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Morphological and genetic diversification of Old-World marbled newts, with the description of a new and 'not-at-all-cryptic' subspecies from the Iberian Peninsula (*Triturus,* Salamandridae)

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Abstract

Morphological and genetic variation of organisms is generally lower in northern than in southern regions of the Palearctic. This ubiquitous geographical pattern has been associated with extinction and recolonization processes in the north versus persistence in the Mediterranean area, as governed by climate change in the Pleistocene. One area of differentiation and complexity is the Iberian Peninsula where two distinct sister-species of marbled newts possess adjoining and geographically sharply delimited ranges over the north (*Triturus marmoratus*) and the south of the peninsula (*T. pygmaeus*). Within the latter species two forms are here documented, with different colouration patterns and non-overlapping ranges. The southern form is striped, much as in *T. marmoratus*, and occurs to the south of the Guadalquivir River. The northern form, here described as a new subspecies, has a reticulated dorsal colouration. It occurs only north of the Guadalquivir and is thus sandwiched in between two striped marbled newt taxa. Both *T. pygmaeus* subspecies engage inside the Doñana National Park in a ca. 2,500 m narrow contact zone. Although an inferred substantial selection against hybrids goes a long way to support species status, a reanalysis of published genetic data is required to solve this issue, along with the wider investigation of the same type of (microsatelite) data for the southern, nominotypical subspecies.

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Keywords

candidate species – clinal variation – coloration pattern – microsatellite genetic data – morphometrics – new subspecies – species status – *Triturus pygmaeus lusitanicus* **ssp. nov.**

Introduction

Biological systematics is the study of the variation and evolution of populations of organisms in relation to their taxonomic classification, with as a supplementary aim the reconstruction of the tree of life. A robust phylogeny for the group at hand allows the study of how *characters* evolve, along with the identification of the selective forces that eventually shaped the organisms over time and space. The insights obtained from such analyses bring us full circle to the enhanced study of biological systematics. Paramount to further progress in this discipline, and biodiversity research in general, is the identification and delimitation of species, here defined as separately evolving sets of populations that retain distinct evolutionary trajectories (Wiley, 1978; Burbrink & Ruane, 2021). The first step for identifying candidate species is to examine the structure of morphological and genetic discontinuities over the landscape and, obviously, the detail at which this exercise is to be carried out depends on how the variation is spatially arranged.

Within the Holarctic, northern regions regularly show low morphological and genetic variability at the population level, whereas southern population groups are often deeply differentiated, even over small geographical distances. This pattern of 'northern purity and southern richness' (Hewitt, 2000) is adequately explained by the history of climate change, in particular the repeated range contractions and northern extinctions resulting from the Pleistocene glaciations. Formerly glaciated or otherwise inhospitable regions in the north were most recently colonized in the Holocene, often via long-range dispersal out of one or few refugia. Fostered by founder effects, this resulted in decreasing genetic variability with increasing distance from the respective refugia. Conversely, southern populations survived the cold periods locally and preserved their ancient genetic diversity (Hewitt, 1996). Palearctic regions of high variation and complexity are the southern peninsulas, from west to east the Iberian, the Apennine and the Balkan peninsula (Schmitt, 2007). Here, the scope for discovery of as yet undescribed taxa is high, but calls for a dense sampling, in the case of ground-dwelling organisms with limited dispersal capability such as amphibians perhaps even more than in other groups.

One amphibian group in which the integrative analysis of comprehensively sampled morphological and genetic data resulted in species discovery and a well-supported phylogeny is the Old-World newt genus *Triturus*, with up to date one northern, two Iberian, two central European-Apennine and four Balkan-Anatolian species (Arntzen et al., 2018; Wielstra et al., 2019). The two species from the Iberian Peninsula, *in casu* the northern marbled newt *T. marmoratus* (Latreille, 1800) and the pygmy marbled newt, *T. pygmaeus* (Wolterstorff, 1905) are morphological and genetically well-differentiated sister-species that engage in a sharp, parapatric contact zone that runs across the western part of the Iberian Peninsula (Arntzen et al., 2014; Arntzen, 2018). Morphological variation is more pronounced within *T. pygmaeus* than within *T. marmoratus*. It is here shown that a northern form of *T. pygmaeus* with a reticulated dorsal colouration and a southern striped form are differentially distributed north and south of the Guadalquivir River, with ranges that only meet in the zone adjacent to the Atlantic Ocean (fig. 1). Published genetic data suggest that the colour forms are genetically isolated at close range within the Doñana National Park, but hypothesis testing is hampered by the unavailability of the constituent data. Even though an inferred lack of gene flow goes a long way in supporting species status of the newly recognized form, it is here conservatively described as a subspecies, in order to safeguard that it is properly labelled. Because the type locality of *T. pygmaeus* is the province of Cadiz (Wolterstorff, 1905), it is the northern taxon that requires recognition, for which the formal description is in Appendix A1. In the remainder of the paper *Triturus p. pygmaeus* and *T. p. lusitanicus* **ssp. nov.** may also be referred to as the southern and northern taxon of pygmy marbled newt, respectively.

Materials and methods

Adult newts were captured by dip-netting or with funnel traps in France, Portugal and Spain under licenses as appropriate (see acknowledgements). All material was investigated for the number of 'links' that they display (Nlinks). Links are dorso-lateral colour pattern character states as described and illustrated by Arntzen (2018). Briefly, links are counted as the number of uninterrupted connections over the green coloured surface,

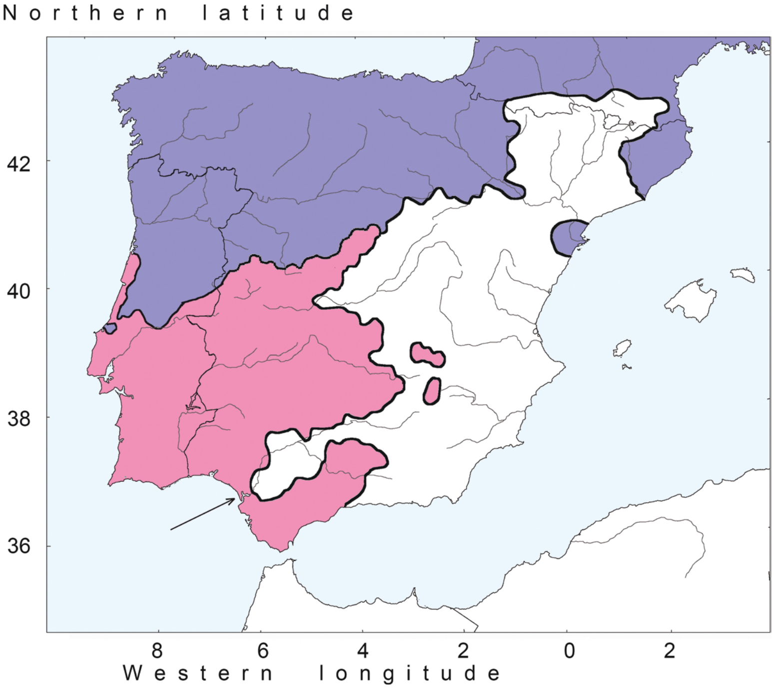

FIGURE 1 Distribution of marbled newts in the Iberian Peninsula with *Triturus marmoratus* in blue and *T. pygmaeus* in red (after Wielstra et al., 2014). The arrow indicates the position of a *T. pygmaeus* subspecies border inside Doñana National Park (present paper).

from the position of the crest in males or the mid-dorsal line in females (occasionally juveniles) 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. Individuals with Nlinks ≤ 5 are likely to be *T. marmoratus* and those with Nlinks > 5 are likely to be *T. pygmaeus*. The model underlying this criterion is supported by an 'area under the curve' value of 0.925 (Arntzen, 2018). All marbled newt populations were assigned to one of five classes, depending on the percentage of inferred *T. marmoratus* (0–20%, 20–40%, 40–60%, etc). With one exception, no special attention was given to museum collections, because in ethanol preserved material the green colour has frequently faded, with Nlinks difficult or impossible to discern. In the often brightly coloured juveniles Nlinks can usually be determined, but few juveniles are stored and it is currently not known if their colouration pattern is stable over lifetime.

Measurements were taken on live adults to investigate a possible morphological differentiation of the southern and northern forms. Body size was taken as the length from the tip of the snout up to and including the insertion of the hind limb (SVl1), or the posterior side of the cloaca (SVl2). Other morphometric characters are: ILd – interlimb distance, FLl – forelimb length, TFl – third finger length, HLl – hindlimb length, FTl – fourth toe length, Hl – head length and Hw – head width. Measurements were taken in mm with a plastic ruler or plasticized graph paper for SVl and ILd, and with Vernier callipers at 0.1 mm precision for the other characters. Extremities were measured at the right side of the body. Because adult size varies markedly over the study area, possibly as a direct response to the conditions of the environment (i.e., phenotypic plasticity) (García-París et al., 1993; Díaz-Paniagua et al., 1996), the standardized residuals of the regression of ln(*character*) to ln(SVl1) were used, with the aim to reduce the effect of overall size in the analysis and to increase the normality of the data. Because of a marked sexual dimorphism (see Results) the sexes were treated separately. Data were analysed by a principal component analysis (PCA) with SPSS 26 (IBM SPSS, 2019), in which populations of the southern taxon (270, 271, 465 and 1003) were compared *a posteriori* to populations that represent the northern taxon (29, 65, 88, 93, 94, 436, 438 and 463) (fig. 2, for precise locality information, see table 1). The analysis was restricted to this set of southern Iberian populations to reduce the possible effects of gradual geographical variation, taxonomic uncertainty in the west of Portugal and admixture with *T. marmoratus* (Espregueiro Themudo et al., 2007; Arntzen & Espregueira Themudo, 2008; Arntzen, 2018; Arntzen et al., 2021; Lopez-Delgado et al., 2021). To facilitate interpretation and for comparability with data and analyses to come, also morphometric indices were calculated as (character)/SVl, i.e., from untransformed data. Values for these indices were compared between southern and northern groups with Mann-Whitney's non-parametric test, also in SPSS.

Microsatellite genetic data have recently been used to investigate population dynamic processes in *T. pygmaeus* in a southern section of the species range (Albert & García-Navas, 2022). Because detailed genetic data used in this paper (11 microsatellite loci) could not be obtained, the approximate reconstruction of the best fitting geographical cline of Structure Q-scores over Doñana National Park (DNP) was performed from printed results (Albert & García-Navas, 2022: Figure 4), with Hybrid Zone Analysis using R (HZAR) software (Derryberry et al., 2014), following a protocol used earlier (Arntzen et al., 2017). The reference point for distance in this analysis is the northern bank of the Guadalquivir River with the coordinates 36.7956 N and 6.3700 W.

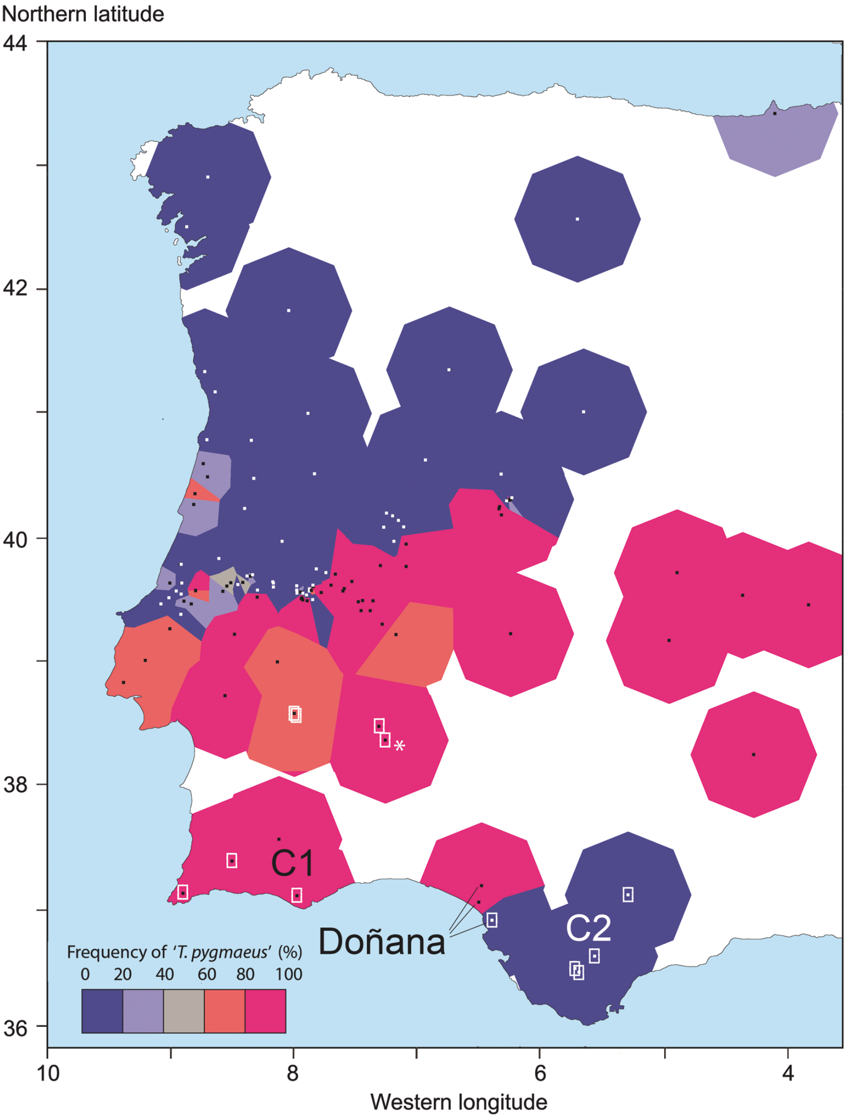

FIGURE 2 The distribution of two species of marbled newts over the western part of the Iberian Peninsula, as inferred from the morphological character Nlinks. *Triturus marmoratus* is shown in two shades of blue, *T. pygmaeus* in two shades of red and four localities with both species are shown in grey (see colour legend). The spatial extrapolation does not exceed ca. 50 km. Note that southern Iberian populations are all *T. pygmaeus*, irrespective of high (cluster C1), low (C2), or bimodal Nlink counts (Doñana National Park). Populations that were studied morphometrically are highlighted by a box. The type locality for *T. pygmaeus lusitanicus* **ssp. nov.** is Granja, Portugal and is marked by an asterisk.

TABLE 1 Link counts for 2152 marbled newts from 128 populations. Shown are average values, standard deviation, range and sample size along with locality number, locality name and geographical coordinates.

Locality number	Locality name	Longitude	Latitude	Sample size	Mean	Standard deviation	Minimum	Maximum
2	Rio Maior, Portugal	−8.921	39.341	20	2.75	2.43	0	9
3	Alqueidão, Portugal	−8.580	39.530	20	5.15	3.17	1	10
4	Gavião 1, Portugal	−7.939	39.477	4	8.75	2.75	6	12
5	Nisa 1, Portugal	−7.604	39.533	5	11.80	3.03	8	16
7	Pedra do Altar, Portugal	−0.821	39.711	56	1.59	1.39	0	5
9	São Domingos 1, Portugal	−8.174	39.603	22	1.64	1.94	0	8
10	Montalvo, Portugal	−8.300	39.481	12	8.75	3.67	1	12
12	Saide, Portugal	−8.329	40.449	15	0.80	1.47	0	5
14	Martinchel 1, Portugal	−8.298	39.538	4	2.00	2.16	0	5
15	Andreus 1, Portugal	−8.170	39.564	1	0		0	0
16	Andreus 2, Portugal	−8.170	39.564	2	4.5	0.71	4	5
19	Domingos da Vinha, Portugal	−7.947	39.523	30	2.90	3.19	0	10
20	Areia 1, Portugal	−7.930	39.515	12	3.00	2.98	0	9
26	Lagoa Seca, Portugal	−9.012	39.596	202	4.87	2.68	0	13
29	Mitra 1, Portugal	−8.001	38.535	23	6.13	2.26	2	11
30	Mora, Portugal	−8.138	38.955	12	6.83	3.33	2	11
31	Mindelo, Portugal	−8.727	41.317	2	1.0	0.00	1	1
32	Gavião 2, Portugal	−7.932	39.459	22	9.64	2.42	4	14
33	Cadafaz, Portugal	−7.943	39.470	6	8.00	4.38	1	13
38	Jublains, France	−0.472	48.253	70	3.87	2.21	0	9

TABLE 1 Link counts for 2152 marbled newts from 128 populations. Shown are average values, standard deviation, range and sample size along with locality number, locality name and geographical coordinates. (*cont.*)

Locality number	Locality name	Longitude	Latitude	Sample size	Mean	Standard deviation	Minimum	Maximum
42	Casas de Ribeira, Portugal	−7.979	39.573	14	2.71	2.33	0	8
46	Dunes SW of Mira, Portugal	−8.807	40.324	4	7.00	4.24	1	10
48	San José de Matos, Portugal	−7.845	39.566	2	9.0	0.00	9	9
51	Gerês – Carris, Portugal	−8.046	41.815	71	1.89	2.20	0	9
52	Zimbreirinha, Portugal	−7.873	39.536	3	7.00	4.36	2	10
53	Vale Pedro Dias 2, Portugal	−7.878	39.533	12	2.42	2.81	0	8
55	Areia 2, Portugal	−7.932	39.514	40	1.78	2.01	0	7
56	Vila Flor, Portugal	−7.781	39.518	22	7.45	2.44	4	15
57	Outeiro Cimeiro, Portugal	−7.901	39.496	48	2.17	2.14	0	8
58	Areia 3, Portugal	−7.934	39.514	28	3.75	1.46	0	8
62	Coimbra, Portugal	−8.404	40.203	10	0.90	1.66	0	5
65	Vila do Bispo – Sagres, Portugal	−8.904	37.072	32	8.50	3.24	1	15
66	Nisa 2, Portugal	−7.593	39.550	17	10.12	3.14	4	17
67	Rosmaninhal, Portugal	−7.091	39.732	40	7.83	2.53	2	12
68	Monforte da Beira, Portugal	−7.300	39.738	1	7		7	7
70	Monte do Conde, Portugal	−7.250	40.167	12	0.67	1.37	0	4
72	João Pires, Portugal	−7.154	40.106	17	0.94	1.52	0	5

TABLE 1 Link counts for 2152 marbled newts from 128 populations. Shown are average values, standard deviation, range and sample size along with locality number, locality name and geographical coordinates. (*cont.*)

Locality number	Locality name	Longitude	Latitude	Sample size	Mean	Standard deviation	Minimum	Maximum
73	Medelim – Penha Garcia nr. Sidral, Portugal	−7.111	40.053	2	2.0	1.41	1	3
76	São João do Deserto, Portugal	−7.198	40.144	5	0.40	0.55	0	1
80	Barragem de Idanha-a-Nova, Portugal	−7.192	39.936	42	2.74	2.05	0	9
84	Zebreira, Portugal	−7.091	39.913	44	9.91	3.09	0	15
87	Soverete, Portugal	−7.286	39.262	4	8.50	2.52	6	12
88	Mourão, Portugal	−7.312	38.429	3	10.67	2.52	8	13
90	Esperança, Portugal	−7.174	39.176	34	8.09	3.06	0	14
91	Castelo Mendo, Portugal	−6.933	40.598	21	3.24	2.32	0	8
92	Mogadouro, Portugal	−6.740	41.332	25	0.60	1.19	0	5
93	Granja, Portugal	−7.262	38.318	14	11.21	1.97	8	15
94	Mitra 2, Portugal	−8.001	38.535	22	8.32	2.82	4	14
95	Aldeia Santa Margarida, Portugal	−7.274	40.050	21	1.71	1.55	0	5
96	Ponte Maceira, Negreira, Spain	−8.703	42.901	10	1.80	1.48	0	4
97	Calvão, Portugal	−8.705	40.460	26	3.65	3.02	0	10
98	Quiaios, Portugal	−8.816	40.237	20	4.15	3.23	0	11

TABLE 1 Link counts for 2152 marbled newts from 128 populations. Shown are average values, standard deviation, range and sample size along with locality number, locality name and geographical coordinates. (*cont.*)

Locality number	Locality name	Longitude	Latitude	Sample size	Mean	Standard deviation	Minimum	Maximum
100	Madeirã, Portugal	−8.101	39.937	31	0.65	1.23	0	4
102	Nespereira de Baixo – Borallal, Portugal	−8.350	40.757	21	0.86	1.53	0	6
103	Mezio, Portugal	−7.885	40.979	28	0.46	0.79	0	2
104	Velada, Portugal	−7.701	39.580	22	9.36	2.94	5	17
107	Vila Velha de Rodão, Portugal	−7.667	39.668	39	8.95	3.27	0	14
108	Vale de Figueira, Portugal	−7.747	39.681	33	0.45	1.06	0	5
114	Torreira, Portugal	−8.710	40.764	4	2.25	1.71	0	4
116	Porto – Botanical Garden, Portugal	−8.642	41.153	18	1.28	1.87	0	6
119	Salir de Matos, Portugal	−9.083	39.427	1	5		5	5
121	Fonte da Pena da Couvinha, Portugal	−9.019	39.477	2	1.5	0.71	1	2
122	Punta Moreiras, Spain	−8.876	42.497	5	4.60	2.51	3	9
127	Mosteiros de Alcanede, Portugal	−8.838	39.426	6	4.00	2.00	1	6
134	Aceituna, Spain	−6.317	40.152	8	11.50	2.07	8	15
135	Santa Cruz de Paniagua, Spain	−6.335	40.201	7	10.00	3.21	5	14

TABLE 1 Link counts for 2152 marbled newts from 128 populations. Shown are average values, standard deviation, range and sample size along with locality number, locality name and geographical coordinates. (*cont.*)

Locality number	Locality name	Longitude	Latitude	Sample size	Mean	Standard deviation	Minimum	Maximum
136	El Bronco, Spain	−6.333	40.218	21	11.43	3.52	5	20
145	Alcuescar, Spain	−6.242	39.184	25	8.76	3.18	0	13
146	Rexaldia 1, Portugal	−8.548	39.572	2	5.5	6.36	1	10
147	Rexaldia 2, Portugal	−8.549	39.571	9	5.89	1.83	3	9
148	Assertiz – Fungalvaz, Portugal	−8.515	39.598	20	6.05	3.15	0	12
150	Covão da Fonte, Portugal	−8.802	39.538	3	11.00	0.00	11	11
152	Molianos, Portugal	−8.920	39.507	2	1.0	0.00	1	1
153	Covas, Portugal	−8.802	39.533	23	5.70	2.64	2	10
155	Casais Monizes, Portugal	−8.896	39.449	30	3.80	2.91	0	11
157	Carrascal – Évora de Alcobaça 2, Portugal	−8.960	39.532	2	4.0	0.00	4	4
160	Juncal 2, Portugal	−8.916	39.597	14	0.43	0.51	0	1
171	Degracia Fundeira nr. Atalaia, Portugal	−7.895	39.451	2	10.0	0.00	10	10
177	Carrascal 2, Portugal	−7.878	39.547	1	0		0	0
186	Zimbreira, Portugal	−7.849	39.576	1	0		0	0
191	Cabanas do Vale da Azinheira, Portugal	−7.851	39.461	1	3		3	3

TABLE 1 Link counts for 2152 marbled newts from 128 populations. Shown are average values, standard deviation, range and sample size along with locality number, locality name and geographical coordinates. (*cont.*)

Locality number	Locality name	Longitude	Latitude	Sample size	Mean	Standard deviation	Minimum	Maximum
196	Serradilla del Llano, Spain	−6.319	40.482	14	1.00	1.41	0	4
202	Mação 1, Portugal	−7.976	39.513	5	0.80	1.10	0	2
203	Mação – Belver, Portugal	−7.980	39.538	1	1		1	1
205	Tomar, Portugal	−8.418	39.602	46	6.87	3.31	2	12
206	Cem Soldos, Portugal	−8.458	39.585	1	0		0	0
209	Sintra 2, Portugal	−9.388	38.788	15	7.80	4.31	2	17
216	Olas, Portugal	−8.339	39.665	2	0.0	0.00	0	0
217	Nelas, Ponte Nove, Portugal	−7.836	40.486	21	0.81	1.17	0	4
218	Casar de Palomera 1, Spain	−6.241	40.278	4	4.00	1.41	2	5
219	Casar de Palomera 2, Spain	−6.251	40.272	3	3.33	3.21	1	7
220	Margachaz North, Spain	−6.250	40.273	11	13.09	2.77	9	18
221	Marchagaz, Spain	−6.270	40.270	1	2		2	2
222	Palomero, Spain	−6.250	40.273	11	13.09	2.95	7	18
224	Santa Catarina, Portugal	−8.357	39.651	4	1.75	2.87	0	6
225	Casais, Portugal	−8.383	39.654	1	0		0	0
243	Praia de Vagueira 2, Portugal	−8.740	40.568	10	3.60	2.46	0	6
244	Albergaria dos Doze, Portugal	−8.612	39.797	2	0.00	0.00	0	0

TABLE 1 Link counts for 2152 marbled newts from 128 populations. Shown are average values, standard deviation, range and sample size along with locality number, locality name and geographical coordinates. (*cont.*)

Locality number	Locality name	Longitude	Latitude	Sample size	Mean	Standard deviation	Minimum	Maximum
245	Montalvao – Spanish border, Portugal	−7.531	39.609	17	9.47	3.02	4	16
247	Castelo de Vide railway line 1, Portugal	−7.479	39.444	13	11.00	2.52	7	14
248	Beira, Portugal	−7.360	39.451	2	7.0	1.41	6	8
250	São Domingos 2, Portugal	−8.171	39.602	11	2.64	2.77	0	8
257	Carreiras, Portugal	−7.457	39.371	2	13.0	1.41	12	14
270	Rio Alberite, Spain	−5.687	36.429	21	1.90	2.02	0	9
271	Doñana, Spain	−0.394	36.853	31	1.29	1.37	0	6
300	Olhos de Agua, Portugal	−7.383	39.371	65	8.75	2.83	2	16
307	Castelo de Vide railway line 2, Portugal	−7.444	39.455	27	9.85	2.18	4	13
340	Venta del Charco, Spain	−4.267	38.200	8	7.38	1.69	4	9
354	Marinha Grande, Portugal	−8.924	39.750	1	2		2	2
436	Umbria near Monchique, Portugal	−8.506	37.335	1	8		8	8
438	Faro University 1, Portugal	−7.979	37.050	2	11.0	4.24	8	14
453	Picarras, Portugal	−8.563	38.680	2	8.0	0.00	8	8
454	Fuenlabrada de los Montes, Spain	−4.956	39.129	2	15.5	4.95	12	19
458	Los Baños del Robledillo, Spain	−4.358	39.496	1	7		7	7

TABLE 1 Link counts for 2152 marbled newts from 128 populations. Shown are average values, standard deviation, range and sample size along with locality number, locality name and geographical coordinates. (*cont.*)

Locality number	Locality name	Longitude	Latitude	Sample size	Mean	Standard deviation	Minimum	Maximum
459	Buenasbodas nr. Belvis de la Jara, Spain	−4.890	39.681	3	9.67	2.08	8	12
461	Los Yebenes – estacion de Urda, Spain	−3.821	39.418	65	10.14	2.24	5	14
463	Villanueva de San Juan – Moron, Spain	−5.289	37.060	1	5		5	5
465	Puerto de Galiz, Spain	−5.560	36.558	17	2.29	2.64	0	9
509	Santillana del Mar, Spain	−4.095	43.419	10	5.10	2.08	2	9
522	Galegos, Portugal	−9.212	38.966	3	5.67	0.58	5	6
533	Cercal, Portugal	−9.012	39.225	12	6.50	2.50	2	11
541	Marianos, Portugal	−8.485	39.179	62	7.94	3.20	0	15
1001	Salamanca, Spain	−5.650	40.990	2	1.5	2.12	0	3
1002	San Miguel del Camino, Spain	−5.700	42.560	1	0		0	0
1003	Alcala de los Gazules, Spain	−5.720	36.460	9	2.11	0.93	1	3
1004	Alcaria Longo, Portugal	−8.124	37.511	2	8.5	0.71	8	9
1005	El Rocio, Spain	−6.501	37.164	3	7.0	1.00	6	8
1005	El Rocio, Spain (juveniles)	−6.501	37.164	15	4.13	2.00	0	7
1006	DNP near field station, Spain	−6.500	37.000	2	7.5	0.71	7	8

Results

Link counts were obtained for 2,137 adult and 15 juvenile newts from altogether 128 populations (table 1). The spatial distribution of morphological groups is tripartite (fig. 2). Inferred *T. marmoratus* are found in France and in the northwest of the Iberian Peninsula whereas inferred *T. pygmaeus* are found in the southwest of the Iberian Peninsula, which is in line with documented species ranges (fig. 1). Marbled newts from the south of Spain show (low) Nlinks values as typical for *T. marmoratus*, yet they unquestionably represent *T. pygmaeus* because the geographical distribution of this coherent group includes the type locality 'Cadiz province'. In line with low (*T. marmoratus* and *T. p. pygmaeus*) and high Nlink values (*T. p. lusitanicus* **ssp. nov.**) individuals have either a dark, horizontally striped or a green, reticulated appearance (fig. 3).

Somewhat atypical link counts were found in northern Spain and France for *T. marmoratus* and in central Portugal for both species. Mixed species allocations were found in four populations at or close to the *T. marmoratus* – *T. pygmaeus* contact zone in central Iberia (fig. 2). The differentiation of southern and northern *T. pygmaeus* has a sharp transition point that is located at the Atlantic coast, adjacent to the mouth of the Guadalquivir River. Link counts are significantly different among groups (Independent samples Mann-Whitney *U*-test, $U = 128$, $P < 0.0001$; fig. 4A). This analysis excludes the juveniles from the preserved Doñana material, that have lower Nlink counts than the adults from this metapopulation (Independent samples Mann-Whitney *U*-test, $U = 41$, $P < 0.05$). It is relevant to note that adults are locally exceptionally small, as observed earlier (García-París et al., 1993; Díaz-Paniagua et al., 1996) and this is illustrated by measurements on preserved material (MNCN 9568, female, SVl1 = 40.5 mm, SVl2 = 44.2 mm; MNCN 9569, male, SVl1 = 37.4 mm, SVl2 = 41.1 mm and MNCN 12067, male, SVl1 = 27.5 mm, SVl2 = 30.0 mm; see also supplementary table S1).

A principal component analysis on males had 42.6% of the total variance explained on the first axis and 21.6% on the second PC-axis and revealed limited morphometric differentiation among the southern and northern *T. pygmaeus* population groups (fig. 5A). Loadings (L) on the first PC-axis were high for extremity lengths (FLl, TFl, HLl and FTl, $L > 0.78$) and low for the other characters ($0.26 < L < 0.32$). On the second axis loadings were high for head size (Hl and Hw, $L > 0.69$) and for interlimb distance ($L < -0.52$) and low for the other characters ($-0.21 < L < 0.22$). In females, morphometric differentiation was marginal (fig. 5B), with 71.6% of the total variance explained on the first axis and 9.4% on the second PC-axis. Loadings on the first axis were high for all characters ($L > 0.77$) whereas on the second axis loadings were differentiated for head size (Hl and Hw, $L > 0.42$) and digit length (TFl and FTl, $L < -0.32$) and not the other characters ($-0.14 < L < 0.14$). In the univariate analyses of relative sizes, significantly shorter digits were observed in southern than in northern *T. pygmaeus*, but only in males (table 2).

The analysis of Structure Q-scores over a densely sampled transect across DNP yields a sharp geographical cline (fig. 4B). The most relevant parameter estimates in the cline model are a width of 2.52 km (95% confidence interval, CI 0.015–4.425) and a position of the cline centre at 16.93 km from the reference point (CI 16.03–17.67 km). The nearest sampled localities at either side of the genetic transition are 3,870 m apart. For details, see supplementary table S2. The transition in microsatellite allelic profile takes place within the area over which the *T. pygmaeus* morphology changes also.

FIGURE 3 Nine marbled newts from three (sub)species each in dorsal and ventral view. Link counts for the left and right side of the body are given in parentheses. Top row – *Triturus marmoratus* from Jublains, France; left male (3, 2) and right female (1, 1). Middle row – *T. pygmaeus lusitanicus* **ssp. nov.** from Cardeña, Spain; from left to right female (2, 4), male (4, 3) and male (4, 5). Bottom row – *T. p. pygmaeus* from Salinas, Spain; from left to right male (2, 1), male (1, 2) male (2,0) and female (3, 1). Note that the colour pattern in *T. marmoratus* and *T. p. pygmaeus* is horizontally banded whereas in *T. p. lusitanicus* **ssp. nov.** it is reticulated.
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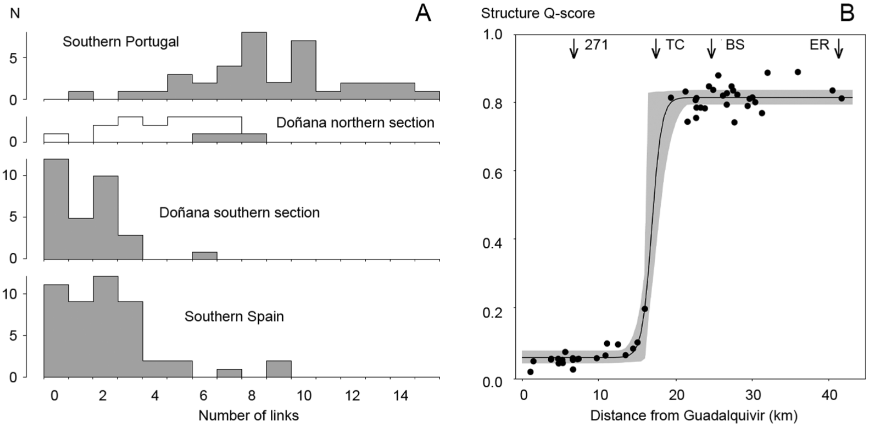

FIGURE 4 Morphological and genetic variation in *Triturus pygmaeus* from in and around Doñana National Park (DNP). A – histograms of Nlink counts for four population groups with, from top to bottom: southwestern cluster of *T. pygmaeus* (localities 65, 436, 438 and 1004 in the south of Portugal, together marked C1 in fig. 2), populations in the northern section of DNP (ER, locality 1005 and BS, locality 1006), population in the southern section of DNP (locality 271) and the Betic cluster of *T. pygmaeus* (localities 270, 463, 465 and 1003 in the very south of Spain, together marked C2 in fig. 1). Exceptionally, the northern Doñana museum material contains 15 juvenile specimens (open bars) along with three adults (shaded bars). B – geographical cline in microsatellite genetic variation. The horizontal axis shows 'Distance from the Guadalquivir River' in km and the vertical axis presents Structure Q-scores. The solid round symbols represent populations and the grey area shows the 95% credibility interval. The Structure Q-scores were extracted from a published figure (Albert & García-Navas, 2022: Figure 4) and are unavoidably imprecise. The position of the 'Torre Carbonero', a cultural landmark at the Doñana beach, is shown by an arrow (TC). Three populations with morphological data available, that can be associated to the transect are El Rocío (arrow marked ER) and the biological station (BS) in northern Doñana and Zacallón C. de los Junqueros in southern Doñana (arrow marked 271). For details, see table 1 and supplementary table S1.

Discussion

The spatial interpretation of marbled newt species allocation by Nlinks corresponds to documented distributions with, however, several areas where morphological data are unconvincing or conflicting (figs. 1 and 2). First, *T. marmoratus* populations in the north of Spain and France show relatively high counts, but more data are needed to support a possible trend. Second, central Portugal displays a variety of values for populations that, from genetic information, are known to mostly be *T. pygmaeus* and few *T. marmoratus* or admixed (Espregueira Themudo & Arntzen, 2007; Arntzen & Espregueira Themudo, 2008; Arntzen et al., 2021; Lopez-Delgado et al., 2021). Third, and most relevant to the present study, is that the Betic region is occupied by *T. pygmaeus* with a horizontally banded pattern and low link counts, as alluded to earlier (García-París et al., 1993). From colour pattern alone all individuals in these populations would erroneously classify as *T. marmoratus*. A similar discrepancy may arise from ontogenetic variation. *Triturus pygmaeus* from DNP has been reported to start reproduction at exceptional small size (males SVL1 ≥ 32.2 mm, females SVL1

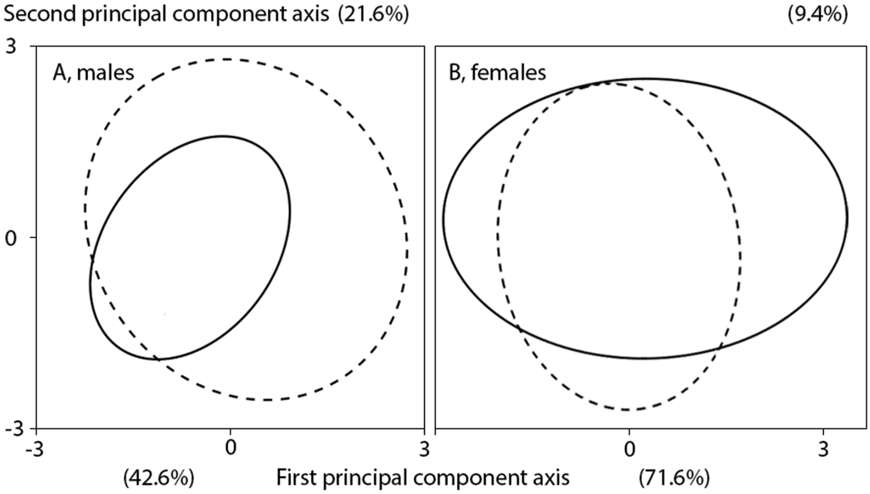

FIGURE 5 Results of a principal component analysis on size corrected morphometric data for *Triturus pygmaeus pygmaeus* (solid lines) and *T. p. lusitanicus* **ssp. nov.** (interrupted lines). The ellipses show the 95% confidence interval of the mean. The left panel is for males and the right panel is for females. Percentages of total variance explained are indicated along the axes. For details, see text.

≥ 31.2 mm) and young age (1–2 years) (Díaz-Paniagua et al., 1996; see also supplementary table S1). Because the museum material represents the same northern Doñana stock, it may be concluded that Nlinks increases over lifetime. This raises the question of how many links adults from northern Doñana would display if they were to reproduce at regular size (ca. 60 mm; García-París et al., 1993; Arntzen, 2018) and age (3–4 years; Castanet & Caetano, 1993).

The southern taxon of *T. pygmaeus* is found in the Betic region whereas the northern taxon is found from north of the Guadalquivir River up to the range of *T. marmoratus* in central Iberia. Atlas data suggest that the *T. pygmaeus* subspecies are currently widely separated on account of the Guadalquivir and the associated low-quality habitats brought about by agricultural land (Pleguezuelos et al., 2002). This distribution is regionally similar to that of fire salamanders (*S.* (*s.*) *longiristris* Joger and Steinfartz, 1994 versus *Salamandra salamandra* (Linnaeus, 1758)), midwife toads (*Alytes dickhilleni* Arntzen and García-Paris, 1995 versus *A. cisternasii* Boscá, 1879 and *A. obstetricans* (Laurenti, 1768)) and parsley frogs (*Pelodytes ibericus* Sánchez-Herráiz, Barbadillo, Machordom and Sanchiz, 2000 versus *P. punctatus* Daudin, 1802 and *P. atlanticus* Diaz-Rodriguez, Gehara, Marquez, Vences, Goncalves, Sequeira, Martinez-Solano and Tejedo, 2017). Whereas these taxa are genetically more or less deeply differentiated (Martínez-Solano et al., 2004; Diaz-Rodriguez et al., 2017; Burgon et al., 2021), the data on genetic differentiation of *T. p. pygmaeus* and *T. p. lusitanicus* **ssp. nov.** are ambiguous.

Substantial nuclear genetic variation has been documented within *T. pygmaeus* with, however, no different signature for Betic populations (Arntzen et al., 2014; Lopez-Delgado et al., 2020). Single populations standing out are from Los Barrios (Cadiz province) that was found to be genetically different from other *T. pygmaeus* (Arntzen et al., 2021) and from Doñana, possibly locality El Rocío, that is characterized by an aberrant chromosomal

TABLE 2 Summary of morphometric data on males (top panel) and females (bottom panel) for two subspecies of the pygmy newt, *Triturus pygmaeus*. N is sample size. Values to the left are averages for the species (in mm) and those to the right are relative values obtained from division by SVl1. Testing for statistically significant differences is done for the relative values, with Mann-Whitney's U-test.

Subspecies	***lusitanicus*** ssp. nov.		***pygmaeus***		**Test**
Males	**N = 60**		**N = 26**		**P**
SVl1	47.88		43.75		
ILd	26.20	0.548	23.58	0.541	NS
FLl	19.67	0.412	17.99	0.412	NS
TFl	7.18	0.151	5.73	0.131	*
HLl	20.35	0.427	18.63	0.426	NS
FTl	8.13	0.171	6.27	0.143	***
Hw	10.08	0.211	8.94	0.205	*
Hl	14.12	0.295	12.68	0.290	NS
Females	**N = 38**		**N = 23**		**P**
SVl1	54.00		55.89		
ILd	29.71	0.550	30.46	0.552	NS
FLl	19.20	0.356	19.89	0.357	NS
TFl	5.93	0.110	6.24	0.112	NS
HLl	19.71	0.365	19.81	0.355	NS
FTl	6.07	0.113	6.26	0.112	NS
Hw	10.95	0.203	10.97	0.197	NS
Hl	15.27	0.283	15.30	0.274	NS

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

C-banding pattern (García-París et al., 1993). In the most detailed genetic study so far (Albert & García-Navas, 2022) different entities were found inside DNP. It is here shown that the data conform to a narrow cline positioned at ca. 17 km north of the Guadalquivir, with a centre that coincides with the position of the 'Torre Carbonero', a cultural hallmark at the adjacent beach (fig. 4B). Populations to the north and the south have different morphologies as well as different microsatellite genetic profiles and it is concluded that DNP harbours the southern as well as the northern subspecies. Mitochondrial DNA variation within the species appears shallow and without a strong geographical component (Wielstra et al., 2013). No data are currently available to allocate the presumably isolated easternmost *T. pygmaeus* occurrences to the one or the other taxon (see fig. 1).

The contact zone of northern and southern *T. pygmaeus* is remarkably narrow. In the absence of selection, the width (w, in km) of a cline can be predicted from a diffusion model as a function of dispersal distance (d, in km per generation) and the length of time since contact (t, in generations), as $w = 2.51*d*\sqrt{t}$ (Barton & Gale, 1993). Dispersal in *Triturus* newts has been estimated at ca. 1 km per year for *T. cristatus* (Laurenti, 1768) (Arntzen & Wallis, 1991; see also Smith & Green, 2005; Trochet et al., 2014). However, local populations of *T. pygmaeus* are charactered by small body size, possibly limiting dispersal capability. At a low dispersal of 100 m per generation, cline width would exceed the observed 2.5 km in ca. 100 generations, i.e., less (or much less) than 300 years. Arguably, the *T. p. pygmaeus* – *T. p. lusitanicus* **ssp. nov.** hybrid zone is much older than this (see discussion below) and its width appears to have been kept in check over thousands of years. In the absence of a strong environmental barrier to dispersal, this may point to a substantial selection against hybrids which, in turn, would assist to support species status for the taxa involved.

Alternatively, a ridge of dunes in between the subspecies has been interpreted as a strong environmental barrier (Albert & García-Navas, 2022), yet these authors report dispersal rates among southern and northern Doñana population groups comparable to, or higher than elsewhere. While I agree that (effective) population size is an important constituent to realized dispersal, I doubt that "The number of sampled individuals … constitutes a representative estimate of the population size (total number of individuals) of each pond" (Albert & García-Navas, 2022: 580). The claim is certainly unfounded for 13 populations for which I collected all material. To complicate matters further, I collected newt larvae in an *ad hoc* manner with a dipnet, therewith deviating from the standardized protocol with funnel traps reported by Albert and García-Navas (2022) for adults and juveniles. Accordingly, Albert's inferences on gene flow, as far as depending on population size and life stage, are yet to be substantiated.

The *T. pygmaeus* subspecies ranges are connected along the Atlantic Ocean and the contact is probably restricted to Doñana National Park. I here suggest that the above-mentioned ridge of dunes marks a former position of the Guadalquivir River and that southern Doñana populations belong to the southern taxon (*T. p. pygmaeus*) that, with a redirection in the course of the river, became disconnected from its main distribution, to eventually connect with the northern taxon (*T. p. lusitanicus* **ssp. nov.).** Archaeological studies acknowledge that the Guadalquivir previously discharged into an inland sea, the Gulf of Tartessos, before reaching the Atlantic Ocean. Bathymetric reconstructions for the 4,000–2,200 BP period suggest the past presence of a spit of land protruding northward from Sanlucar de Barrameda into the Tartessian gulf. This spit of land is not only a candidate location for the lost city of Tartessos (Abril et al., 2013), but may also have housed the southern taxon of *T. pygmaeus*.

Similar patterns of genetic variation within the DNP as for *T. pygmaeus* have been reported for the salamander *Pleurodeles waltl* Michahelles, 1830 and for the frog *Pelophylax perezi* (López-Seoane, 1885) (G. Sánchez-Montes, pers. comm., 2023). The occurrence of three microsatellite clines in parallel suggests that the three species involved have been subject to a common spatio-temporal regime, to which the most recent major change in the course of the Guadalquivir may have been a key event, i.e., by disrupting the overland connection to the south and establishing an overland connection to the north. A hierarchy in population genetic differentiation with a profound separation over the DNP transect and not the river would support the proposed vicariant scenario, but data for adjacent populations south of the lower Guadalquivir are yet to be gathered.

With *T. pygmaeus* as a prime candidate, it might be worthwhile to also collect morphological data over the wider DNP-transect, to investigate to what extent geographical clines from genetic and morphological markers are coincident and concordant. Furthermore, the existence of multiple contact zones within the Doñana National Park calls for studies on the relative contribution of ecological, behavioural and genetic incompatibilities of differentiated and possibly independently evolving, speciating lineages. A further hypothesis to be tested is that other organisms with a low dispersal capability have been subject to the same vicariant scenario. Fortuitously, the entire region is characterized by high levels of diversification among and within species (Hewitt, 2011; Molina-Venegas et al., 2017), so that the baseline genetic variation needed for this test will often be available. Finally, I amplify the call to journal editors to make data sharing prescriptive (Gabelica et al., 2022; Watson, 2022).

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Version of Record

The electronic version of this article is considered a published work according to the International Commission on Zoological Nomenclature (ICZN). Therefore, the new name contained in the electronic version is officially published under the ICZN code. The work has also been registered in ZooBank (http://zoobank.org/) and can be accessed under http://zoobank.org/urn:lsid:zoobank.org:pub: A6340CA8-57CD-4F2F-81A0-0E0C6422BE90.

Supplementary material

Supplementary material is available online at: https://doi.org/10.6084/m9.figshare.24420589

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Appendix A1

Triturus pygmaeus

The description of the species' external morphology below largely follows García-París et al. (1993: 5), albeit that in my terminology marbled newts have a dark dorsal side with a green colouration pattern. The male crest is lowered over the pelvic area and males from the northern part of the species range (with the reticulated colour pattern) were found to have not shorter but longer digits that those in the south.

Large head, longer than wide, gradually sharpened, ending abruptly in a narrow, blunt nose. Small eyes placed in a forward position. Gular fold moderately conspicuous. Elongated body with long, thin legs. Digits long, especially in males. Toe webbing absent. Relatively short tail, its cross-section being almost cylindrical at the base and progressively compressed towards the tip. Finely granulated skin, ridged in the gular region, with large granules unevenly spread over the dorsal surface. A general north to south decrease in overall size, be it that individuals of extremely small size have been reported in and around the Doñana National Park, irrespective of intraspecific taxonomic affiliation.

During the terrestrial phase the dorsal colour pattern consists of an irregular pattern of olive-green spots on a dark background. These spots can create a reticulum covering the side of the body or be sparse or absent in the middle, to the effect of a dark, more or less wide band running along the side of the body (fig. 3). In females, the dorsal midline appears as a continuous orange stripe whereas males have a line with alternating orange-yellow and black narrow bands. The dorsal part of the tail shows the same colour as the dorsum. Ventrally, the colouration is that of an off-white, crème to grey background, sometimes with an orange hue towards the cloaca, on which large blackish spots and small white dots may be found. These white dots may show a higher density on the flanks and occasionally completely cover the background colouration. White dots are also present on the base of the flanks, spreading over the sides of the neck and the cheeks, here alternating with black spots. The ventral black spots are usually large and roundish in males and small and more elliptically shaped in females. The ventral background colour continues over the gular region, often at a lighter shade of pale. The distribution of black and white dots does not seem to follow any obvious geographical trend (fig. 3, Appendix fig. A1). Juveniles show a brighter colour than adults, frequently with a conspicuous, fluorescent-like orange dorsal line and fluorescent-like green dorso-lateral colouration, and a yellowish ventral colouration.

During the breeding period the males have a moderately high dorsal crest, which continues along the tail with an inflexion in the pelvic area. Male crest blotched by alternating black and white vertical bands. In the female, the mid-dorsal line usually shows orange-yellowish, or greyish tones. Male cloaca exposed, swollen and deep black. Female cloaca walnut-shaped, bordered with papillae. Well-developed labial folds during the aquatic phase, especially in females. While the intensity of the dorso-lateral colouration pattern may change during the aquatic phase, especially in males, the reticulation itself remains. Some individuals may become very dark and melanic in appearance, with the colouration pattern difficult to discern. Post-metamorphic juveniles are rarely if ever found in the water.

Description of Triturus pygmaeus lusitanicus *ssp. nov.*

Description of the type specimen – adult male in breeding condition. Preserved on ethanol in excellent storage condition at the Museo Nacional de Ciencias Naturales, Madrid, under catalogue number MNCN 51799. Measurements are SVl1 51 mm, SVl2 57 mm, ILd 25 mm, Hw 10.3 mm, Hl 14.9 mm, FLl 20.0 mm, TFl 6.0 mm, HLl 20.8 mm and FTl 7.7 mm. Total length 105 mm. Relative finger lengths 1 < 4 < 2 < 3. Relative toe lengths 5 < 1 < 2 < 3 = 4. Nlinks left 5, right 6, total 11; colour pattern reticulated. Thirteen crème-white bands over the head and body part of the mid-dorsal crest. Light grey ventral colouration, with an orange hue towards the swollen, deep black cloaca. Several roundish but vague ventral dark spots and many irregularly shaped medium large white spots. The type specimen is shown in Appendix fig. A2.

Description of paratypes

First paratype – MNCN 51800 Female. Excellent storage condition. SVl1 52 mm, SVl2 55 mm, ILd 27 mm, Hw 10.0 mm, Hl 14.7 mm, FLl 18.4 mm, TFl 5.8 mm, HLl 18.7 mm and FTl 6.8 mm. Total length 105 mm. Relative finger lengths 1 < 4 < 2 < 3. Relative toe lengths 5 < 1 < 2 < 4 < 3. Nlinks left 3, right 6, total 9; colour pattern reticulated. Medium grey ventral colouration with no black spots and a medium number of small white spots.

Second paratype – MNCN 51801. Female. Excellent storage condition. SVl1 58 mm, SVl2 61 mm, ILd 29 mm, Hw 10.8 mm, Hl 15.8 mm, FLl 20.0 mm, TFl 7.1 mm, HLl 21.3 mm and FTl 7.6 mm. Total length 120 mm. finger lengths 4 < 1 < 2 < 3. Relative toe lengths 5 < 1 < 2 < 3 = 4. Nlinks left 6, right 6, total 12; colour pattern reticulated. Grey ventral colouration with few small black spots and few small white spots.

Other material from the type locality – Additional morphological data on 11 individuals from the type locality are in the Appendix table A1.

Locality and date of collecting – Granja, Portugal, at 7.262 western longitude and 38.318 northern latitude, elevation 160 m above sea level. Collected by J. W. Arntzen on 11 March 2023.

Diagnostic features – the newly recognized subspecies is morphologically distinguishable from the southern, nominotypical subspecies by a high number of green coloured transversal bands (also referred to as 'links'), by long fingers and toes in males and possibly by a relatively big head. Microsatellite data for a section of the range show a genetic profile that is drastically different from that of the adjacent southern form, as studied in the southern section of Doñana National Park.

Taxonomic status – The northern form is described as a subspecies. This decision is debatable because the currently available microsatellite genetic data suggest that genetic exchange with the nominotypical form in the short contact zone is absent or rare. The low level of genetic exchange may be due to a local environmental barrier, or to strong selection against hybrids.

Derivatio nominus – the subspecies name 'lusitanicus' acknowledges that the range of the newly recognized taxon coincides with the former Roman province of Lusitania.

Suggested vernacular name – Lusitanian pygmy newt.

Distribution – The south-western corner of the Iberian Peninsula with the exception of the Betic Cordillera. Approximate latitudinal range borders are the range of *T. marmoratus* at ca. 40 degrees north and the Guadalquivir to the south. In the east ranging till ca. 3.0 degrees west, perhaps 1.5 degrees west. Widely allopatric to *T. p. pygmaeus*, yet presumably with a narrow and sharp contact zone located inside the Doñana National Park. See figs 1 and 2.

FIGURE A1 Variation in ventral colouration of *Triturus pygmaeus*. Top row – *T. p. lusitanicus* **ssp. nov.**: male (with dark tail underside) and two females from Villalba and male from Cardeña. Middle row – *T. p. pygmaeus*: male from Salinas, male and female from Los Barrios and male from Rio Alberite. Bottom row – *T. p. pygmaeus*: two males and two females from Rio Alberite. PHOTOGRAPHY L. A. VAN DER LAAN

FIGURE A2 Holotype of *Triturus pygmaeus lusitanicus* **ssp. nov.** in live condition, at the left (top) and right dorso-lateral view (middle), and in ventral view (bottom). The scale bar equals 10 mm.

TABLE A1 Morphological data for 11 individuals from type locality Granja, Portugal. Measurements are in mm. For abbreviations and counts on the number of links, see the main text and Arntzen (2018).

Field number	Sex	SVl1	SVl2	ILd	FLl	TFl	HLl	FTl	Hw	Hl	Number of links		
											Left	Right	Total
1617	Male	52	60	28	20.5	6.0	20.8	7.7	10.6	15.1	6	9	15
1618	Female	56	61	29	18.3	4.8	20.8	5.1	10.7	16.0	6	5	11
1619	Male	49	56	24	18.2	4.7	18.8	6.5	9.9	14.3	8	4	12
1620	Female	52	59	27	18.4	5.5	19.7	5.7	11.4	14.9	6	6	12
1621	Male	50	59	27	20.0	7.6	22.0	6.7	11.2	15.5	6	6	12
1622	Female	56	62	35	18.2	5.9	20.2	5.0	11.1	15.9	6	3	9
1623	Female	60	66	33	22.8	6.3	23.3	7.3	12.1	17.5	4	4	8
1624	Female	55	60	28	19.4	6.4	20.7	6.7	11.4	16.1	4	6	10
1625	Female	59	63	35	18.3	5.4	19.7	5.3	11.3	14.7	6	6	12
1626	Female	55	61	29	18.2	5.7	20.9	6.1	10.8	15.2	5	3	8
1627	Female	60	66	33	19.7	5.3	22.2	5.5	11.9	16.0	5	8	13