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Morphological and genetic diversification of pygmy and marbled newts, with the description of a new species from the wider Lisbon Peninsula (*Triturus*, Salamandridae)

Jan W. Arntzen | ORCID: 0000-0003-3229-5993
Institute of Biology, Leiden University, 2333 BE Leiden, The Netherlands
Naturalis Biodiversity Center, 2333 CR Leiden, The Netherlands
pim.arntzen@gmail.com

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Abstract

Iberian populations of large-bodied newts, with *Triturus marmoratus* in the north and *T. pygmaeus* in the south of the peninsula, were studied for external morphology, mitochondrial DNA and for a panel of single nucleotide polymorphisms. This confirmed the species' low level of interspecific hybridization and their parapatric, mosaic-like mutual range border across the peninsula. The genetic data also revealed substantial variation within *T. pygmaeus*, with narrow (0.43–35.2 km) clinal transitions in the very centre of Portugal. Similar clines were observed for body size and colouration pattern. Pygmy newts in the west of Portugal are larger, with a more striped (less reticulated) green dorso-lateral colouration pattern than those in the east and south of the country. The western group of populations is described as a new species, *Triturus rudolfi* **sp. nov.**, on account of a long, ca. 2.5 Ma, independent evolutionary history and limited hybridization with its sister-species *T. pygmaeus*, suggesting selection against hybrid offspring. The range of the newly described species may be restricted to the wider Lisbon Peninsula, stretching northwards along the Atlantic coast to the river Vouga estuary. Inland, the range border may be set by the lower Tejo River, or by the currently wide area of agricultural land at either side of that river, that may accommodate a residual hybrid zone. The close contact between both pygmy newt species is effectively limited to a ca. 20 × 40 km area directly north of the town Entroncamento, where *T. rudolfi* **sp. nov.** is sandwiched in between *T. marmoratus* and the river Tejo.

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Keywords

clinal variation – colouration pattern – hybrid zone – Lisbon pygmy newt – mitochondrial DNA – morphometrics – Portugal – single nucleotide polymorphisms – *Triturus marmoratus* – *Triturus pygmaeus* – *Triturus rudolfi* **sp. nov.**

Introduction

We do not know, and probably never will know, the number of species in Iberia or Siberia. Apart from essentially unsolvable conceptual questions of 'what is a species?', or what criteria to employ for species recognition and delimitation (de Queiroz, 2007; Wilkins, 2011; Burbrink & Ruane, 2021), a frequently overlooked issue is that we are dealing with a moving target, with standards now set higher than before, procured by technological innovation. Once the 'low hanging fruit' of species was harvested in the early days – say from Aristotle and Theophrastus (Mayr, 1982), through Linnaeus (1758) to Mayr (1969) – increasingly species are described that are morphologically cryptic, with small ranges and from difficult-to-explore parts of the planet, or that are (or were) problematic to survey such as cave organisms and internal parasites. While, for example, Mertens and Wermuth (1960) might have thought that their checklist of European amphibians and reptiles was pretty complete, recognized species numbers have more than doubled (N close to 300; Speybroeck et al., 2020) and the end is not in sight (e.g., Bassitta et al., 2020; Recknagel et al., 2023).

Recent taxonomic advance is often achieved through phylogenetic and phylogeographic studies that employ multi-locus nuclear genetic data. Such studies are, however, prone to 'taxonomic inflation' because exhaustive datasets tend to resolve consistent divergences deep within species boundaries (Arntzen and Bauer, 1996; Sukumaran and Knowles, 2017; Leaché et al., 2019; Burbrink et al., 2022; Dufresnes et al., 2023). In response, it has been advocated to subject candidate species to population genetic tests, increasing the workload even further. One acid test would be hybrid zone analysis, to affirm that candidates experience some degree of reproductive isolation, consistent with selection against hybrid offspring (Mayr, 1942; Dufresnes et al., 2020; Burbrink and Ruane, 2021).

Much of the increase in documented species richness is in the southern Holarctic – thus more in Iberia than in Siberia – in line with the well understood 'southern richness, northern purity' paradigm (Hewitt, 1996, 2000). The present study follows on from analyses that deal with morphological and genetic differentiation of large-bodied newts (genus *Triturus*) in southwestern Europe, i.e., France, Spain and Portugal (Arntzen, 2018, 2024). The data so far show the existence of three, spatially coherent, morphologically differentiated groups. Briefly, to the north of ca. 40 degrees northern latitude (northern) marbled newts are large and robust, with dark coloured bellies and a horizontally banded dorso-lateral colouration pattern. To the south of the Guadalquivir River pygmy newts as they are called, are small, with light coloured undersides and with a banded pattern also. These two groups enclose a third group in the southwest of the Iberian Peninsula, that is characterised by a reticulated dorso-lateral colouration pattern and light bellies. The northern group corresponds to *T. marmoratus* (Latreille, 1800) and to the south the species is *T. pygmaeus* (Wolterstorff, 1905), for which the central group has been described (*T. pygmaeus lusitanicus* Arntzen, 2023) as different from the southernmost nominotypical subspecies (fig. 1). When,

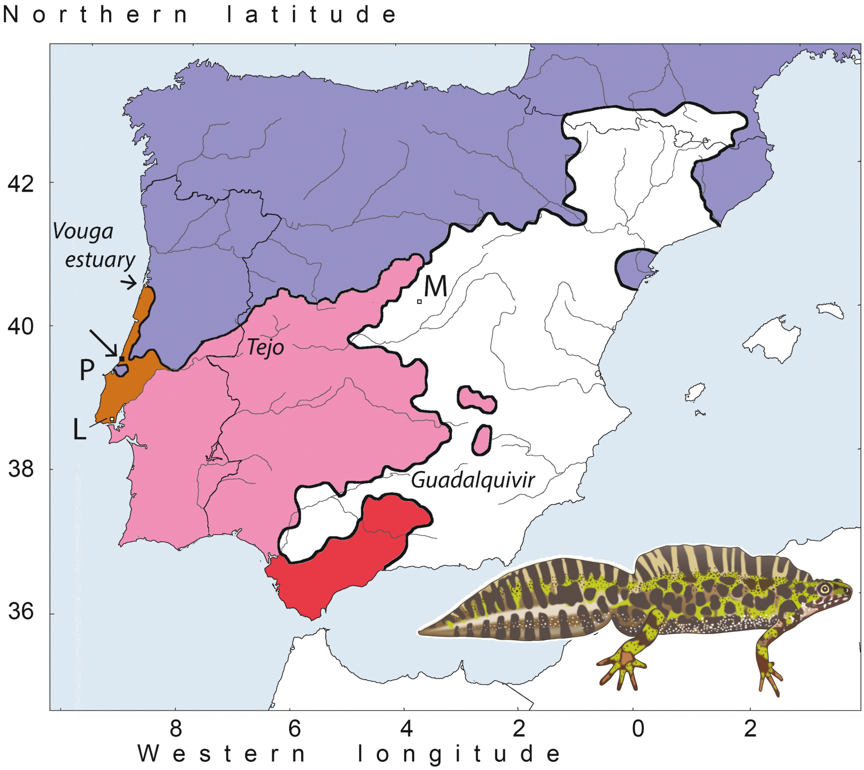

FIGURE 1 The Iberian Peninsula with the approximate distributions of four taxa of large-bodied newts as described in table 1. Colour codes are: blue – *Triturus marmoratus,* dark red – *T. pygmaeus pygmaeus*, light red – *T. p. lusitanicus* and brown – *T. rudolfi* **nov. sp.**, i.e., the newly described species from the wider Lisbon Peninsula. Letters indicate the capital cities Lisbon, Portugal (L) and Madrid, Spain (M) as well as Peniche (P) at the Atlantic coast. Major rivers partially coinciding with (sub)species borders are the Guadalquivir, the Vouga and the Tejo. The new species' type locality Lagoa Seca near Valado dos Frades is indicated by a long arrow. The insert shows an adult male *T. marmoratus*.
ANIMAL DRAWING BY BAS BLANKEVOORT

in this paper, *T. pygmaeus* is written with quotation marks ('*T. pygmaeus*'), this refers to the taxon before the here presented case for *T. rudolfi* **sp. nov.** as different from *T. pygmaeus*. For convenience of the reader the present-day knowledge is summarized in table 1.

Material and methods

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. The morphological characters measured are: snout-vent length up to and including the insertion of the hindlegs (SVl1), and up to and including the cloaca (SVl2, for descriptive purposes only), head length (Hl), head width (Hw), interlimb distance (ILd), forelimb length (FLl), third finger length (TFl), hindlimb length (HLl) and fourth toe length (FTl). Measurements were taken on live adults, with a plastic ruler in mm (SVl, ILd) or with plastic Vernier callipers at 0.1 mm precision (the other characters, for details, see Arntzen, 2018). Extremities

TABLE 1 Summary of information on taxonomic status, phenotype and distribution of four taxa of Iberian large-bodied newts (genus *Triturus*).

Species	***T. marmoratus***	***T. rudolfi* sp. nov.**	***T. pygmaeus* #**		**References**
Subspecies			*lusitanicus*	*pygmaeus*	
Range across Iberian Peninsula	North	Wider Lisbon Peninsula	South	South of Guadalquivir	
Type locality	Paris, France	Lagoa Seca, Valado dos Frades, Portugal	Granja, Portugal	Province Cadiz, Spain	1, 2
Author	Latreille, 1800	Present paper	Arntzen, 2023	Wolterstorf, 1905	
Vernacular name	Marbled newt	Lisbon pygmy newt	Lusitanian pygmy newt	Southern pygmy newt	
Character					
Body size	Large	Intermediate large	Intermediate small	Small	2, 3, 4
Dorso-lateral colour pattern	Banded	Variable, intermediate	Reticulated	Banded	2, 3, 4
Number of links	Low	Intermediate	High	Low	2, 4
Dorsal colouration	Hard green	Intermediate	Olive green	Olive green	2, 3, 4
Ventral colouration	Grey or black	Light grey	Light grey	Light grey	2, 3, 4
Climatic conditions	Relatively cool and wet	Relatively cool and wet	Relatively warm and dry	Not studied	

Originally described as a subspecies of *T. marmoratus*, but raised to species level by García-París et al. (2001).
References: 1 – Mertens and Müller (1928), 2 – Arntzen (2024), 3 – García-París et al. (1993), and 4 – Arntzen (2018).

were measured at the right side of the body. Because of a marked dimorphism (see Results) the sexes were treated separately. In salamanders, age and size at reproduction may vary from one population to the other, possibly as a direct response to conditions of the environment, a phenomenon especially prominent in pygmy newts (García-París et al., 1993; Díaz-Paniagua et al., 1996). To reduce the effect of overall size and to increase normality of the data, the standardized residuals of the regression of ln(character) to ln(SVl1) were used in multivariate analysis. To facilitate data interpretation and for comparability with published data, I also calculated body proportions from untransformed data (R_*character* = *character*/SVl1). Individuals were also inspected for the number of 'links' that they display, with most data taken from Arntzen (2024). Links are dorso-lateral colour pattern character states. They were counted as the number of uninterrupted connections following the green coloured surface at the lateral sides of the body, in between the insertion of the fore- and hindlegs, summed for the left and right side of the body (N_{links}). For a further explanation, and for illustrated examples and discussion of how the counting is done see Arntzen (2018). Although the morphological differentiation between *T. marmoratus* and *'T. pygmaeus'* has already been analysed (Arntzen, 2018), data on the former species are included as a reference. Data for genetically admixed populations (see below) were excluded.

Tissues for molecular genetic analyses were larval or adult tail tips. Alternatively, recently deposited eggs (embryos) were raised in 5l buckets until hatching. DNA was extracted from 95% ethanol preserved material under standard protocols with the KingfisherTM (Thermo Scientific) and DNeasy extraction kits (Qiagen, Valencia, CA, USA). Single nucleotide polymorphism (SNP) data were gathered for 54 markers developed for the system (Lopez-Delgado et al., 2021), with data for 2622 individuals from 258 populations. Genotyping took place at the Institute of Biology Leiden SNP line facility of the Institute of Biology, Leiden, using the Kompetitive Allele-Specific PCR system (KASP, LGC genomics, UK). A principal component analysis (PCA) of the SNP-data was performed with Adegenet (Jombart, 2008). In a later phase of the study, five populations were additionally studied to narrow the observation gap along the lower Tejo River. This material was studied for 14 of the most informative markers selected out of the original 54 (see supplementary table S1).

A fragment of the mitochondrially encoded NADH dehydrogenase 4 gene (ND4) was amplified and sequenced with established primers following a set protocol (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 typical for '*T. pygmaeus*' were identified as belonging to the 'western' (henceforth 'southwestern') and the 'eastern' haplotype group, based on a single differentiated nucleotide that corresponds to position 11241 in the full mitochondrial genome of *Salamandra salamandra* (Linnaeus, 1758) with Genbank accession number KX094971 (Mulder et al., 2016). '*Triturus pygmaeus*' haplotypes found in pure or admixed populations of *T. marmoratus* and *vice versa* (as determined from SNP and published nuclear genetic data (present paper, Arntzen, 2018) or, if so needed, inferred from the documented species distributions (fig. 1)) were omitted, because introgression, hybrid zone dynamics, population demographic analyses and species replacement are the topics of a forthcoming paper.

Blanket distribution maps composed of so-called Dirichlet cells (Matérn, 1979; Burrough et al., 2015) were obtained by spatial data interpolation of the extracted information with the 'NearestPoint' procedure in ILWIS 3.8.6 (ILWIS 2019). Characters of interest were analysed for spatial variation, with the focus on '*T. pygmaeus*' populations along the northern edge of its range (see transect described below). The best fitting geographical clines were estimated with the Hybrid Zone Analysis under R (HZAR) software (Derryberry et al., 2014), with a protocol used earlier (Arntzen et al., 2017). The reference point for longitudinal distance in this analysis is Entroncamento railway station at -8.478 E, 39.456 N, the putative centre of the hybrid zone (see below).

Finally, environmental data were extracted for *Triturus* newt populations with species identity known. Parameters available for selection were elevation and 19 climate variables obtained from the BioClim data base at 30 arc seconds spatial resolution (Fick and Hijmans, 2017). This data set was reduced to five largely uncorrelated variables (Spearman's correlation coefficient |rho| < 0.7) following clustering with the UPGMA-procedure in Primer 7 (Clarke and Gorley, 2015). The number of 258 populations in three species groups was brought back to N = 232, due to positional overlap of neighbouring populations and because genetically admixed ones were excluded. For details on the parameter selection procedure, see supplementary fig. S1. Other statistical analyses were with SPSS 26 (IBM SPSS, 2021).

Results

A principal component analysis of SNP data for 54 marker loci reveals a bimodal distribution of scores along the first PCA-axis, composed of 821 *T. marmoratus* (31.3%) and 1689 *'T. pygmaeus'* (64.4%). The latter cluster is subdivided in two groups along the second PCA-axis that conform to *T. rudolfi* **sp. nov.** (see below) and *T. pygmaeus*. At the population level the corresponding numbers are 89 *T. marmoratus,* 154 *'T. pygmaeus'* and 15 intermediate populations along the first axis and 123 *T. pygmaeus* and 26 *T. rudolfi* **sp. nov.** along the second axis with five intermediate populations (fig. 2). The spatial distribution of the genotypic clusters is geographically coherent, with *T. marmoratus* in the northern half of the Iberian Peninsula, the group of western pygmy newts (*T. rudolfi* **sp. nov.**) in the Lisbon Peninsula and along the adjacent Atlantic coast and the group of eastern pygmy newts (*T. pygmaeus*) inland and to the south (figs. 1 and 3). Genetically intermediate populations are all positioned at species range borders, so that it seems appropriate to interpret these as (rare) hybrid populations. The transition from *T. marmoratus* in the north to '*T. pygmaeus'* in the south of the Iberian Peninsula is abrupt. The contact zone is parapatric with aspects of a mosaic, suggesting that species replacement has taken place under competitive exclusion, without extensive hybridization. The other main transition is from *T. rudolfi* **sp. nov.** in the west to *T. pygmaeus* in the southeast of the Iberian Peninsula. Analysis with HZAR revealed a steep geographical cline for PCA2 of the panel of SNP markers (fig. 4). It has a central position at 0.3 km (95% confidence interval, CI -1.0–7.5 km) and a width of 35.2 km (CI 20.4–58.4 km). Details on the geographical clines are in supplementary table S2.

A total of 1977 mitochondrial DNA (mtDNA) sequences was split in two, about equally sized haplotype groups (N = 1638 when presumably introgressed haplotypes were excluded). The southwestern haplotype is dominant across the entire *T. rudolfi* **sp. nov.** and *T. p. pygmaeus* ranges as well as in the southern section of the *T. p. lusitanicus* range whereas the eastern haplotype is dominant in the northern section of the *T. p. lusitanicus* range. The spatial separation within *T. pygmaeus* is detailed in supplementary fig. S4. The haplotype distribution inside the transect is strongly bimodal. The geographical cline has a central position at 15.2 km (CI 12.9–16.1 km) and a width of 0.434 km (CI 0.048–8.659 km) (fig. 4).

Data on N_{links} were available for 2145 adult newts (N = 1953 when data for hybrid populations were excluded). Numbers of links are different for the (sub)species with low values for *T. marmoratus* and *T. p. pygmaeus,* intermediate values for *T. rudolfi* **sp. nov.** and high values for *T. p. lusitanicus* (fig. 5), with a statistically significant overall differentiation (Kruskal-Wallis test statistic 1177.3, P < 0.0001). Post-hoc comparisons of taxa show that pairwise comparisons are also significant (P < 0.0001), except for *T. marmoratus* and *T. p. pygmaeus* (P > 0.05). An analysis

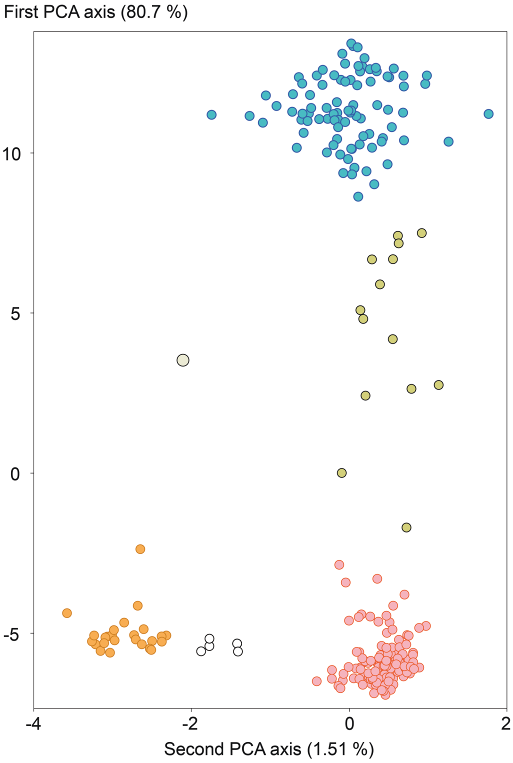

FIGURE 2 Genetic differentiation in 258 marbled newt populations for 54 nuclear SNPs. Plotted are population averages for scores along the first and second principal component axis. The first axis explains 80.7% of the observed variation and separates *Triturus marmoratus* (blue symbols) from '*T. pygmaeus*'. The second axis explains 1.51% of the observed variation and separates western pygmy newts (*T. rudolfi* **nov. sp.**, brown symbols) from southeastern pygmy newts (*T. pygmaeus*, red symbols). Note the unequal scales applied to the axes (for explanation see main text). Populations with intermediate positions are genetically admixed and presumed to contain hybrids between *T. marmoratus* and *T. pygmaeus* (14 grey symbols), *T. marmoratus* and *T. rudolfi* **nov. sp.** (one large, light grey symbol) and between *T. pygmaeus* and *T. rudolfi* **nov. sp.** (five open symbols).

with logistic regression indicates that the optimal classification criterion for *T. rudolfi* **sp. nov.** versus *T. pygmaeus lusitanicus* is at N_{links} = 6.8. The fit of the classification, as expressed by the Area Under the Curve (AUC) statistic is 0.851 ± 0.120 at the individual level and 0.929 ± 0.056 when data are averaged for populations. The geographical cline for N_{links} has a central position at -2.8 km (CI -15.5–18.0 km) and a width of 208.9 km (CI 20.4–425.0 km) (fig. 3).

Body sizes as measured by SVl1 are large for *T. marmoratus*, medium for *T. rudolfi* **sp. nov.** and small for *T. pygmaeus* in both sexes (table 2) which is for the latter two species aptly illustrated by the geographical clines over the transect (fig. 4). The overall differentiation of species is significant (Independent samples Kruskal-Wallis test: males N = 714, test statistic 407.9, df = 2, P < 0.0001; females N = 876, test statistic 387.8, df = 2, P < 0.0001). Post-hoc tests show that pairwise species comparisons are also significant (P < 0.0001 in all combinations for both sexes). Analyses with logistic regression indicate that the optimal classification criterion for *T. rudolfi* **sp. nov.** versus *T. pygmaeus lusitanicus* is at SVl1 = 51.2 mm for males with AUC = 0.671 ± 0.030 at the individual level and AUC = 0.686 ± 0.107 when data are averaged for populations. For females the corresponding value is SVl1 = 57.3 mm, with AUC = 0.680 ± 0.030 and AUC = 0.743 ± 0.092, respectively. Geographical clines for body size have central positions of 65.7 km (CI 25.5–82.8) for males and 65.9 km (CI 22.9–89.2 km) for females. Cline widths are 56.4 km (CI 1.95–178.5 km) for males and 98.8 km (CI 47.7–246.2 km) for females (fig. 4).

Body proportions are different for the three species, except for R_FTl in both sexes (Kruskal-Wallis test, P > 0.05). Post-hoc comparisons suggest that relative extremity lengths (R_FLl, R_HLl) decrease in the order *T. marmoratus* – *T. rudolfi* **sp. nov.** – *T. pygmaeus*, that *T. pygmaeus* has a longer head (R_Hl) and *T. marmoratus* has a shorter body (R_ILd) compared to the other species

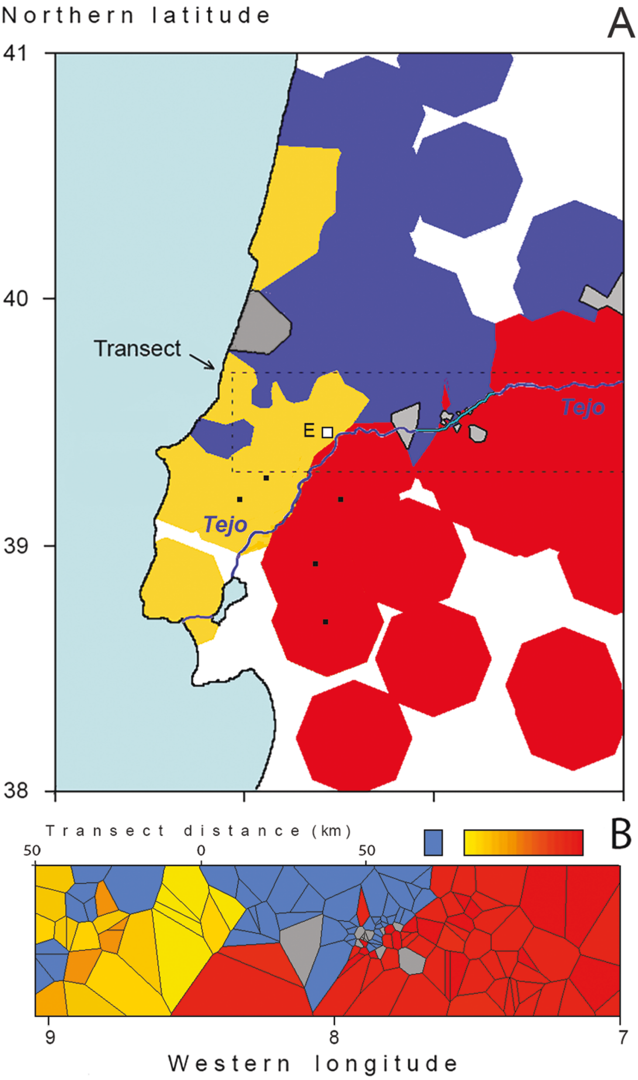

FIGURE 3 (A) Genetically investigated populations of large-bodied newts from the western part of the Iberian Peninsula plotted with the nearest point method, with a maximum spatial extrapolation of ca. 25 km. Colour codes are: blue – *T. marmoratus*, red – *T. pygmaeus* and ochre – *T. rudolfi* **sp. nov.** Inferred hybrid populations of *T. marmoratus* and '*T. pygmaeus*' are shown in grey (as in fig. 2). Note that populations from densely sampled areas do not always stand out separately. Five populations from along the lower Tejo area added *a posteriori* are shown by black dots. The boxed area in between 39.3-39.7 N encompasses the transect studied for spatial variation in morphometrics (35 populations), N_{links} (35 populations), mitochondrial DNA (69 populations) and nuclear genetic composition (82 populations) with HZAR software (for results see fig. 4). The nominal centre of the *T. pygmaeus* – *T. rudolfi* **sp. nov.** hybrid zone is at Entroncamento railway station (E, coordinates -8.478 E, 39.456 N). (B) Dirichlet tessellation of populations studied for variation in nuclear genetic composition, with colours like above. The yellow to red colour bar corresponds to the one shown in fig. 4. Distances in km are relative to Entroncamento railway station.

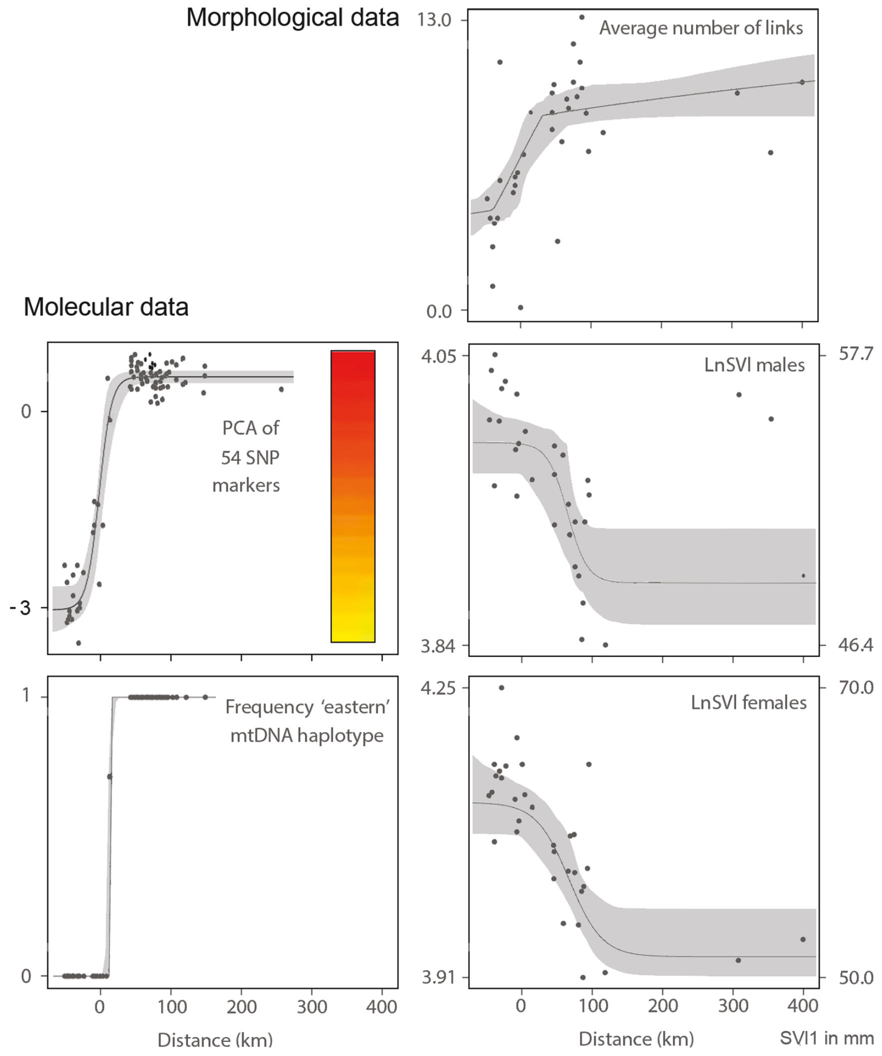

FIGURE 4 Geographical clines observed for *'Triturus pygmaeus'* in a longitudinal transect across central Portugal (see fig. 3), with *T. rudolfi* **nov. sp.** in the west and *T. pygmaeus* in the east. The horizontal axis is distance in km, measured from the nominal centre of the contact zone at Entroncamento railway station. The vertical axes are from top to bottom, left column – PCA2 for 54 SNP markers and the frequency of the eastern mtDNA haplotype, and in the right-hand column – the number of links and body size (lnSVl1) of adult males and females. Solid dots represent populations and the grey areas represent the 95% credibility intervals. Note that the colour bar is applied to fig. 3B. The formal cline descriptions are in supplementary table S3.

(table 2). A principal component analysis on size-corrected morphometric data shows some differentiation among *T. marmoratus* and '*T. pygmaeus*' as well as the near-complete overlap of scores for *T. pygmaeus* and *T. rudolfi* **sp. nov.**, for males and for females (fig. 6). Percentages of variance explained are 41.4% and 15.2% for males and 36.4% and 17.4% for females, along the first and second axis, respectively. Measurements mostly

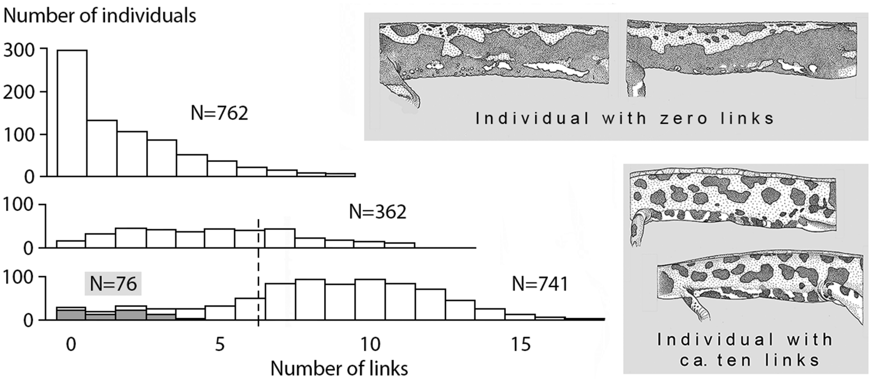

FIGURE 5 Histogram for the number of links (N_{links}) observed in Iberian large-bodied newts, with low values for *Triturus marmoratus* (top panel), intermediate values for *T. rudolfi* **sp. nov.** (middle panel) and low to high values for *T. pygmaeus* (bottom panel). A distinction is made between *T. p. pygmaeus* from the Betic region (grey bars) and *T. p. lusitanicus* from the remainder of the species range (open bars) (see Arntzen, 2024). The optimal separation of *T. rudolfi* **nov. sp.** versus *T. p. lusitanicus* is achieved at $N_{links} = 6.8$, as shown by an interrupted line. To the right examples are shown of individuals with low and high link counts. Animals are facing left, with *T. marmoratus* from Gerês, northern Portugal at the top and *T. p. lusitanicus* from Sagres, southern Portugal at the bottom. Links are counted over the left and right side of the newts' bodies, in between the insertion of the fore- and hind leg.
THE IMAGERY IS REPRODUCED FROM ARNTZEN (2018)

contributing to the first axis concern the extremities, with loadings (L) $0.81<L<0.75$ in males and $0.78<L<0.71$ in females. Both head measurements and ILd (in females only) are more strongly contributing to the second axis, with $0.47<L<0.55$ in males and $0.52<L<0.57$ in females. The unprocessed morphometric data are available in supplementary table S3.

Non-parametric Kruskal-Wallis tests revealed significant ecological differentiation between the three species for each of the selected parameters (bio01, bio02, bio03, bio 12 and bio15; table 3). In post-hoc tests no significant differences were found for bio 15 (seasonality of precipitation) whereas for bio02 (mean diurnal temperature range) the species were all significantly different from one another. For bio01 (annual mean temperature), bio03 (isothermality) and bio12 (annual precipitation) climatic profiles were similar for *T. marmoratus* and *T. rudolfi* **sp. nov.**, and significantly different for *T. pygmaeus,* suggesting that both former species share temperate, cooler and wetter environmental conditions, as different from *T. pygmaeus.*

The consistent genetic, morphological and ecological differentiation of western and eastern pygmy newt population groups warrants the description of a new taxon. The sharp genetic transition, in combination with a readily diagnosable differential morphology, particularly in colouration pattern and body size, justifies description at the species level. Because the type locality of '*T. pygmaeus*' is the province of Cadiz, it is the western taxon that needs recognition. The formal description for *T. rudolfi* **sp. nov.** is in the Appendix, the taxonomic status at the species level is briefly discussed below and three individuals photographed alive are shown in Appendix Figure A2.

TABLE 2 Summary of morphometric data on males (top panel) and females (bottom panel) for three Iberian species of large-bodied newts (genus *Triturus*). N is sample size with the number of populations involved in parentheses. Values to the left are averages for the species (in mm) and those to the right are relative values obtained from division by SVl1 (R_ILd, R_FLl, etc.). Post-hoc tests are pairwise comparisons, indicating the species for which relative size is larger. 'About equal' signs (≈) are to be read as 'not significantly different from'.

		T. marmoratus **(m)**		***T. rudolfi*** **sp. nov. (r)**		***T. pygmaeus*** **(p)**		**Kruskal-Wallis test**	
Males		**N = 319 (44)**		**N = 115 (12)**		**N = 280 (37)**		**Overall significance #**	**Post-hoc tests**
	SVl1	61.63		53.08		49.73			
	ILd	33.45	0.544	29.32	0.552	27.34	0.550	*	r>m
	FLl	25.48	0.414	22.03	0.415	19.96	0.402	***	m≈r>p
	TFl	8.16	0.133	6.68	0.126	6.35	0.128	***	m>r≈p
	HLl	26.01	0.423	22.87	0.432	20.76	0.419	***	m≈r>p
	FTl	8.72	0.142	7.38	0.139	7.13	0.144	NS	
	Hw	12.92	0.210	10.96	0.207	10.44	0.210	**	m≈p>r
	Hl	17.61	0.287	15.26	0.288	14.60	0.294	**	p>m≈r
Females		**N = 419 (44)**		**N = 116 (15)**		**N = 341 (41)**			
	SVl 1	65.59		59.59		55.57			
	ILd	35.83	0.546	33.98	0.570	31.22	0.560	***	r≈p>m
	FLl	24.32	0.371	21.46	0.361	19.79	0.356	***	m>r≈p
	TFl	7.23	0.110	6.22	0.105	5.90	0.106	***	m>r≈p
	HLl	24.12	0.369	21.50	0.361	20.02	0.360	***	m>r≈p
	FTl	7.11	0.109	6.25	0.105	5.98	0.108	NS	
	Hw	13.42	0.205	12.03	0.202	11.22	0.202	**	m>p
	Hl	17.87	0.273	16.10	0.271	15.53	0.279	***	p>m≈r

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

Discussion

The combined analysis of morphological and genetic data confirms that the Iberian Peninsula is inhabited by more than one species of *Triturus* newt, as is summarized in table 1 and fig. 1. The prime distinction is between *Triturus marmoratus* in the north and '*T. pygmaeus*' in the south. The species border runs from Peniche at the Atlantic coast to the Madrid area, east of which both species cease to exist. Hybridization is rare and introgression is limited for most genetic markers (Espregueira Themudo and Arntzen, 2007a; Arntzen and Espregueira Themudo, 2008; Wielstra et al., 2013; Arntzen et al., 2014; Arntzen, 2018; Rancilhac et al., 2021; Gaczorek et al., 2023; Kazilas et al., 2023). The strongly parapatric and mosaic distribution has been interpreted as the signature of species replacement in northerly direction, most pronounced so along the Atlantic coast, by *T. rudolfi* **sp. nov.** at the expense of *T. marmoratus* (Espregueira Themudo and Arntzen, 2007b; Arntzen et al., 2021).

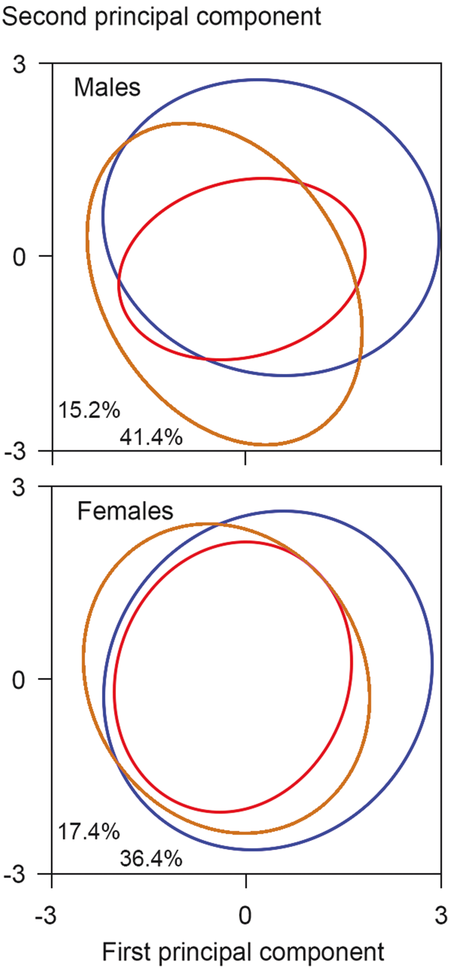

FIGURE 6 Results of a principal component analysis on size corrected morphometric data for three species of Iberian *Triturus* newts. The ellipses show the 95% confidence interval of the mean. Colours represent: blue – *T. marmoratus*, brown – *T. rudolfi* **sp. nov.**, and red – *T. pygmaeus*. Top panel males and bottom panel females. Percentages of variance explained are indicated along the axes.

The southern half of the Iberian Peninsula accommodates more than one morphologically and genetically differentiated group of pygmy newts. *Triturus p. pygmaeus* from the Betic region differs from *T. p. lusitanicus* from north of the Guadalquivir by low link scores (fig. 5). Microsatellite genetic data revealed a narrow and steep transition between these subspecies, but information is limited to the Doñana National Park and the area of contact is small (Arntzen, 2024). For an intriguing hypothesis on how *T. p. pygmaeus*, along with presumably southern population groups of the salamander *Pleurodeles waltl* Michahelles, 1830 and the frog *Pelophylax perezi* (López Seoane, 1885) managed to colonize the southern stretches of Doñana National Park see Arntzen (2024). More work is needed to clarify the situation within Doñana National Park and across the Guadalquivir.

The Lisbon Peninsula and the Atlantic coast to the north are the territory of *T. rudolfi* **sp. nov.** The species meets up with *T. marmoratus* along the coastal dunes up to the river Vouga estuary (Arntzen et al., 2021). To the northeast *T. rudolfi* **sp. nov.** is wedged in between *T. marmoratus* and the Tejo River. To the east and southeast *T. rudolfi* **sp. nov.** and *T. pygmaeus* are probably widely separated by the Tejo River and the currently low-quality habitats brought about by agricultural land, with possibly some widespread introgression from *T. rudolfi* **sp. nov.** into *T. pygmaeus* (Kazilas et al., 2023). The area where the *T. pygmaeus* – *T. rudolfi* **sp. nov.** contact zone can most profitably be studied appears to be limited to a ca. 20 × 40 km stretch of land north of Entroncamento (fig. 3).

The geographical cline for mtDNA is sharper than the one for the nuclear genetic data and displaced to the east by 14.9 km (figure 4, supplementary table S2). Non-coincident and non-concordant clines in mtDNA versus nuDNA have generally been explained by differential introgression, for instance in scenarios where the demographic expansion of one species leads to range overlap and admixture in the advancing front, or

TABLE 3 Summary of environmental data extracted for 232 genetically identified populations in three species of *Triturus* newts in the Iberian Peninsula. Post-hoc tests are pairwise comparisons, indicating the species for which values are larger. 'About equal' signs (≈) are to be read as 'not significantly different from'.

Species	***Triturus***			**Kruskal-Wallis test**	
	marmoratus **(m)**	***pygmaeus*** **(p)**	***rudolfi*** **sp. nov. (r)**	**Overall significance**	**Post-hoc tests**
Populations studied	84	120	28		
Environmental variable					
Temperature					
Bio001 – annual average	15.0	15.7	15.3	***	p<m≈r
Bio002 – mean diurnal range	9.5	10.6	8.3	***	p<m<r
Bio003 – isothermality	41.6	39.9	42.6	***	m≈r<p
Precipitation					
Bio012 – annual total	919.8	675.4	785.1	***	m≈r<p
Bio015 – seasonality	56.1	57.8	57.7	*	m≈p≈r

Significance levels are: * $P < 0.05$ and *** $P < 0.001$.

when sex-biased dispersal promotes faster diffusion of the mitochondrial genome (Currat et al., 2008; Wielstra et al., 2017). The clines for morphology are substantially wider than for the genetic data, yet in approximately the same position for N_{links} and displaced to the east by ca. 65 km for body size, in both sexes. It therefore seems that a morphology-based estimate for the position of the species' genetic transition is more readily, and considering the obtained AUC-support values also more reliably, obtained from colouration pattern than from body size.

One point to be emphasized is that SNP-marker development was geared up to the analysis of the *T. marmoratus* – '*T. pygmaeus*' contact zone (Arntzen et al., 2021; Lopez-Delgado et al., 2021) and that the markers that are somewhat less informative for that purpose are those that perform best in distinguishing *T. pygmaeus* and *T. rudolfi* **sp. nov.** Accordingly, the separation of species achieved over the first (*T. marmoratus* – '*T. pygmaeus*') and second axes of the principal component analysis (*T. pygmaeus* – *T. rudolfi* **sp. nov.**) (fig. 2) are not at the same scale so that inferences on the absolute level of differentiation would be invalid. Unbiased genetic data (Kazilas et al., 2023) resolve *T. marmoratus* and '*T. pygmaeus*' as sister taxa, and *T. rudolfi* **sp. nov.** and *T. pygmaeus* as sister species within that clade. Under reference to an age of 24–16 Ma for the most recent ancestor in the genus (Steinfartz et al., 2007;

Marjanović and Laurin, 2014), the date for these splits is estimated at ca. 5.0–3.3 Ma and 2.0–1.3 Ma, respectively. A phylogenetic analysis with a large panel of loci uncovered by restriction site-associated DNA sequencing yields somewhat different relative branch lengths, resulting in the substantially higher estimates of ca. 8.0–5.3 Ma and 4.0–2.7 Ma, respectively (cf. Rancilhac et al., 2023), yet it has been argued that molecular phylogenetic inference requires careful attention to model assumptions, especially where it concerns the reconstruction of branch length (Leaché et al., 2015).

The present paper recognizes a new species of pygmy newt, *T. rudolfi* **sp. nov.** from the wider Lisbon Peninsula as different from *T. pygmaeus* in the south of the Iberian Peninsula. Independent data revealed the long-lasting, reciprocal monophyly of independently evolving *T. pygmaeus* and *T. rudolfi* **sp. nov.** population groups (Kazilas et al., 2023), supporting species status under the phylogenetic (Eldredge and Cracraft, 1980) as well as evolutionary species concept (Wiley, 1978; de Queiroz, 2007). The newly gathered genetic data provide ample evidence for partial reproductive isolation within a narrow zone of intergradation, consistent with some selection against hybrids. These observations support species status under the biological species concept as well (Mayr, 1942). *Triturus rudolfi* **sp. nov.**, while phylogenetically more closely related to *T. pygmaeus* than to *T. marmoratus* (Kazilas et al., 2023), deviates towards the latter species in terms of geographical position, climate conditions, overall size and colouration pattern.

Although Mertens and Wermuth (1960) only recognized *T. marmoratus* and the crested newt, *T. cristatus* (Laurenti, 1768), they had another five taxa listed (four subspecies and one 'variety') that are by now accepted as species, namely *T. carnifex* (Laurenti, 1768), *T. dobrogicus* (Kiritzescu, 1903), *T. karelinii* (Strauch, 1870), *T. macedonicus* (Karaman, 1922) and *T. pygmaeus*. Two crested newt species have since been described from the southeastern parts of the genus' range (*T. anatolicus* Wielstra and Arntzen, 2016 and *T. ivanbureschi* Arntzen and Wielstra, 2013), to which it is now added *T. rudolfi* **sp. nov.** from Portugal, bringing the total to ten *Triturus* species.

The coastal zone of Portugal has for long gone unnoticed for a high level of amphibian diversity. However, with the uprise of molecular systematics, two endemic amphibian species, the newt *Lissotriton maltzani* (Boettger, 1879) and the frog *Pelodytes atlanticus* Díaz-Rodríguez, Gehara, Márquez, Vences, Gonçalves, Sequeira, Martínez-Solano and Tejedo, 2017 were resolved as different from *L. boscai* (Lataste, 1879) and *P. ibericus* Sánchez-Herráiz, Barbadillo, Machordom and Sanchiz, 2000 (Díaz-Rodríguez et al., 2017; Dufresnes et al., 2020; Sequeira et al., 2020). Whereas the former are morphologically cryptic taxa, *T. rudolfi* **sp. nov.** is readily diagnosable because the signal from nuclear and mitochondrial DNA is paralleled by morphological differentiation, most prominently in the species' dorso-lateral coloration pattern.

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Data accessibility

Data on *Triturus* colouration patterns and the localities studied are available from a previous publication (Arntzen, 2024). The morphometric data are presented as supplementary table S3. The molecular genetic data here employed 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.

Supplementary material

Supplementary material is available online at: https://doi.org/10.6084/m9.figshare.25158365

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.25264903, courtesy of Dr. Tijana Vučić.

References

Arntzen, J. W. (2018). Morphological and molecular characters to describe a marbled newt hybrid zone in the Iberian peninsula. *Contributions to Zoology* 87: 167–185. https://doi.org/10.1163/18759866-08703003.

Arntzen, J. W. (2024). 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). *Contributions to Zoology* 1–26. https://doi.org/10.1163/18759866-bja10055.

Arntzen, J. W., Bauer, A. (eds.) (1996). Species and species concepts: too many or too few? *Amphibia-Reptilia* 17: 321–323. https://doi.org/10.1163/156853896X00045.

Arntzen, J. W., Espregueira Themudo, G. (2008). Environmental parameters that determine species geographical range limits as a matter of time and space. *Journal of Biogeography* 35: 1177–1186. https://doi.org/10.1111/j.1365-2699.2007.01875.x.

Arntzen, J. W., Wielstra, B., Wallis, G. P. (2014). The modality of nine *Triturus* newt hybrid zones assessed with nuclear, mitochondrial and morphological data. *Biological Journal of the Linnean Society* 113: 604–622. https://doi.org/10.1111/bij.12358.

Arntzen, J. W., de Vries, W., Canestrelli, D., Martínez-Solano, I. (2017). Hybrid zone formation and contrasting outcomes of secondary contact over transects in common toads. *Molecular Ecology* 26: 5663–5675. https://doi.org/10.1111/mec.14273.

Arntzen, J. W., López-Delgado, J., van Riemsdijk, I., Wielstra, B. (2021). A genomic footprint of a moving hybrid zone in marbled newts. *Journal of Zoological Systematics and Evolutionary Research* 59: 459–465. https://doi.org/10.1111/jzs.12439.

Bassitta, M., Buades, J. M., Pérez-Cembranos, A., Pérez-Mellado, V., Terrasa, B., Brown, R. P., Navarro, P., Lluch, J., Ortega, J., Castro, J. A., Picornell, A., Ramon, C. (2020). Multilocus and morphological analysis of south-eastern Iberian Wall lizards (Squamata, *Podarcis*). *Zoologica Scripta* 49: 668–683. https://doi.org/10.1111/zsc.12450.

Burbrink, F. T., Ruane, S. (2021). Contemporary philosophy and methods for studying speciation and delimiting species. *Ichthyology and Herpetology* 109: 874–894. https://doi.org/10.1643/h2020073.

Burbrink, F. T., Crother, B. I., Murray, C. M., Smith, B. T., Ruane, S., Myers, E. A., Pyron, R. A. (2022). Empirical and philosophical problems with the subspecies rank. *Ecology and Evolution* 12: e9069. https://doi.org/10.1002/ece3.9069.

Burrough, P. A., McDonnell, R. A., Lloyd, C. D. (2015). *Principles of Geographical Information Systems*. Oxford University Press, Oxford, UK.

Clarke, K. R., Gorley, R. N. (2015). *PRIMER v7: User Manual/Tutorial*. PRIMER-E, Plymouth, UK.

Currat, M., Ruedi, M., Petit, R. J., Excoffier, L. (2008). The hidden side of invasions: massive introgression by local genes. *Evolution* 62: 1908–1920. https://doi.org/10.1111/j.1558-5646.2008.00413.x.

Derryberry, E. P., Derryberry, G. E., Maley, J. M., Brumfield, R. T. (2014). HZAR: hybrid zone analysis using an R software package. *Molecular Ecology Resources* 14: 652–663. https://doi.org/10.1111/1755-0998.12209.

Díaz-Paniagua, C., Mateo, J. A., Andreu, A. C. (1996). Age and size structure of populations of small marbled newts (*Triturus marmoratus pygmaeus*) from Doñana National Park (SW Spain). A case of dwarfism among dwarfs. *Journal of Zoology* 239: 83–92. https://doi.org/10.1111/j.1469-7998.1996.tb05438.x.

Díaz-Rodríguez, J., Gehara, M., Márquez, R., Vences, M., Gonçalves, H., Sequeira, Martínez-Solano, I., Tejedo M. (2017). Integration of molecular, bioacoustical and morphological data reveals two new cryptic species of *Pelodytes* (Anura, Pelodytidae) from the Iberian Peninsula. *Zootaxa* 4243: 1–41. https://doi.org/10.11646/zootaxa.4243.1.1.

Dufresnes, C., Pribille, M., Alard, B., Gonçalves, H., Amat, F., Crochet, P. A., Dubey, S., Perrin, N., Fumagalli, L., Vences, M., Martínez-Solano, I. (2020). Integrating hybrid zone analyses in species delimitation: lessons from two anuran radiations of the Western Mediterranean. *Heredity* 124: 423–438. https://doi.org/10.1038/s41437-020-0294-z.

Dufresnes, C., Poyarkov, N. Jablonski, D. (2023). Acknowledging more biodiversity without more species. *Proceedings of the National Academy of Sciences* 120: e2302424120 https://doi.org/10.1073/pnas.2302424120.

Eldredge, N., Cracraft, J. (1980). *Phylogenetic Patterns and the Evolutionary Process*. Columbia University Press, New York, USA.

Espregueira Themudo, G., Arntzen, J. W. (2007a). Molecular identification of marbled newts and a justification of species status for *Triturus marmoratus* and *T. pygmaeus*. *Herpetological Journal* 17: 24–30.

Espregueira Themudo, G., Arntzen, J. W. (2007b). Newts under siege: range expansion of *Triturus pygmaeus* isolates populations of its sister species. *Diversity and Distributions* 13: 580–586. https://doi.org/10.1111/j.1472-4642.2007.00373.x.

Fick, S. E., Hijmans, R. J. (2017). WorldClim 2: new 1-km spatial resolution climate surfaces for global land areas. *International Journal of Climatology* 37: 4302–4315. https://doi.org/10.1002/joc.5086.

Gaczorek, T. S., Marszałek, M., Dudek, K., Arntzen, J. W., Wielstra, B., Babik, W. (2023). Interspecific introgression of MHC genes in *Triturus* newts: Evidence from multiple contact zones. *Molecular Ecology* 32: 867–880. https://doi.org/10.1111/mec.16804.

García-París, M., Herrero, P., Martín, C., Dorda, J., Esteban, M., Arano, B. (1993). Morphological characterization, cytogenetic analysis, and geographical distribution of the Pygmy marbled newt *Triturus marmoratus pygmaeus* (Wolterstorff, 1905) (Caudata: Salamandridae). *Contributions to Zoology* 63: 3–14. https://doi.org/10.1163/26660644-06301001.

García-París, M., Arano, B., Herrero, P. (2001). Molecular characterization of the contact zone between *Triturus pygmaeus* and *T. marmoratus* (Caudata: Salamandridae) in central Spain and their taxonomic assessment. *Revista Española de Herpetología* 15: 115–126.

Hewitt, G. M. (1996). Some genetic consequences of ice ages, and their role in divergence and speciation. *Biological Journal of the Linnean Society* 58: 247–276. https://doi.org/10.1006/bijl.1996.0035.

Hewitt, G. M. (2000). The genetic legacy of the quaternary ice ages. *Nature* 405: 907–913. https://doi.org/10.1038/35016000.

IBM SPSS (2021). IBM SPSS *Statistics for Windows*. IBM Corporation, Armonk, New York, USA.

ILWIS (2019). *Integrated Land and Watershed Management Information System*. International Institute for Aerospace Survey and Earth Sciences, Enschede, The Netherlands.

Jombart, T. (2008). Adegenet: a R package for the multivariate analysis of genetic markers. *Bioinformatics* 24: 1403–1405. https://doi.org/10.1093/bioinformatics/btn129.

Kazilas, C., Dufresnes, C., France, J., Kalaentzis, K., Martínez-Solano, I., de Visser, M. C., Arntzen, J. W., Wielstra, B. (2023). Spatial genetic structure in European marbled newts revealed with target enrichment by sequence capture. *Molecular Phylogenetics and Evolution.* https://doi.org/10.1016/j.ympev.2024.108043.

Leaché, A. D., Banbury, B. L., Felsenstein, J., De Oca, A. N. M., Stamatakis, A. (2015). Short tree, long tree, right tree, wrong tree: new acquisition bias corrections for inferring SNP phylogenies. *Systematic Biology* 64: 1032–1047. https://doi.org/10.1093/sysbio/syv053.

Leaché A. D., Zhu T., Rannala B., Yang Z. (2019). The spectre of too many species. *Systematic Biology* 68: 168–181. https://doi.org/10.1093/sysbio/syy051.

Linnaeus, C. (1758). *Systema Naturae*. Laurentii Salvii, Stockholm, Sweden.

López-Delgado, J., van Riemsdijk, I., Arntzen, J. W. (2021). Tracing species replacement in Iberian marbled newts. *Ecology and Evolution* 11: 402–414. https://doi.org/10.1002/ece3.7060.

Malkmus, R. (2004). *Amphibians and Reptiles of Portugal, Madeira and the Azores-Archipelago*. A. R. G. Gantner Verlag Kommanditgesellschaft, Ruggell, Liechtenstein.

Marjanović, D., Laurin, M. (2014). An updated paleontological timetree of lissamphibians, with comments on the anatomy of Jurassic crown-group salamanders (Urodela). *Historical Biology* 26, 535–550. https://doi.org/10.1080/08912963.2013.797972.

Matérn, B. (1979). The analysis of ecological maps as mosaics. Pp. 271–287 *in* Cormack, R. M. and Ord, J. K. (eds.) *Spatial and Temporal Analysis in Ecology*. International Co-operative Publishing House, Fairland, MD, USA.

Mayr, E. (1942). *Systematics and the Origin of Species from the Viewpoint of a Zoologist.* Harvard University Press, Cambridge, Massachusetts, USA.

Mayr, E. (1969). *Principles of Systematic Zoology*. McGraw-Hill, New York, USA.

Mayr, E. (1982). *The Growth of Biological Thought: Diversity, Evolution, and Inheritance*. Harvard University Press, Cambridge, Massachusetts, USA.

Mertens, R., Müller, L. (1928). *Liste der Amphibien und Reptilien Europas*. Senckenbergische Naturforschende Gesellschaft, Frankfurt, Germany.

Mertens, R., Wermuth, H. (1960). *Die Amphibien und Reptilien Europas*. Senckenberg Buch Nr. 38. Waldemar Kramer, Frankfurt am Main, Germany.

Mulder, K. P., Lourenço, A., Carneiro, M., Velo-Antón, G. (2016). The complete mitochondrial genome of *Salamandra salamandra* (Amphibia: Urodela: Salamandridae). *Mitochondrial* DNA *Part B* 1: 880–882. https://doi.org/10.1080/23802359.2016.1253042.

de Queiroz, K. (2007). Species concepts and species delimitation. *Systematic Biology* 56: 879–886. https://doi.org/10.1080/10635150701701083.

Rancilhac, L., Irisarri, I., Angelini, C., Arntzen, J. W., Babik, W., Bossuyt, F., Künzel, S., Lüddecke, T., Pasmans, F., Sanchez, E., Weisrock, D., Veith, M., Wielstra, B., Steinfartz, S., Hofreiter, M., Philippe, H., Vences, M. (2021). Phylotranscriptomic evidence for pervasive ancient hybridization among Old World salamanders. *Molecular Phylogenetics and Evolution* 155: 106967. https://doi.org/10.1016/j.ympev.2020.106967.

Rancilhac, L., Sylvestre, F., Hutter, C. R., Arntzen, J. W., Babik, W., Crochet, P.-A., Deso, G., Duguet, R., Galan, P., Pabijan, M., Policain, M., Priol, P., Sabino-Pinto, J., Capstick, M., Elmer, K. R., Dufresnes. C., Vences, M. (2023). Exploring the impact of read clustering thresholds on RADseq-based systematics: an empirical example from European amphibians. BioRxiv, 2023–04. https://doi.org/10.1101/2023.04.19.537466.

Recknagel, H., Zakšek, V., Delić, T., Gorički, Š., Trontelj, P. (2023). Multiple transitions between realms shape relict lineages of *Proteus* cave salamanders. *Molecular Ecology*. https://doi.org/10.1111/mec.16868.

Sequeira, F., Bessa-Silva, A., Tarroso, P., Sousa-Neves, T., Vallinoto, M., Gonçalves, H., Martínez-Solano, I. (2020). Discordant patterns of introgression across a narrow hybrid zone between two cryptic lineages of an Iberian endemic newt. *Journal of Evolutionary Biology* 33: 202–216. https://doi.org/10.1111/jeb.13562.

Speybroeck, J., Beukema, W., Dufresnes, C., Fritz, U., Jablonski, D., Lymberakis, P., Martínez-Solano, I., Razzetti, E., Vamberger, M., Vences, M., Vörös, J., Crochet, P.-A. (2020). Species list of the European herpetofauna – 2020 update by the Taxonomic Committee of the Societas Europaea Herpetologica. *Amphibia-Reptilia* 41: 139–189. https://doi.org/10.1163/15685381-bja10010.

Steinfartz, S., Vicario, S., Arntzen, J. W., Caccone, A. (2007). A Bayesian approach on molecules and behavior: Reconsidering phylogenetic and evolutionary patterns of the Salamandridae with emphasis on *Triturus* newts. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution* 308: 139–162. https://doi.org/10.1002/jez.b.21119.

Sukumaran, J., Knowles, L. L. (2017). Multispecies coalescent delimits structure, not species. *Proceedings of the National Academy of Sciences* 114: 1607–1612. https://doi.org/10.1073/pnas.1607921114.

Wielstra, B., Arntzen, J. W. (2011). Unraveling the rapid radiation of crested newts (*Triturus cristatus* superspecies) using complete mitogenomic sequences. BMC *Evolutionary Biology* 11: 162. https://doi.org/10.1186/1471-2148-11-162.

Wielstra, B., Crnobrnja-Isailović, J., Litvinchuk, S. N., Reijnen, B. T., Skidmore, A. K., Sotiropoulos, K., Toxopeus, A. G., Tzankov, N., Vukov, T., Arntzen, J. W. (2013). Tracing glacial refugia of *Triturus* newts based on mitochondrial DNA phylogeography and species distribution modeling. *Frontiers in Zoology* 10: 1–14. https://doi.org/10.1186/1742-9994-10-13.

Wielstra, B., Burke, T., Butlin, R. K., Arntzen, J. W. (2017). A signature of dynamic biogeography: enclaves indicate past species replacement. *Proceedings of the Royal Society B: Biological Sciences* 284: 20172014. https://royalsocietypublishing.org/doi/10.1098/rspb.2017.2014.

Wiley, E. O. (1978). The evolutionary species concept reconsidered. *Systematic Zoology* 27: 17–26. https://doi.org/10.2307/2412809.

Wilkins, J. S. (2011). Philosophically speaking, how many species concepts are there? *Zootaxa* 2765: 58–60. https://doi.org/10.11646/zootaxa.2765.1.5.

Appendix A1

Description of ***Triturus rudolfi*** *sp. nov.*
Descriptions were made from preserved material, except for the number of links that was determined in the field. Dorsal colouration invariably dark, without patterning clear enough to allow a detailed description.

Description of type specimen – preserved on ethanol in excellent storage condition at the Museo Nacional de Ciencias Naturales, Madrid, Spain under catalogue number 51784. Adult male in breeding condition with a well-developed crest and a large black cloaca. SVl1 58 mm, SVl2 68 mm, ILd 30 mm, Hw 11.6 mm, Hl 19.8 mm, FLl 23.7 mm, TFl 7.0 mm, HLl 24.5 mm and FTl 10.5 mm. Total length 125 mm. Relative finger lengths 4 < 1 < 2 < 3. Relative toe lengths 1 < 5 < 2 < 4 < 3. N_{links} left 2, right 2, total 4. Fourteen crème-white bands over the head and body part of the mid-dorsal crest. Grey ventral colouration with many large, irregular shaped dark dots and many evenly distributed white spots, denser in the central part than towards the gular and cloacal regions. Colouration of throat region continuous with that of the belly. Underside of legs with dark dots. See fig. A1.

Description of paratypes – Preserved on ethanol in excellent storage condition at the Museo Nacional de Ciencias Naturales, Madrid, Spain under catalogue numbers 51785-54791, sequentially.

First paratype. Adult male in breeding condition with a well-developed crest and a large black cloaca. SVl1 43 mm, SVl2 52 mm, ILd 21 mm, Hw 9.5 mm, Hl 13.7 mm, FLl 19.1 mm, TFl 6.8 mm, HLl 19.9 mm and FTl 7.2 mm. Total length 90 mm. Relative finger lengths 1 = 4 < 2 < 3. Relative toe lengths 1 < 5 < 2 < 4 < 3. N_{links} left 4, right 4, total 8. Thirteen crème-white bands over the head and body part of the mid-dorsal crest. Continuous crème ventral colouration from the throat to the cloacal region and underside of fore- and hindlegs.

Second paratype. Adult male in breeding condition with a well-developed crest and a large black cloaca. SVl1 50 mm, SVl2

FIGURE A1 Holotype of *Triturus rudolfi* **sp. nov.** at right (top) and left lateral view (middle), and in ventral view (bottom). Size bar is 1 cm. Stored at the Museo Nacional de Ciencias Naturales, Madrid, Spain under catalogue number 51784.

FIGURE A2 *Triturus rudolfi* **sp. nov.** from Serra de Sintra (top) and from the Colares and Janas region (middle and bottom).
PHOTOGRAPHY R. MALKMUS

58 mm, ILd 26 mm, Hw 11.0 mm, Hl 15.1 mm, FLl 21.4 mm, TFl 7.6 mm, HLl 22.6 and FTl 8.4 mm. Total length 107 mm. Relative finger lengths 4 < 1 < 2 < 3. Relative toe lengths 1 < 5 < 2 < 4 < 3. N_{links} left 2, right 4, total 6. Fifteen crème-white bands over the head and body part of the mid-dorsal crest. Grey ventral colouration with many large, irregular shaped dark dots and many evenly distributed white spots. Throat with few dots and spots. Region towards cloaca and underside legs crème coloured.

Third paratype. Adult male in breeding condition with a well-developed crest and a large black cloaca. SVl1 53 mm, SVl2 61 mm, ILd 26 mm, Hw 11.2 mm, Hl 17.2 mm, FLl 23.2 mm, TFl 8.0 mm, HLl 24.4 and FTl 10.0 mm. Total length 110 mm. Relative finger lengths 1 < 4 < 2 < 3. Relative toe lengths 1 < 5 < 2 < 3 = 4. N_{links} left 0, right 4, total 4. Sixteen crème-white bands over the head and body part of the mid-dorsal crest. Light grey ventral colouration, with large, reticulated dark dots and white spots over half the surface. Colouration of throat continuous with belly. Crème colour towards cloaca. Underside hindlegs crème coloured with dark dots.

Fourth paratype. Adult female in breeding condition with ovarian eggs shining through. SVl1 66 mm, SVl2 72 mm, ILd 35 mm, Hw 12.6 mm, Hl 18.4 mm, FLl 23.6 mm, TFl 7.5 mm., HLl 23.1 and FTl 7.3 mm. Total length 134 mm. Relative finger lengths 1 < 4 < 2 < 3. Relative toe lengths 1 = 5 < 2 < 4 < 3. N_{links} left 2, right 3, total 5. Light grey ventral colouration with few small dark dots. Colouration of throat continuous with belly with small white spots. Crème coloured towards cloaca and underside hindlegs.

Fifth paratype. Adult female in breeding condition with ovarian eggs shining through. SVl1 57 mm, SVl2 63 mm, ILd 29 mm, Hw 11.3 mm, Hl 15.4 mm, FLl 21.9 mm, TFl 7.7 mm, HLl 22.0 and FTl 8.0 mm. Total length 120 mm. Relative finger lengths 1 = 4 < 2 < 3. Relative toe lengths 5 < 1 < 2 < 3 = 4. N_{links} left 0, right 1, total 1. Grey ventral colouration with few small dark dots. Colouration of throat continuous with belly. Crème coloured towards cloaca and underside hindlegs.

Sixth paratype. Adult female in breeding condition with ovarian eggs shining through. SVl1 63 mm, SVl2 68 mm, ILd 32 mm, Hw 11.8 mm, Hl 18.5 mm, FLl 22.0 mm, TFl 7.6 mm, HLl 21.0 and FTl 7.2 mm (measured at left side, because toe at right side is either malformed or regenerating). Total length 127 mm.

Relative finger lengths 1 < 4 < 2 < 3. Relative toe lengths 1 < 5 < 2 < 3 = 4 (left side). N_{links} left 1, right 1, total 2. Upper side of tail damaged, possibly a bite mark. Grey ventral colouration with medium number of dark dots and few white spots. Colouration of throat continuous with belly. Crème coloured towards cloaca and underside hindlegs.

Seventh paratype. Adult female in breeding condition with ovarian eggs shining through. SVl1 60 mm, SVl2 66 mm, ILd 32 mm, Hw 11.7 mm, Hl 17.2 mm, FLl 22.5 mm, TFl 7.6 mm, HLl 21.2 and FTl 7.8mm. Total length 121 mm. Relative finger lengths 1 < 4 < 2 < 3. Relative toe lengths 1 = 5 < 2 < 3 < 4. N_{links} left 0, right 2, total 2. Light grey ventral colouration, with few small dark dots. Colouration of throat region similar to that of belly, but lighter and with small white spots. Crème coloured towards cloaca and on underside hind legs.

Other material from the type locality – ZMA.RENA.9332 (N = 1, leg. J. W. Arntzen) and ZMA.RENA.19271 (N = 4, leg., J. W. Arntzen and E. Froufe).

Locality and date of collecting – Lagoa Seca, Valado dos Frades near Nazaré, Portugal at 39.596 northern latitude and 9.012 western longitude. Elevation 27 m a.s.l. Date of collecting 20 March 2013, leg. J. W. Arntzen. Date of deposition at the Museo Nacional de Ciencias Naturales 16 August 2023.

Diagnostic features – the newly recognized species is most closely related to, yet morphologically distinguishable from *T. pygmaeus*, on account of a lower number of green coloured dorso-lateral transversal bands ('links'). Body size larger than in *T. pygmaeus*. Significant genetic differences for population groups at either side of the cline near Entroncamento were found at 34 out of 54 investigated nuclear markers, as well as for mitochondrial DNA. Body size smaller than in *T. marmoratus*, with a higher number of links and with a light rather than dark coloured underside.

Derivatio nominis – the species name is chosen in honour of Mr. Rudolf Malkmus, in recognition of his contribution to the knowledge of the Portuguese herpetofauna. Mr. Malkmus placed the Portuguese herpetofauna on the map, not just as a figure of speech, but also literally (Malkmus, 2004).

Suggested vernacular name – Malkmus' pygmy newt or Lisbon pygmy newt. I further advocate to restrict the name 'marbled newt' to *T. marmoratus* and to use 'pygmy newts' for *T. pygmae*us and *T. rudolfi* **sp. nov.** (see also table 1).

Distribution – the Lisbon Peninsula in Portugal, reaching northward along the Atlantic Ocean up to the river Vouga estuary. Approximate range borders: bounded by *T. marmoratus* in the north at ca. 39.5 N, separated from *T. pygmaeus* in the east by the Tejo River and in the northeast by a narrow hybrid zone that is wedged in between *T. marmoratus* territory and the river Tejo.

Conservation status – vulnerable, on account of the small range probably more so than other Iberian *Triturus* species.

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:D6D62940-6B0E-42E5-8212-383D28DC3D6A.

TABLE A1 Morphological data for 43 individuals from type locality Lagoa Seca, Valado dos Frades, Portugal. Measurements are in mm. For abbreviations and for counting the number of links (N_{links}) see the main text and Arntzen (2018).

Field number											
	Sex	**SVl1**	**SVl2**	**ILd**	**FLl**	**TFl**	**HLl**	**FTl**	**Hw**	**Hl**	**N_{links}**
154	Female	62	68	34	21.4	5.9	21.0	6.9	13.1	18.1	4
155	Female	63	69	33	22.4	7.6	21.2	7.1	14.1	19.3	2
156	Male	53	61	31	24.1	7.2	22.0	7.0	12.0	16.7	9
157	Female	61	67	35	22.5	6.6	21.6	6.7	14.7	18.1	2
158	Female	63	69	33	24.1	6.6	21.0	6.5	13.2	15.9	5
159	Female	59	65	35	21.3	6.6	21.6	6.5	12.3	16.9	5
160	Female	62	68	32	21.0	6.5	20.4	6.2	12.3	12.3	4
161	Female	63	69	36	21.0	6.5	21.0	6.5	12.6	17.3	7
162	Female	54	60	31	20.0	5.6	20.0	6.2	13.0	16.4	4
163	Female	58	64	34	19.7	5.9	19.0	6.2	12.1	16.0	5
164	Female	54	60	32	17.5	5.0	17.1	6.0	12.1	15.4	5
165	Male	54	62	28	22.9	7.0	23.0	8.0	11.0	15.0	7
166	Male	56	64	33	24.0	7.1	30.0	8.5	12.4	16.3	2
167	Male	51	59	28	22.3	7.0	21.0	8.9	12.0	15.0	13
168	Male	51	59	26	24.0	8.0	22.1	9.5	12.4	16.1	6
169	Male	54	62	28	22.4	8.5	24.0	10.0	12.1	17.4	6
170	Male	62	70	30	22.1	7.0	20.4	8.4	11.6	16.1	6
171	Male	52	60	28	22.0	6.5	22.0	8.0	12.1	16.3	1
172	Male	54	62	31	24.0	7.9	24.7	9.0	12.2	16.7	9
173	Female	61	67	32	22.1	5.3	20.0	6.1	13.4	17.1	0
977	Female	65	71	35	26.0	8.7	25.8	9.0	13.1	14.6	1
2794	Female	68	73	41	23.1	6.5	23.2	6.4	12.6	18.3	5
2795	Female	65	71	36	25.0	7.8	25.3	6.5	13.5	17.9	9
2796	Female	56	61	31	17.4	5.3	21.0	6.2	11.4	15.9	7
2797	Female	67	73	39	23.5	6.3	22.5	6.3	13.7	18.5	5
2798	Female	65	70	37	23.9	6.8	22.9	6.9	12.1	17.1	7
2799	Female	60	66	33	20.8	7.0	20.5	6.5	9.7	15.4	2
2800	Female	64	70	41	22.4	7.1	22.6	6.5	12.3	16.8	5
2801	Female	60	67	33	22.8	6.3	23.9	6.0	12.2	15.6	6
2802	Female	65	70	37	22.0	7.0	21.2	6.6	11.6	16.2	7
2803	Female	58	63	31	20.3	5.8	20.3	6.1	11.2	15.1	6
2804	Female	67	73	37	23.7	6.8	24.2	7.2	14.2	19.3	4
2805	Female	67	73	37	22.9	6.0	24.0	7.4	13.0	18.2	1
2806	Female	58	63	36	20.0	6.3	20.3	6.0	11.0	14.9	3
2807	Female	58	62	34	22.4	5.3	20.2	6.0	11.3	16.4	5

TABLE A1 Morphological data for 43 individuals from type locality Lagoa Seca, Valado dos Frades, Portugal. Measurements are in mm. For abbreviations and for counting the number of links (Nlinks) see the main text and Arntzen (2018). (*cont.*)

Field number											
	Sex	**SVl1**	**SVl2**	**ILd**	**FLl**	**TFl**	**HLl**	**FTl**	**Hw**	**Hl**	**N_{links}**
2808	Female	65	70	38	20.6	6.8	21.5	6.7	12.7	17.4	6
2809	Female	69	76	36	24.7	7.1	23.7	6.7	13.5	17.6	5
2810	Female	57	63	35	20.5	6.2	25.0	5.5	11.2	13.8	6
2811	Male	58	65	32	23.5	7.8	25.3	8.0	11.8	17.2	2
2812	Male	52	60	29	23.9	7.3	24.1	8.1	11.2	15.9	6
2813	Male	57	66	33	23.7	7.5	25.3	7.8	12.0	16.6	7
2814	Male	60	66	34	24.6	7.3	26.7	8.2	11.9	14.9	4
2815	Male	57	65	31	22.9	6.7	24.5	7.8	11.4	16.7	10