninsula (ca. 500 km), it should be relatively straightforward to find areas in which latitudinal transects can be studied in detail.

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and Portugal – Instituto da Conservação da Natureza, by letters dated 26/10/1998, 19/4/2000 and 19/3/2002 and by permit numbers 397/2007/CAPT, 102/2010/CAPT, 103/2010/CAPT and 107/2012/CAPT. I thank Bastian Reijnen and Notis Theodoropoulos for assistance with the mtDNA sequencing.

Data availability

Many data used in this study are available from published works – for morphology and localities see Arntzen (2023, 2024) and for molecular data see Wielstra et al. (2013) and Kazilas et al. (2024). New mtDNA sequences were collected for the purpose of a study on hybrid zone dynamics and species replacement that is *in statu nascendi* and are therefore not yet released.

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Appendix

Description of *Triturus marmoratus harmanis* ssp. nov.

Descriptions were made from preserved material, except for the N_{links} character state that was determined in the field. Dorsal colouration invariably dark, yet with the green colouration pattern discernible. Morphological data retrieved for a series of live animals are in Table A1.

Description of type specimen – preserved on ethanol in excellent storage condition at the ‘Museo Nacional de Ciencias Naturales’, Madrid, Spain under catalogue number MNCN 51792. Adult male in breeding condition with a well-developed crest and a large back cloaca. Thirteen crème-white bands over the head and body part of the crest. SV1 64.5 mm, SV2 73.5 mm, ILD 32.5 mm, FL1 28.3 mm, TFL 8.5 mm, HLL 28.1 mm, FT1 11.6 mm, Hw 14.1 mm and Hl 19.7 mm. Total length 136.5 mm. Relative finger lengths $1 < 4 < 2 < 3$. Relative toe lengths $1 < 5 < 2 < 4 < 3$. N_{links} left 0, right 0, total 0. Solid dark grey ventral colouration with many more or less evenly distributed white spots, denser in the middle part than towards the gular and cloacal regions. Throat region light grey with many small white dots. Underside of the legs and cloacal region with light and dark regions. The holotype is shown in fig. A1. A three-dimensional model of the entire skeleton of the type specimen obtained with CT-scanning is available for inspection at <https://doi.org/10.6084/m9.figshare.25358890>, courtesy of Dr. Tijana Vučić.

Description of paratypes – Preserved on ethanol in excellent storage condition at the ‘Museo Nacional de Ciencias Naturales’, Madrid, Spain.

TABLE A1 Morphometric data for live adult *Triturus marmoratus harmannis* ssp. nov. from the type locality. Characters measured are described in the text.

Males											
Number	SVl ₁	SVl ₂	ILd	FLl	TFl	HlL	Ftl	Hw	Hl	Total length	N _{links}
1	73	83	38	30.3	8.9	32.6	9.3	15.1	20.1	151	1
2	58	67	31	25.3	7.8	25.1	7.3	12.5	17.9	123	0
3	65	75	32	28.8	7.4	27.0	8.3	13.3	19.0	137	0
4	70	79	39	27.4	7.8	28.7	7.9	14.3	20.8	145	0
5	70	80	38	29.4	8.4	28.7	8.8	14.6	18.0	147	0
6	66	75	37	27.9	7.6	30.0	8.5	13.8	18.8	139	1
7	59	63	30	25.8	6.7	25.3	7.2	13.2	17.5	126	0
8	65	75	37	27.3	7.2	27.9	7.8	13.8	18.0	136	0
9	74	83	42	31.7	9.2	33.0	9.6	15.5	21.1	139 #	0
10	67	75	33	30.5	8.6	30.2	9.1	14.2	16.8	140	0
11	66	75	34	30.0	8.8	28.5	8.3	14.5	18.5	137	0
12	62	70	36	28.3	8.8	28.8	9.3	13.6	16.1	132	0
Females											
13	73	81	39	27.2	7.8	27.5	6.3	15.6	19.4	151	0
14	73	81	40	27.7	6.7	28.2	7.1	14.2	20.5	152	0
15	75	82	41	19.3	8.0	28.9	7.7	14.7	19.6	156	0
16	76	84	43	28.4	7.4	28.0	7.1	15.6	21.3	155	0
17	76	83	42	28.9	8.4	28.5	7.8	15.1	18.9	155	0
18	71	78	40	26.5	7.4	26.7	6.6	15.5	20.6	148	3
19	75	84	40	28.1	6.4	26.5	6.2	15.4	21.5	155	0
20	75	83	44	28.5	6.7	27.4	6.6	15.6	19.3	155	4
21	72	80	40	29.0	7.5	28.8	6.6	15.5	19.5	150	0
22	76	83	40	28.6	6.5	28.8	7.7	14.4	22.2	155	3
23	74	80	42	28.2	6.8	26.3	6.3	14.6	19.1	152	3
24	71	78	38	25.3	7.1	25.5	6.4	14.3	19.5	147	0

Tail damaged

First paratype MNCN 51793. Adult male in breeding condition with a well-developed crest and a large back cloaca. Seventeen crème-white bands over the head and body part of the crest. SVl₁ 65.0 mm, SVl₂ 73.5 mm, ILd 34.0 mm, FLl 26.2 mm, TFl 9.3 mm, HlL 27.0 mm, Ftl 11.5 mm, Hw 13.6 mm and Hl 19.9 mm. Total length 135 mm. Relative finger lengths 1=4<2<3. Relative toe lengths

1<5<2<4<3. N_{links} left 0, right 1, total 1. Grey ventral colouration with many dark dots. Many white spots mostly positioned on the undotted sections. Colour of the throat continuous with that of the belly. Underside of the legs and cloacal region with light and dark regions.

Second paratype MNCN 51794. Adult male in breeding condition with a low crest and a



FIGURE A1 Holotype of *Triturus marmoratus harmannis* ssp. nov. at right and ventral view. Size bar is 1 cm. Stored at the Museo Nacional de Ciencias Naturales, Madrid, Spain under catalogue number 51792.

large back cloaca. Eleven crème-white bands over the head and body part of the low crest. SV1 56.5 mm, SV2 64.0 mm, ILd 28.5 mm, FL1 23.4 mm, TFl 7.7 mm, HL1 23.6 mm, FTl 9.6 mm, Hw 12.6 mm and Hl 17.7 mm. Total length 118 mm. Tail tip regenerating. Relative finger lengths $1 < 4 < 2 < 3$. Relative toe lengths $1 < 5 < 2 < 4 < 3$. N_{links} left 1, right 2, total 3. Solid black ventral colouration with many, evenly distributed small white spots. Throat colouration continuous with belly with equally dense but larger white spots. Underside of the legs and cloacal region light with few dark regions.

Third paratype MNCN 51795. Adult female in breeding condition with ovaries shining through. SV1 64.0 mm, SV2 70.0 mm, ILd 33.0 mm, FL1 25.1 mm, TFl 8.3 mm, HL1 23.7 mm, FTl 7.6 mm, Hw 14.5 mm and Hl 19.6 mm. Total length 140.5 mm. Relative finger lengths $1 < 4 < 2 < 3$. Relative toe lengths $1 = 5 < 2 < 4 < 3$. N_{links} left 2, right 2, total 4. Solid, medium-grey ventral colouration with few, evenly distributed small white spots. Throat coloration continuous with that of the belly.

Underside of the legs and cloacal region solid light grey.

Fourth paratype MNCN 51796. Adult female in breeding condition with ovaries shining through. SV1 73.0 mm, SV2 79.5 mm, ILd 39.5 mm, FL1 28.1 mm, TFl 9.3 mm, HL1 26.4 mm, FTl 8.8 mm, Hw 16.5 mm and Hl 21.7 mm. Total length 149 mm. Relative finger lengths $1 < 4 < 2 < 3$. Relative toe lengths $1 < 5 < 2 < 4 < 3$. N_{links} left 0, right 0, total 0. Dark grey ventral colouration with few small dark dots and few white spots. Colouration of throat continuous with belly. Underside of the legs and cloacal region light with few dark regions.

Fifth paratype MNCN 51797. Adult female in breeding condition with ovaries shining through. SV1 71.0 mm, SV2 77.5 mm, ILd 37.5 mm, FL1 27.4 mm, TFl 9.1 mm, HL1 26.0 mm, FTl 7.2 mm, Hw 15.4 mm and Hl 22.2 mm. Total length 150.5 mm. Relative finger lengths $1 = 4 < 2 < 3$. Relative toe lengths $1 = 5 < 2 < 4 < 3$. N_{links} left 1, right 2, total 3. Light grey ventral colouration with large dark

dots and few white spots towards the flanks. Colouration of throat continuous with belly. Underside of the legs and cloacal region light with few dark regions.

Sixth paratype MNCN 51798. Adult female in breeding condition with ovaries shining through. SVl₁ 72.0 mm, SVl₂ 78.5 mm, ILd 38.5 mm, FLl 28.0 mm, TFl 9.0 mm, HLL 27.2 mm, FTl 8.7 mm, Hw 16.1 mm and Hl 21.1 mm. Total length 146 mm. Relative finger lengths $1=4<2<3$. Relative toe lengths $1<5<2<3=4$. N_{links} left 2, right 2, total 4. Light grey ventral colouration with medium number of large dark dots and few white spots. Colouration of throat continuous with belly. Underside of the legs and cloacal region light with few dark regions.

Locality and date of collecting – Arrochela, near Madeirã, Portugal at 39.9386 northern latitude and 8.1025 western longitude. Elevation 396 m a.s.l. Date of collecting 20 March 2013, leg. J. W. Arntzen. Date of deposition at MNCN 16 August 2023.

Diagnostic features – the newly recognized subspecies is closely related to and morphometrically similar to *T. m. marmoratus*, yet at the population level characterized by a lower number of green coloured dorso-lateral transversal bands ('links'). In comparison with *T. m. marmoratus*, it has a small body size, significantly shorter extremities in females and a relatively big head in both sexes. The distinctiveness of the subspecies is supported by nuclear and mitochondrial DNA data.

Derivatio nominis – the subspecies name is chosen to commemorate the Dutch couple

Harm and Annie (or Ann) Walen, who lived from 1926–2005 (Mr. H. C. Walen) and 1928–2018 (Mrs. A. A. van Silfhout). After Harm's retirement from his taxidermist job at the Zoological Museum in Amsterdam, the two-some eventually landed in Nisa, Portugal where they constructed their own 'quinta' and felt enormously at place. Without 'harmann's' hospitality and moral support, my extensive fieldwork in Portugal and adjacent Spain late last century would hardly have been sustainable. The new subspecies' name refers to a lifelong couple and only indirectly to individual people so that, matrimony being gender neutral, the third declension is used.

Suggested vernacular name – Harmann's marbled newt, or central Iberian marbled newt Distribution – central-western Iberia. The southern range border is determined by a sharp, parapatric, yet mosaic range border with two pygmy newt species, namely *T. rudolfi* in the west and *T. pygmaeus* in the centre of the Iberian Peninsula. The northern edge of the range is positioned at ca. 41.5N. The transition area with the nominotypical subspecies may be wide.

Nomenclatorial act – the electronic 'on-line early' version of this article is considered a published work according to the International Code of Zoological Nomenclature. The new name has also been registered in ZooBank (<http://zoobank.org/>) where it can be accessed under <http://zoobank.org/urn:lsid:zoobank.org:pub:6DE32B0B-FB92-4F45-80BB-15EBC49DD23E>.