Morphological and molecular characters to describe a marbled newt hybrid zone in the Iberian peninsula

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Abstract

I document the contiguous distribution of the marbled newt species Triturus marmoratus and T. pygmaeus over the western part of the Iberian peninsula with a suite of morphological and molecular genetic data from altogether 141 populations. Morphological characters that identify the species are body size and the colour pattern character ‘Links’. Links is the number of transversal connections following the green surface at the lateral sides of the newts’ body. Large adults with few Links are T. marmoratus and small adults with many Links are T. pygmaeus. However, no morphological identification criterion is entirely adequate. Eight molecular genetic markers show markedly bimodal character state distributions that give rise to sharp species range descriptions, with T. marmoratus in the north of the Iberian peninsula and T. pygmaeus in the south and along most of the Atlantic coast of Portugal. I encountered ten genetically admixed populations that are all located at the T. marmoratus - T. pygmaeus species range interface, suggesting widespread but limited interspecific hybridization. A latitudinal transect across Portugal confirmed the narrow and steep transition from one to the other species for morphological and molecular characters alike. In central Portugal the position of the hybrid zone coincides with the river Tejo. However, the cline for mitochondrial DNA is relatively wide and shallow and its centre is positioned south of the river. In view of published data that reconstruct the northward advance of T. marmoratus along the Portuguese coast at the expense of T. marmoratus, I propose that T. marmoratus had a wider range in central Portugal too, where it was eventually superseded by T. pygmaeus. I hypothesize that ‘marmoratus’ mtDNA haplotypes found south of the Tejo constitute a ‘genetic footprint’ left behind in T. pygmaeus by the receding species T. marmoratus.

Introduction

Hybrid zones figure prominently in evolutionary research. They are seen as ‘windows on evolutionary process’ (Harrison, 1990) and ‘natural laboratories’ (Hewitt, 1988). One particular asset of hybrid zones is the opportunity they provide to evaluate the performance of new, previously untested genetic combinations of differentiated genomes in nature. Possible outcomes range from the (near-)complete merger of previously allopatric lineages to a (near-)complete genetic isolation. Debated aspects are the modes by which post-zygotic isolation accumulates and how much gene flow can be tolerated before the speciation process goes in reverse. A good group to investigate these research questions would be monophyletic, species-rich, show contiguous species ranges where many closely as well as distantly related species engage in long hybrid zones. One group that qualifies is the European newt genus Triturus. The species group inner distribution ranges form a patchwork and most species are involved in intra-generic hybridization (Wielstra et al., 2014). The analyses over nine hybrid zones support the idea that in Triturus post-zygotic hybrid incompatibilities accumulate gradually (Arntzen et al., 2014). Results may, however, be dependent on local conditions. Moreover, for one species combination - that of the northern marbled newt Triturus marmoratus (Latreille, 1800) and the pygmy marbled newt Triturus pygmaeus (Wolterstorff, 1905) - the studied transect near Madrid, Spain may be allotopic and lack hybridization altogether. I here use morphological and molecular characters to describe the marbled newt species contact across Portugal and into Spain. I show that T. marmoratus and T. pygmaeus engage in limited but widespread hybridization, not unlike crested newts in the same genus at approximately the same level of divergence (Arntzen et al., 2014). The approximately
600 km long hybrid zone provides ample opportunities for further evolutionary research.

**Material and methods**

The morphological characters measured in marbled newts are SVL - snout-vent length up to including the cloaca, HL - head length, Hw - head width, ILd - interlimb distance, FL1 - fore limb length, TF1 - third finger length, HLI - hind limb length and FT1 - fourth toe length. Measurements were taken in mm with a plastic ruler (SVL, ILd) and with Vernier calipers at 0.1 mm precision (the other characters). Ventral side colouration characters are Bc - belly colour, WSn and WSs - number and size of white spots, and BSn and BSs - number and size of black spots. These characters were scored at a 1-4 or 1-5 scale, from off-white to black (Bc), from absent or few to many (WSn and BSn) and from absent or tiny to large (WSs and BSs). To guide
Table 1. Studied populations of marbled newts (*Triturus marmoratus* and *T. pygmaeus*) in France, Spain and Portugal, with sample sizes and results of morphological analyses. Populations marked by a hash (#) have molecular data and are listed in Tables 2 and 3. Populations are labelled after the pond they were found, with pond numbers 2-436.

| Pond eq. population number | Locality         | Country     | Longitude | Latitude | Sample size morphology | Mean discriminant score | Number of newts with toe abnormalities |
|----------------------------|------------------|-------------|-----------|----------|------------------------|-------------------------|----------------------------------------|
| 2 #                        | Rio Maior        | Portugal    | -8.921    | 39.341   | 65                     | 0.705                   |                                        |
| 3 #                        | Alqueidão        | Portugal    | -8.580    | 39.530   | 20                     | 0.713                   |                                        |
| 4 #                        | Gavião 1         | Portugal    | -7.939    | 39.477   | 32                     | 0.941                   |                                        |
| 5 #                        | Nisa 1           | Portugal    | -7.604    | 39.533   | 4                      | 0.996                   |                                        |
| 6                          | Sobral do Campo  | Portugal    | -7.549    | 39.998   | 1                      | 0.990                   |                                        |
| 7 #                        | Pedra do Altar   | Portugal    | -7.821    | 39.711   | 56                     | 0.030                   |                                        |
| 9                          | São Domingos 1   | Portugal    | -8.174    | 39.603   | 22                     | 0.060                   |                                        |
| 10                         | Montalvo         | Portugal    | -8.300    | 39.481   | 15                     | 0.687                   |                                        |
| 11                         | Alfena           | Portugal    | -8.525    | 41.238   | 1                      | 0.001                   |                                        |
| 12 #                       | Saide            | Portugal    | -8.329    | 40.449   | 49                     | 0.018                   |                                        |
| 13                         | Vimeiro          | Portugal    | -9.019    | 39.468   | 1                      | 0.631                   |                                        |
| 14                         | Martinchel 1     | Portugal    | -8.298    | 39.358   | 4                      | 0.012                   |                                        |
| 15                         | Andreus 1        | Portugal    | -8.170    | 39.564   | 1                      | 0.004                   |                                        |
| 16                         | Andreus 2        | Portugal    | -8.170    | 39.564   | 5                      | 0.064                   |                                        |
| 19 #                       | Domingos da Vinha| Portugal    | -7.947    | 39.523   | 30                     | 0.322                   |                                        |
| 24                         | Gois             | Portugal    | -8.111    | 40.160   | 1                      | 0.005                   |                                        |
| 25                         | Envidos          | Portugal    | -7.883    | 39.582   | 4                      | 0.059                   |                                        |
| 26 #                       | Lagoa Seca       | Portugal    | -9.012    | 39.596   | 43                     | 0.598                   |                                        |
| 28 #                       | Serra de St Antonio| Portugal | -8.731    | 39.516   | 7                      | 0.437                   |                                        |
| 29 #                       | Mitra 1          | Portugal    | -8.001    | 38.535   | 23                     | 0.992                   |                                        |
| 30 #                       | Mora             | Portugal    | -8.138    | 38.955   | 13                     | 0.997                   |                                        |
| 31                         | Mindelo          | Portugal    | -8.727    | 41.317   | 2                      | 0.482                   |                                        |
| 32                         | Gavião 2         | Portugal    | -7.932    | 39.459   | 22                     | 0.974                   |                                        |
| 33                         | Cadafaz          | Portugal    | -7.943    | 39.470   | 6                      | 0.980                   |                                        |
| 34                         | Martinchel 2     | Portugal    | -8.291    | 39.540   | 4                      | 0.151                   |                                        |
| 38 #                       | Jublains         | France      | -0.472    | 48.255   | 40                     | 0.052                   |                                        |
| 39 #                       | Dunes SW of Mira | Portugal    | -8.785    | 40.389   |                         |                         |                                        |
| 42 #                       | Casas de Ribeira | Portugal    | -7.979    | 39.573   | 7                      | 0.008                   |                                        |
| 46                         | dunes SW of Mira | Portugal    | -8.807    | 40.324   | 4                      | 0.987                   |                                        |
| 48                         | San Jose de Matos| Portugal    | -7.845    | 39.566   | 2                      | 0.958                   |                                        |
| 51 #                       | Gerês - Carris   | Portugal    | -8.046    | 41.815   | 71                     | 0.024                   |                                        |
| 52                         | Zimbreirinha     | Portugal    | -7.873    | 39.536   | 3                      | 0.966                   |                                        |
| 53                         | Vale Pedro Dias 2| Portugal    | -7.878    | 39.533   | 9                      | 0.151                   |                                        |
| 55 #                       | Areia 2          | Portugal    | -7.932    | 39.514   | 50                     | 0.132                   |                                        |
| 56                         | Vila Flor        | Portugal    | -7.781    | 39.518   | 22                     | 0.970                   |                                        |
| 57                         | Outeiro Cimeiro  | Portugal    | -7.901    | 39.496   | 28                     | 0.042                   |                                        |
| 58 #                       | Areia 3          | Portugal    | -7.934    | 39.514   | 11                     | 0.137                   |                                        |
| 60                         | Maxial           | Portugal    | -7.966    | 39.563   | 1                      | 0.001                   |                                        |
| 62 #                       | Coimbra          | Portugal    | -8.404    | 40.203   | 10                     | 0.040                   |                                        |
| 64 #                       | Degracias - Casas de S. Jorge| Portugal | -8.521    | 40.007   |                         |                         |                                        |
| 65 #                       | Vila do Bispo - Sagres | Portugal | -8.904    | 37.072   | 31                     | 0.964                   |                                        |
| 66 #                       | Nisa 2           | Portugal    | -7.593    | 39.550   | 17                     | 0.962                   |                                        |
| 67                         | Rosmanínhal      | Portugal    | -7.091    | 39.732   | 40                     | 0.976                   |                                        |
| 68                         | Monforte da Beira| Portugal    | -7.300    | 39.738   | 1                      | 0.841                   |                                        |
| 69 #                       | Soure            | Portugal    | -8.639    | 40.032   |                         |                         |                                        |
| 70 #                       | Monte do Conde   | Portugal    | -7.250    | 40.167   | 12                     | 0.015                   |                                        |
| 72 #                       | João Pires       | Portugal    | -7.154    | 40.106   | 17                     | 0.024                   |                                        |
Table 1 continued

| Pond cq. population number | Locality                     | Country            | Longitude | Latitude | Sample size morphology | Mean discriminant score | Number of newts with toe abnormalities |
|---------------------------|-----------------------------|--------------------|-----------|----------|------------------------|-------------------------|----------------------------------------|
| 73                        | Medelim - Penha Garcia nr. Sidral | Portugal           | -7.111    | 40.053   | 2                      | 0.067                   |                                        |
| 76                        | São João do Deserto          | Portugal           | -7.198    | 40.144   | 5                      | 0.005                   |                                        |
| 80                        | Barragem de Idanha-a-Nova    | Portugal           | -7.192    | 39.936   | 42                     | 0.222                   |                                        |
| 81                        | Sra. do Almortão 1           | Portugal           | -7.206    | 39.907   |                        |                         |                                        |
| 84                        | Zebreira                     | Portugal           | -7.091    | 39.913   | 44                     | 0.968                   |                                        |
| 87                        | Soverete                      | Portugal           | -7.286    | 39.262   | 4                      | 0.886                   |                                        |
| 88                        | Mouriño                       | Portugal           | -7.312    | 38.429   | 3                      | 0.996                   |                                        |
| 90                        | Esperança                     | Portugal           | -7.174    | 39.176   | 34                     | 0.905                   |                                        |
| 91                        | Castelo Mendo                 | Portugal           | -6.933    | 40.598   | 21                     | 0.060                   |                                        |
| 92                        | Mogadouro                    | Portugal           | -6.740    | 41.332   | 25                     | 0.011                   |                                        |
| 93                        | Granja                        | Portugal           | -7.262    | 38.318   | 11                     | 0.986                   |                                        |
| 94                        | Mitra 2                       | Portugal           | -8.001    | 38.535   | 22                     | 0.978                   |                                        |
| 95                        | Aldeia Santa Margarida        | Portugal           | -7.274    | 40.050   | 21                     | 0.082                   |                                        |
| 96                        | Ponte Maceira, Negreira       | Spain              | -8.703    | 42.901   | 10                     | 0.010                   |                                        |
| 97                        | Calvão                        | Portugal           | -8.705    | 40.460   | 26                     | 0.847                   | 1                                      |
| 98                        | Quiaios                       | Portugal           | -8.816    | 40.237   | 20                     | 0.890                   |                                        |
| 99                        | Praia de Vagueira             | Portugal           | -8.740    | 40.569   |                        |                         |                                        |
| 100                       | Madeirã                       | Portugal           | -8.101    | 39.937   | 24                     | 0.000                   |                                        |
| 102                       | Nespereira de Baixo - Borallal| Portugal           | -8.350    | 40.757   | 21                     | 0.007                   |                                        |
| 103                       | Mezio                         | Portugal           | -7.885    | 40.977   | 28                     | 0.002                   |                                        |
| 104                       | Velada                        | Portugal           | -7.701    | 39.580   | 22                     | 0.965                   |                                        |
| 105                       | Fratel - Carepa                | Portugal           | -7.761    | 39.630   |                        |                         |                                        |
| 106                       | Carepa                        | Portugal           | -7.773    | 39.626   |                        |                         |                                        |
| 107                       | Vila Velha de Rodão           | Portugal           | -7.667    | 39.668   | 40                     | 0.899                   |                                        |
| 108                       | Vale de Figueira              | Portugal           | -7.747    | 39.681   | 31                     | 0.005                   |                                        |
| 109                       | Vilar de Boi                  | Portugal           | -7.736    | 39.672   |                        |                         |                                        |
| 110                       | Zebreira West                 | Portugal           | -7.112    | 39.839   |                        |                         |                                        |
| 114                       | Torreira                      | Portugal           | -8.710    | 40.764   |                        |                         |                                        |
| 116                       | Porto - Botanical Garden      | Portugal           | -8.642    | 41.153   |                        |                         |                                        |
| 119                       | Salir de Matos                | Portugal           | -9.083    | 39.427   | 1                      | 0.000                   |                                        |
| 121                       | Fonte da Pena da Covinha      | Portugal           | -9.019    | 39.477   | 2                      | 0.001                   |                                        |
| 122                       | Punta Moreiras                | Spain              | -8.876    | 42.497   | 8                      | 0.040                   |                                        |
| 127                       | Mosteiros de Alcanede         | Portugal           | -8.838    | 39.426   | 6                      | 0.523                   |                                        |
| 134                       | Aceitunas                     | Spain              | -6.317    | 40.152   | 8                      | 1.000                   |                                        |
| 135                       | Santa Cruz de Paniagua        | Spain              | -6.335    | 40.201   | 7                      | 0.937                   | 2                                      |
| 136                       | El Bronco                     | Spain              | -6.333    | 40.218   | 21                     | 0.991                   | 1                                      |
| 138                       | Pedro Mañoz 2                 | Spain              | -6.343    | 40.275   |                        |                         |                                        |
| 139                       | Pedro Mañoz 3                 | Spain              | -6.340    | 40.257   |                        |                         |                                        |
| 141                       | Puerto Nuevo                  | Spain              | -6.536    | 40.361   |                        |                         |                                        |
| 145                       | Alcuescar                     | Spain              | -6.242    | 39.184   | 25                     | 0.925                   | 1                                      |
| 146                       | Rexaldia 1                    | Portugal           | -8.548    | 39.572   | 2                      | 0.951                   |                                        |
| 147                       | Rexaldia 2                    | Portugal           | -8.549    | 39.571   | 10                     | 0.960                   | 1                                      |
| 148                       | Assertiz - Fungalvaz         | Portugal           | -8.515    | 39.598   | 20                     | 0.868                   |                                        |
| 150                       | Covão da Fonte                | Portugal           | -8.802    | 39.538   | 2                      | 1.000                   |                                        |
| 151                       | Carrascal - Évora de Alcobaca 1| Portugal          | -8.961    | 39.531   | 2                      | 0.804                   |                                        |
| 152                       | Molianos                      | Portugal           | -8.920    | 39.507   | 1                      | 0.185                   |                                        |
| 153                       | Covas                         | Portugal           | -8.802    | 39.533   | 16                     | 0.559                   |                                        |
Table 1 continued

| Pond eq. number | Locality                        | Country | Longitude | Latitude | Sample size (morphology) | Mean discriminant score | Number of newts with toe abnormalities |
|----------------|---------------------------------|---------|-----------|----------|--------------------------|-------------------------|----------------------------------------|
| 155            | Casais Monizes                  | Portugal| -8.896    | 39.449   | 11                       | 0.459                   |                                        |
| 157            | Carrascal - Évora de Alcobaça 2 | Portugal| -8.960    | 39.532   | 2                        | 0.723                   |                                        |
| 160            | Junçal 2                         | Portugal| -8.916    | 39.597   | 7                        | 0.266                   |                                        |
| 165            | Andam                           | Portugal| -8.897    | 39.619   | 1                        | 0.734                   |                                        |
| 177            | Carrascal 2                      | Portugal| -7.878    | 39.547   | 1                        | 0.010                   |                                        |
| 186            | Zimbreira                        | Portugal| -7.849    | 39.576   | 1                        | 0.000                   | 1                                      |
| 196 #           | Serradilla del Llano             | Spain   | -6.319    | 40.482   | 14                       | 0.080                   | 3                                      |
| 202            | Mação 1                          | Portugal| -7.976    | 39.513   | 2                        | 0.259                   |                                        |
| 203 #           | Mação - Belver                   | Portugal| -7.980    | 39.538   | 1                        | 0.001                   |                                        |
| 205             | Tomar                            | Portugal| -8.418    | 39.602   | 46                       | 0.841                   | 6                                      |
| 206             | Cem Saldos                       | Portugal| -8.458    | 39.585   | 1                        | 0.594                   |                                        |
| 208 #           | Sintra 1                         | Portugal| -9.418    | 38.793   |                          |                         |                                        |
| 209 #           | Sintra 2                         | Portugal| -9.388    | 38.788   | 15                       | 0.660                   | 2                                      |
| 216             | Olas                             | Portugal| -8.339    | 39.665   | 2                        | 0.000                   |                                        |
| 217 #           | Nelas, Ponte Nove                | Portugal| -7.836    | 40.486   | 21                       | 0.014                   | 1                                      |
| 218 #           | Casar de Palomera 1              | Spain   | -6.241    | 40.278   | 4                        | 0.010                   |                                        |
| 219 #           | Casar de Palomera 2              | Spain   | -6.251    | 40.272   | 3                        | 0.110                   |                                        |
| 221             | Marchagaz                        | Spain   | -6.270    | 40.270   | 1                        | 0.002                   |                                        |
| 222 #           | Palomero                         | Spain   | -6.250    | 40.273   | 11                       | 0.997                   |                                        |
| 224 #           | Santa Catarina                   | Portugal| -8.357    | 39.651   | 4                        | 0.075                   |                                        |
| 225             | Casais                           | Portugal| -8.383    | 39.654   | 1                        | 0.002                   |                                        |
| 231             | Braia                            | Portugal| -8.740    | 40.568   | 10                       | 0.714                   |                                        |
| 244             | Alberga dos Doze                 | Portugal| -8.612    | 39.797   | 2                        | 0.064                   |                                        |
| 245             | Montalva - Spanish border        | Portugal| -7.531    | 39.609   | 17                       | 0.973                   |                                        |
| 247             | Castelo de Vide railway line 1   | Portugal| -7.479    | 39.444   | 13                       | 0.995                   |                                        |
| 248             | Beira                            | Portugal| -7.360    | 39.451   | 2                        | 0.871                   |                                        |
| 250             | São Domingos 2                   | Portugal| -8.171    | 39.602   | 11                       | 0.101                   |                                        |
| 257             | Carreiras                        | Portugal| -7.457    | 39.371   | 2                        | 1.000                   |                                        |
| 271             | Doñana                           | Spain   | -6.394    | 36.853   | 31                       | 0.949                   |                                        |
| 273 #           | Archidona, Loja                  | Spain   | -4.287    | 37.130   |                          |                         |                                        |
| 300             | Olhos de Agua                    | Portugal| -7.383    | 39.371   | 16                       | 0.974                   |                                        |
| 307             | Castelo de Vide railway line 2   | Portugal| -7.444    | 39.455   | 10                       | 0.954                   |                                        |
| 336 #           | Confolens                        | France  | 0.667     | 46.017   |                          |                         |                                        |
| 337 #           | El Berruco                       | Spain   | -3.883    | 40.900   |                          |                         |                                        |
| 338 #           | Puerto de Galiz                  | Spain   | -5.450    | 36.683   |                          |                         |                                        |
| 339 #           | Rochechouart                     | France  | 0.833     | 45.817   |                          |                         |                                        |
| 340 #           | Venta del Charco                 | Spain   | -4.267    | 38.200   |                          |                         |                                        |
| 341 #           | Zafarraya                        | Spain   | -4.133    | 36.967   |                          |                         |                                        |
| 354             | Marinha Grande                   | Portugal| -8.924    | 39.750   | 1                        | 0.005                   |                                        |
| 346             | Umbria near Monchique            | Portugal| -8.506    | 37.335   | 1                        | 0.996                   |                                        |
| 348             | Faro University 1                | Portugal| -7.979    | 37.050   | 2                        | 1.000                   |                                        |
| 453             | Picarras                         | Portugal| -8.563    | 38.680   | 2                        | 0.990                   |                                        |
| 454             | Fuenlabrada de los Montes        | Spain   | -4.956    | 39.129   | 2                        | 1.000                   |                                        |
| 458             | Los Baños del Robledillo         | Spain   | -4.358    | 39.496   | 1                        | 0.987                   |                                        |
| 459             | Buenasbodas nr. Belvis de la Jara| Spain   | -4.890    | 39.681   | 3                        | 0.974                   |                                        |
| 461             | Los Yebenes - estacion de Urda   | Spain   | -3.821    | 39.418   | 20                       | 0.992                   |                                        |
| 463             | Villanueva de San Juan - Moron   | Spain   | -5.289    | 37.060   | 1                        | 0.966                   |                                        |
the reader of what the colour classifications are like see Figure 1. Note that the image in black and white captures nearly all ventral ‘colour’ information. Since these characters are continuous, borderline cases are not infrequent. Dorso-lateral colour pattern characters are ‘Links’ and ‘Toplinks’. Links is the number of uninterrupted transversal connections following the green coloured surface at the lateral side of the body. Links were counted over both body sides independently, over the section of the body covered by ILd. Toplinks is the number of dark spots that reach up to the mid-dorsal line and make it at least half way down the side and are also counted over both sides of the body. Illustrations of these characters are in Figure 2.

The morphometric data were ln-transformed. In order to analyze predominantly shape and not size, I calculated the standardized residuals of the regression of ‘ln character’ on lnSVl. Males and females were

Figure 2. Line drawings of the dorso-lateral sides of adult marbled newts. The dominant colours green and brown are shown by light and dark shading, respectively. On the left are *Triturus marmoratus* from Gerês in northern Portugal (A-C, locality 51) and Casas de Ribeira at the northern side of the contact zone (D-F, locality 42). On the right are *T. pygmaeus* from Gavião at the southern side of the contact zone (G, H and J, locality 32) and from Vale de Bispo - Sagres in southern Portugal (K-M, locality 65). The imagery illustrates how Links (numbers) and Toplinks (T) are counted. Links are the transversal connection following the green surface at the lateral side of the newts’ body and are indicated by numbers (1-1, 2-2, etc.). Tick marks (^) to the left and right indicate genuine Links, but these are not counted because they fall (partly) outside the area covered by the measure interlimb distance. Toplinks are the dark spots that touch the mid-dorsal line and reach at least halfway down the newt’s lateral side. Ambiguities in counting Links include the often vague colour transition from the lateral to the ventral side. This did not preclude recognizing links, such as Link 3 in specimen E. In specimen K Links 8 and 9 could also have been scored as a single one, because the Link paths share a short section in the middle. In counting Toplinks ambiguities may also arise, such as in specimen A where for the first Toplink the connection over the side is thin and in specimen J where it is arbitrary if the second Toplink reaches the mid-dorsal line. Reticulated (or ‘wavy’) colour patterns such as in specimen E and to a lesser extent in specimen D were only observed in the zone of contact and are tentatively associated with interspecific hybridization. Also ‘colour islands’ (I) such as in specimen J were found exclusively in and around the contact zone.
analyzed separately because of a documented sexual size dimorphism (García-París et al., 1993). Altogether data were gathered for 1690 adult individuals in one French, 17 Spanish and 101 Portuguese populations (average N=14.2, range 1-71). The populations studied are listed in Table 1. However, colouration character states were defined during the project and are missing for 143 individuals (8.5%). Occasionally, Links or Topleinks could not be counted due to albinism (N=1, population 51), melanism (N=9, populations 122, 151, 157, 165 and 206), or because of an aberrant, highly fragmented colouration pattern (N=2, population 307). The green dorso-lateral colour is overall darker and harder in T. marmoratus than the softer, olive shades of green that are prevalent in T. pygmaeus, but I was unable to score this character consistently. The tuberculate versus smooth skins and thick versus thin arms and legs for respectively T. marmoratus and T. pygmaeus were difficult to describe and also remained unrecorded. Finally, notes were taken on morphological abnormalities of fingers and toes, because these may be indicative of hybridization, as is the case of T. marmoratus and T. cristatus (Laurenti, 1768) in central France (Valléé, 1959; Arntzen and Wallis, 1991). To obtain an indication of measurement error 21 marbled newts were measured by myself in duplicate and by two volunteer students. To estimate measuring error the untransformed data were analyzed by principal component analysis, in SPSS v. 20 (IBM SPSS, 2016).

To be able to evaluate the diagnostic properties of the morphological data I also gathered data for enzyme genetic markers with proven performance for species identification, following Espregueira Themudo and Arntzen (2007a). The four marker loci that differentiated T. marmoratus from T. pygmaeus are PepA with reported \( \kappa \) values (Cohen’s kappa, Cohen, 1960) \( \kappa=0.92 \), PepB with \( \kappa=0.97 \), PepD with \( \kappa=0.12 \) and Ldh2 with \( \kappa=0.56 \). A total of 896 individuals was studied for ten Spanish and 46 Portuguese populations (average N=16.0, range 1-41). Tissues investigated were tail tips taken from adults. Missing data amounted to 3.4%, with no more than one locus with missing data for any individual. The individual genotypes are in Online Supplementary Information SI. DNA sequences of the nuclear genes \( \beta \)-fibrinogen intron 7 (FGBIT7, or BF), calreticulin intron 3 (CALRIT3, or CALR), platelet-derived growth factor receptor a-intron 11 (PDGFRAIT11, or PDG) and the mitochondrial gene NADH dehydrogenase subunit 4 (ND4) were taken from the literature (Espregueira Themudo et al., 2012). A total of 1325 sequences were downloaded from Genbank and aligned with Clustal \( \Omega \) (Sievers and Higgins, 2014). Median joining haplotype networks were constructed for each sequenced marker with PopArt (Bandelt et al., 1999; Leigh and Bryant, 2015). The internal node was located that maximized the separation between T. marmoratus and T. pygmaeus individuals from non-admixed populations. The fit of the haplotype groups to the species was \( \kappa=0.87 \) for BF, \( \kappa=0.96 \) for CALR, \( \kappa=0.66 \) for PDG and \( \kappa=0.93 \) for ND4. A total of 79 populations have sequence data available.

Species differentiation, population diversity and admixture were explored with the Bayesian clustering algorithm Structure (Pritchard et al., 2000) with a predetermined \( K=2 \) number of groups, reflecting the species to be analyzed and the known diagnostic properties of the marker loci. The data were also analyzed with NewHybrids (Anderson and Thompson, 2002) under default settings, to identify possible cases of hybridization. The classes recognized were T. marmoratus, T. pygmaeus and a hybrid class composed of pooled F\(_1\)’s, F\(_2\)’s and backcross hybrid individuals. Structure \( Q \)-values in the 0.2 - 0.8 range and NewHybrid \( P_{NH} \) values > 0.5 were interpreted as
evidence for recent (NewHybrids) or possibly more ancient (Structure) hybridization and interspecific gene flow. The basic population genetic analyses for Hardy-Weinberg and linkage (dis)equilibrium were carried out with GenePop (Raymond and Rousset, 1995) under population-wide Bonferroni correction.

The morphological data were analyzed by univariate and stepwise discriminant analysis in SPSS with Structure individual allocations to T. marmoratus ($Q_p < 0.2$), T. pygmaeus ($Q_p > 0.8$) or unknown. The ‘unknowns’ were either genetically admixed with $Q$-scores in the 0.2 - 0.8 range, or had no genetic data available. The unknowns were classified a posteriori from their morphology discriminant scores as T. marmoratus ($P_{Dap} < 0.2$), T. pygmaeus ($P_{Dap} > 0.8$) or intermediate ($0.2 < P_{Dap} < 0.8$). The fit of the morphological to the genetic species identification was assessed with the AUC (area under the curve) statistic in SPSS. Ellipses summarizing variation around the mean in bivariate plots were produced with Mystat 12 software (Mystat, 2007). Spatial contour maps were also made with Mystat, under default settings and with population data weighted for sample size.

Geographical clines were calculated for populations in a south to north transect across Portugal, encompassed by the coordinates 37.0 - 42.0 N and 7.8 - 8.2 W. I performed maximum likelihood geographical cline analyses with the R package HZAR (Derryberry et al., 2014). Clines were fitted on probabilities of species assignment for morphological data from discriminant analysis ($P_{Dap}$, 31 populations), on Structure scores for the enzyme loci ($Q_p$, 14 populations) and on the
frequencies of species indicative haplotype groups for sequence data ($F_p$, eight populations) with values running from zero (T. marmoratus) to unity (T. pygmaeus). Clines were also fitted for the best performing morphological characters individually (SV1 and Links, 27-29 populations, see below). The shape of each cline is described as a sigmoidal curve with a centre (maximum slope) and two tails (one in each side) modelled as an exponential decay, following Szymura and Barton (1986). Five different models were tested, varying on the presence/absence of left and right tails and their symmetry/asymmetry. Additionally, minimum ($P_{\text{min}}$) and maximum ($P_{\text{max}}$) character values were used to scale cline models in three different ways: with empirical estimates (using lower and higher observed values in the data), with best-fit values, and with no scaling. The combination of cline shapes and scaling thus produces a total of 15 different models for each cline, plus a null model (see for instance Prada and Hellberg, 2014). The Metropolis-Hasting algorithm in HZAR was run for the default value of 100,000 steps with a randomly selected seed and a burn-in of 10%. Given the uneven sample sizes it is important to note that HZAR cline estimates are made with sample size taken into account. After each run, I recorded the corrected Akaike Information Criterion score for each model within each cline and chose the one with the lowest score to infer cline width, cline centre and other cline shape parameters.

Table 2. Structure Q-scores derived from four enzyme loci (LDH2, PEPA, PEPB and PEPD) along with sample sizes and classification results, with m - Triturus marmoratus (27 populations with $Q_p < 0.20$), p - T. pygmaeus (28 populations with $Q_p > 0.8$) and x - admixed (one populations with intermediate $Q_p$). For locality information see Table 1; for individual genotypes see Online Supplementary Information S1; for Q-scores along with NewHybrid results see Figure 3.

| Population | Structure Q-score | Sample size | Species classification |
|------------|-------------------|-------------|------------------------|
| 2          | 0.958             | 20          | p                      |
| 3          | 0.949             | 19          | p                      |
| 4          | 0.935             | 22          | p                      |
| 5          | 0.989             | 17          | p                      |
| 6          | 0.926             | 36          | m                      |
| 7          | 0.038             | 10          | m                      |
| 8          | 0.215             | 14          | x                      |
| 9          | 0.977             | 22          | p                      |
| 10         | 0.988             | 23          | p                      |
| 11         | 0.977             | 7           | p                      |
| 12         | 0.885             | 10          | p                      |
| 13         | 0.036             | 7           | m                      |
| 14         | 0.038             | 25          | m                      |
| 15         | 0.083             | 10          | m                      |
| 16         | 0.054             | 7           | m                      |
| 17         | 0.032             | 5           | m                      |
| 18         | 0.989             | 10          | p                      |
| 19         | 0.942             | 34          | p                      |
| 20         | 0.017             | 7           | m                      |
| 21         | 0.055             | 10          | m                      |
| 22         | 0.015             | 5           | m                      |
| 23         | 0.037             | 38          | m                      |
| 24         | 0.925             | 41          | p                      |
| 25         | 0.990             | 4           | p                      |
| 26         | 0.991             | 3           | p                      |
| 27         | 0.978             | 27          | p                      |
| 28         | 0.081             | 21          | m                      |
| 29         | 0.014             | 25          | m                      |
| 30         | 0.988             | 11          | p                      |
| 31         | 0.016             | 21          | m                      |
| 32         | 0.013             | 10          | m                      |
| 33         | 0.970             | 26          | p                      |
| 34         | 0.973             | 20          | p                      |
| 35         | 0.108             | 24          | m                      |
| 36         | 0.031             | 21          | m                      |
| 37         | 0.033             | 28          | m                      |
| 38         | 0.963             | 22          | p                      |
| 39         | 0.940             | 40          | p                      |
| 40         | 0.053             | 31          | m                      |
| 41         | 0.908             | 1           | m                      |
| 42         | 0.014             | 2           | m                      |
| 43         | 0.017             | 8           | m                      |
| 44         | 0.980             | 6           | p                      |
| 45         | 0.960             | 8           | p                      |
| 46         | 0.981             | 7           | p                      |
| 47         | 0.982             | 21          | p                      |
| 48         | 0.978             | 24          | p                      |
| 49         | 0.974             | 2           | p                      |
| 50         | 0.982             | 10          | p                      |
| 51         | 0.978             | 20          | p                      |
| 52         | 0.073             | 14          | m                      |
| 53         | 0.030             | 1           | m                      |
| 54         | 0.024             | 21          | m                      |
| 55         | 0.012             | 4           | m                      |
| 56         | 0.151             | 3           | m                      |
| 57         | 0.983             | 11          | p                      |

Results

Deviations from Hardy-Weinberg equilibrium with deficiencies of heterozygotes were found for the loci PepB and PepD in population 56. Significant linkage disequilibrium was not observed. With the Bayesian clustering algorithm Structure 393 individuals were classified as T. marmoratus ($Q_p < 0.2$), 466 were classified as T. pygmaeus ($Q_p > 0.8$) and 35 were admixed. At the population level 27 were T. marmoratus, 28 were T. pygmaeus and one was admixed (population 19, Table 2, Figure 3). With NewHybrids individuals and populations were allocated to the non-hybrid class (either T. marmoratus or T. pygmaeus), though four individuals (and no populations) showed a weak signal with $P_{\text{NH}}$ values in the 0.2 - 0.5 range. The DNA sequence data allocated 34 populations to T. marmoratus, 36 to T. pygmaeus and nine were admixed (Table 3). The resulting species distribution is strongly partitioned with T. marmoratus...
Table 3. Frequency of species-indicative haplotypes for three nuclear genes (BF, CALR and PDG) and the mitochondrial ND4 gene, along with sample sizes in brackets and classification results, with m – *Triturus marmoratus* (34 populations with $F_p < 0.20$), p – *T. pygmaeus* (36 populations with $F_p > 0.8$) and x – admixed (nine populations with $F_p$ intermediate). For locality information see Table 1.

A .kml file for visualization with e.g. Google Earth is provided as Online Supplementary Information S3.

| Population | BF (N) | CALR (N) | PDG (N) | ND4 (N) | Classification |
|------------|--------|----------|---------|---------|----------------|
| 2          | 1.00 (5) | 1.00 (5) | 0.70 (5) | 1.00 (6) | p              |
| 3          | 1.00 (1) | 1.00 (4) | 0.50 (5) | 1.00 (5) | p              |
| 4          | 0.33 (3) | 1.00 (5) | 1.00 (6) | 0.50 (8) | x              |
| 7          | 1.00 (3) | 1.00 (3) | 1.00 (5) | 1.00 (5) | m              |
| 12         | 0.50 (2) | 0.25 (2) | 0.00 (2) | 0.00 (5) | m              |
| 19         | 0.00 (5) | 1.00 (5) | 1.00 (5) | 1.00 (5) | p              |
| 26         | 1.00 (3) | 1.00 (5) | 0.50 (5) | 1.00 (5) | p              |
| 30         | 1.00 (4) | 1.00 (5) | 1.00 (4) | 0.75 (4) | p              |
| 32         | 0.00 (4) | 0.00 (4) | 0.00 (5) | 0.00 (5) | m              |
| 38         | 0.00 (4) | 0.00 (4) | 1.00 (4) | 1.00 (4) | p              |
| 39         | 1.00 (4) | 0.13 (4) | 0.38 (4) | 0.00 (5) | m              |
| 42         | 0.10 (5) | 0.13 (4) | 0.38 (4) | 0.00 (5) | m              |
| 51         | 0.50 (4) | 0.00 (5) | 0.00 (7) | 0.20 (5) | m              |
| 55         | 0.25 (4) | 0.20 (5) | 0.50 (5) | 0.40 (5) | x              |
| 58         | 0.60 (5) | 0.60 (5) | 0.60 (5) | 0.60 (5) | x              |
| 62         | 0.00 (3) | 0.00 (3) | 0.00 (3) | 0.00 (3) | m              |
| 66         | 0.00 (5) | 0.00 (5) | 0.00 (5) | 0.00 (5) | m              |
| 67         | 0.67 (3) | 1.00 (7) | 0.90 (5) | 1.00 (5) | p              |
| 69         | 1.00 (5) | 1.00 (5) | 1.00 (5) | 1.00 (5) | p              |
| 70         | 0.00 (3) | 0.00 (3) | 0.00 (3) | 0.00 (3) | m              |
| 72         | 0.00 (3) | 0.00 (3) | 0.00 (3) | 0.00 (3) | m              |
| 76         | 0.00 (5) | 0.13 (4) | 0.00 (5) | 0.00 (5) | m              |
| 80         | 0.20 (5) | 0.20 (5) | 0.00 (5) | 0.00 (4) | m              |
| 81         | 1.00 (4) | 1.00 (5) | 0.50 (9) | 0.80 (5) | x              |
| 84         | 1.00 (2) | 1.00 (3) | 0.75 (4) | 0.50 (4) | x              |
| 87         | 1.00 (3) | 1.00 (3) | 1.00 (3) | 1.00 (3) | p              |
| 88         | 1.00 (1) | 1.00 (1) | 1.00 (1) | 1.00 (2) | p              |
| 90         | 1.00 (7) | 1.00 (9) | 1.00 (10) | 0.63 (8) | p              |
| 91         | 0.33 (3) | 0.00 (3) | 0.00 (5) | 0.00 (5) | m              |
| 92         | 0.30 (5) | 0.00 (5) | 0.00 (5) | 0.00 (5) | m              |
| 93         | 1.00 (5) | 1.00 (4) | 1.00 (4) | 1.00 (5) | p              |
| 94         | 1.00 (5) | 1.00 (5) | 1.00 (5) | 1.00 (5) | p              |
| 95         | 0.00 (5) | 0.00 (5) | 0.00 (5) | 0.00 (5) | m              |
| 96         | 0.38 (4) | 0.00 (5) | 0.00 (4) | 0.00 (5) | m              |
| 97         | 1.00 (3) | 1.00 (3) | 1.00 (3) | 1.00 (3) | p              |
| 98         | 1.00 (4) | 1.00 (4) | 1.00 (4) | 1.00 (4) | p              |
| 99         | 1.00 (10) | 1.00 (10) | 0.30 (10) | 1.00 (10) | p              |
| 100        | 1.00 (5) | 1.00 (5) | 1.00 (5) | 1.00 (5) | p              |
| 102        | 0.10 (5) | 0.00 (5) | 0.00 (5) | 0.00 (5) | m              |
| 103        | 0.00 (1) | 0.00 (4) | 0.00 (4) | 0.00 (5) | m              |
| 104        | 1.00 (5) | 1.00 (5) | 0.75 (2) | 1.00 (5) | p              |
| 105        | 0.00 (5) | 0.00 (5) | 0.20 (5) | 0.00 (5) | m              |
| 106        | 0.20 (5) | 0.00 (5) | 0.60 (5) | 0.00 (5) | x              |
in the north and *T. pygmaeus* in the south of the Iberian peninsula. The transition between the species is sharp. The admixed populations are located at the species contact area in central Portugal (N=6), eastern Portugal (N=2) and western Spain (N=2, Figure 4; for details see Online Supplementary Information S2 and S3). Notable is the northerly distribution of *T. pygmaeus* along the Atlantic coast and the isolated occurrence of *T. marmoratus* directly north of the Lisbon peninsula. Because these results closely follow earlier reconstructions (e.g. Arntzen and Espregueira Themudo, 2008) and link up to the species borders recorded for Spain (García-París et al., 1993; 2001; Pleguezuelos et al., 2002) the results set the scene for an evaluation of the performance of morphological data for species identification.

The replicated morphology measures were analyzed by principal component (PC) analysis. The first and second axes explained 28.1 % and 18.8 % of the total explained variance, respectively. The analysis revealed correspondence among three data sets out of four (Figure 5), suggesting that one observer measured in a different manner than the others. The outlying data shifted along the second PC-axis that has particularly high loadings for digit lengths (TFl and FTl) and the characters describing the extent of the black ventral colouration. The mean coefficient of variation (*V*) per character for the three

| Population | BF (N) | CALR (N) | PDG (N) | ND4 (N) | Classification |
|------------|--------|----------|---------|---------|----------------|
| 107        | 0.67 (3) | 0.33 (3) | 0.75 (4) | 0.25 (4) | x              |
| 108        | 0.00 (5) | 0.00 (5) | 0.00 (5) | 0.00 (5) | m              |
| 109        | 1.00 (5) | 0.10 (5) | 0.30 (5) | 0.00 (5) | m              |
| 110        | 0.00 (2) | 0.17 (3) | 0.00 (3) | 0.00 (3) | p              |
| 114        | 0.30 (5) | 0.00 (5) | 0.00 (5) | 0.00 (5) | m              |
| 127        | 1.00 (4) | 1.00 (4) | 0.50 (4) | 0.00 (4) | p              |
| 134        | 1.00 (4) | 1.00 (4) | 0.75 (4) | 1.00 (4) | p              |
| 135        | 1.00 (3) | 1.00 (4) | 0.50 (4) | 1.00 (5) | p              |
| 136        | 1.00 (5) | 1.00 (5) | 0.70 (5) | 1.00 (5) | p              |
| 138        | 0.00 (2) | 1.00 (1) | 0.50 (1) | 0.00 (3) | m              |
| 139        | 1.00 (1) | 1.00 (1) | 1.00 (1) | 1.00 (4) | p              |
| 141        | 0.00 (2) | 0.00 (3) | 0.00 (1) | 0.00 (4) | m              |
| 145        | 1.00 (4) | 1.00 (5) | 0.50 (5) | 1.00 (5) | x              |
| 146        | 1.00 (1) | 1.00 (1) | 1.00 (1) | 1.00 (1) | p              |
| 147        | 1.00 (2) | 1.00 (3) | 0.50 (4) | 1.00 (4) | p              |
| 148        | 1.00 (5) | 1.00 (5) | 0.90 (5) | 1.00 (5) | p              |
| 196        | 0.00 (5) | 0.00 (5) | 0.00 (5) | 0.00 (5) | m              |
| 205        | 1.00 (5) | 1.00 (5) | 0.70 (5) | 1.00 (5) | p              |
| 208        | 1.00 (4) | 1.00 (3) | 0.50 (1) | 1.00 (2) | p              |
| 209        | 1.00 (4) | 0.90 (5) | 0.40 (5) | 1.00 (4) | p              |
| 217        | 0.00 (5) | 0.00 (5) | 0.00 (5) | 0.00 (5) | m              |
| 218        | 0.00 (4) | 0.00 (4) | 0.00 (4) | 0.25 (4) | m              |
| 219        | 1.00 (1) | 0.00 (1) | 0.00 (1) | 0.00 (1) | x              |
| 222        | 1.00 (5) | 1.00 (5) | 0.70 (5) | 0.80 (5) | p              |
| 224        | 0.00 (3) | 0.00 (4) | 0.25 (4) | 0.25 (4) | m              |
| 273        | 1.00 (4) | 1.00 (4) | 1.00 (3) | 1.00 (3) | p              |
| 336        | 0.00 (1) | 0.00 (1) | 0.00 (1) | 0.00 (1) | m              |
| 337        | 0.00 (3) | 0.00 (4) | 0.00 (4) | 0.00 (4) | m              |
| 338        | 1.00 (4) | 1.00 (5) | 1.00 (5) | 1.00 (5) | p              |
| 339        | 1.00 (1) | 0.00 (3) | 0.00 (3) | 0.00 (3) | m              |
| 340        | 1.00 (3) | 1.00 (3) | 1.00 (3) | 1.00 (3) | p              |
| 341        | 1.00 (3) | 1.00 (3) | 1.00 (3) | 1.00 (3) | p              |
and scores for Toplinks are higher in *T. marmoratus*. SVL and Links are the prime characters selected in the discriminant analysis. These characters stand out for efficient species discrimination to the extent that model fit on the basis of these two characters (0.97 < AUC < 0.98) is only marginally less than for the set of nine characters selected by discriminant analysis (0.98 < AUC < 0.99, Table 4). In two populations out of 59, species frequencies from enzyme genetic and morphological characters are significantly different (*G*-test for independence, *P* < 0.05 for population 3, and *P* < 0.001 for population 80). These populations are located at or close to (<20 km) the centre of the hybrid zone (see Table 1 and Figure 4).
The morphological identification criteria were also applied to populations for which enzyme data were unavailable. The species distributions on the basis of external morphology closely follows that of the molecules (Figure 4). However, in the contour plot of discriminant species probabilities, the transition zone is wide, in particular north of the Lisbon peninsula where the species distribution forms a mosaic. Along the coast the species transition zone reaches up to Aveiro. In central Portugal it follows the Tejo river and runs through the town Idanha-a-Nova into Spain where it follows the northern edge of Extremadura and, presumably, the southern edge of Castile and León, and into the province of Madrid where the species contact discontinues. Digital deformities I observed in 19 individuals in ten populations, located inside as well as outside the species transition zone (Table 1).

At the transect the *T. marmoratus* – *T. pygmaeus* species transition is sharp and coincides with the position of the Tejo river, for most characters studied (Table 5, Figures 6 and 7). With the pooled enzyme genetic data set as a reference, the clines are congruent, with the exception of female SVl and PDG for which clines are marginally wider than the reference cline. The cline for mtDNA stands out on the basis of its width and southerly position. For the characters lnSVl and Links the clines revealed a prevalence of intermediate values at the very centre of the contact zone (Figure 6CDE). This raised the question if these morphologically intermediate populations were composed of genetically admixed individuals. Because the sample in the transect was small, I performed analyses with all data, with the *Q*-scores classification as above, and with admixture set as a wider class (0.1 < *Q* < 0.9). An ANOVA indicated significant differences for the three groups, with the genetically admixed group as morphologically intermediate (Table 6).

**Discussion**

I reconstructed the distribution of the hybridizing newt species *T. marmoratus* and *T. pygmaeus* over the western part of the Iberian Peninsula from morphological and molecular data. I report on ten admixed populations that all are located at the *T. marmoratus* – *T. pygmaeus* range interface. Yet, the molecular data show a strong bimodality with few admixed individuals, suggesting that interspecific hybridization is limited, possibly with strong selection against hybrids (Barton and Hewitt, 1985; Mallet, 2005). The level of hybridization is comparable to that in *Triturus* crested newts at the same level of divergence (Arntzen et al., 2014). Some introgression appears to have taken place as shown by admixed individuals in e.g. populations 100, 104 and 107 (Figure 3). However, with few partially diagnostic markers ‘admixed’ genotypes may have arisen by chance recombination. This may be the case in population 100 that is located away from the species contact zone and figures as an outlier in cline analysis (Figure 6f). Morphologically intermediate individuals are more numerous than genetically admixed individuals, suggesting that the molecular markers do not reflect the full scope of *T. marmoratus* × *T. pygmaeus* hybridization. To document introgression and to elucidate hybrid zone structure at a fine scale, more genetic markers are required.

Morphological characters that stand out for species identification are body size (SVl) and the dorso-lateral
Table 5. Parameter estimates for the maximum-likelihood geographical clines for morphological data (top panel), nuclear genetic data (middle panel) and nuclear and mitochondrial sequence data (bottom panel) over the south to north transect shown in Figure 4. Cline position is relative to Tejo river at 8° W and width is 1/maximum slope. Confidence intervals are based upon the log-likelihood unit support limits. δ and τ are the shape parameters for the tails, with fitting at a mirror (M), at the right (R) or none (N). The parameters \( P_{\min} \) and \( P_{\max} \) are the estimated values at either end of the transect and are set to minimum and maximum values (typ models), fixed to their empirical values (fix models), or \( P_{\min} \) and \( P_{\max} \) are fitted (opt models). Clines are shown in Figures 6 and 7.

| Character | Model | Position (km) # | Confidence interval | Width (km) | Confidence interval | Mirror | Right | \( P \) | Cline shown in Figure |
|-----------|-------|-----------------|---------------------|------------|---------------------|---------|--------|---------|----------------------|
| Morphological data analysed with stepwise discriminant analysis - 31 populations | | | | | | | | | |
| All populations | OptR | 0.73 | -0.09 | 1.65 | 1.17 | 0.12 | 2.81 | 0.387 | 0.031 | 0.017 | 0.985 |
| Populations with genetic data | OptR | 2.15 | 0.83 | 3.05 | 4.75 | 1.83 | 4.91 | 1.683 | 0.140 | 0.018 | 0.991 |
| Populations without genetic data | OptN | 1.25 | -1.07 | 1.91 | 0.23 | 0.00 | 2.73 | 0.119 | 0.979 | 6B |
| Morphology, single characters - 29 populations | | | | | | | | | |
| lnSVl, males | OptN | 0.07 | -24.47 | 5.03 | 16.54 | 0.28 | 109.92 | 4.028 | 4.263 | 6C |
| lnSVl, females | OptN | 0.53 | -11.64 | 6.00 | 14.65 | 5.60 | 86.51 | 4.055 | 4.299 | 6D |
| Links | OptN | 1.68 | -0.15 | 1.85 | 0.49 | 0.02 | 2.22 | 1.718 | 8.650 | 6E |
| Nuclear genetic data analysed with Structure - 14 populations | | | | | | | | | |
| Four loci | FixM | 2.14 | 0.14 | 3.47 | 1.23 | 0.15 | 5.21 | 0.759 | 0.009 | 0.024 | 0.988 |
| PepA | TypM | 1.24 | 0.25 | 3.42 | 1.80 | 0.20 | 8.63 | 0.727 | 0.003 | 0 |
| PepB | OptN | -1.17 | -4.20 | 0.23 | 5.01 | 1.42 | 13.02 | 0.067 | 0.789 | 6H |
| PepD | OptN | 0.77 | -0.20 | 3.82 | 1.39 | 0.00 | 5.23 | 0.155 | 0.786 | 6J |
| Ldh2 | FixN | 26.72 | -124 | > 125 | > 400 | 4.54 >> 400 | 0.079 | 0.869 |
| DNA sequence data, analyzed from species indicative haplotype frequencies - eight populations | | | | | | | | | |
| BF | OptN | -7.49 | -57.62 | 2.67 | 18.63 | 0.002 | 106.71 | 0.180 | 0.997 | 7L |
| CALR | TypN | 3.23 | -0.49 | 7.29 | 7.53 | 2.91 | 34.15 | 0 | 1 |
| PDG | TypN | 5.99 | 1.73 | 19.47 | 12.21 | 5.34 | 81.86 | 0 | 1 |
| ND4 mitochondrial | TypR | -19.28 | -51.29 | -5.54 | 78.86 | 26.24 | 182.44 | 51.88 | 0 | 1 |

# Relative to the Tejo river at 8.0° W. Note that the transect crosses the Tejo river at an angle, at positions varying from 39.446° to 39.543° N, amounting to a latitudinal difference of 10.8 km.

colour pattern (Links). Marbled newts with SVI > 63 (males), SVI > 65 (females) and Links ≤ 5 are likely to be *T. marmoratus*, whereas marbled newts smaller than that with higher link counts are likely to be *T. pygmaeus*. *Triturus pygmaeus* was described as a small sized ('pygmy') subspecies of *T. marmoratus* by Wolterstorff (1905) with Algeciras, Cadiz as type locality. Body size was considered a diagnostic character on the basis of material from the same region (García-París et al., 1993). However, southern populations of *T. pygmaeus* may be of particular small body size (Diaz-Paniagua et al., 1996). Body size may also be affected by local feeding conditions and other environmental variables. Moreover, newts show indeterminate growth (Arntzen, 2000) so that individual age is a confounding factor. The character Links has higher diagnostic power than body size and is equally applicable to males and females. However, diagnostic power appears to break down in the very south of Spain where link counts for *T. pygmaeus* are close to zero (García-París et al., 1993). Observer bias may be an issue in morphological species identification, but was not shown to affect SVI or Links. Counting links may be ambiguous if there are many, but with a threshold set at 5.5 this is not an identification issue. Marbled newts with aberrant colour patterns were found at the hybrid zone. In some ways similar ‘reticulate’ or ‘wavy’ colouration patterns have been observed in *Bombina bombina* (Linnaeus, 1761) x *B. variegata* (Linnaeus, 1758) reciprocal crosses (Héron-Royer, 1891; Michalowski and Madej, 1969). Given the observations of toe abnormalities well outside the hybrid zone it is unclear if these have to do with hybridization, as was
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Modelling has shown that even limited hybridization can result in substantial introgression, when one species is competitively displacing the other (Currat et al., 2008). This genetic footprint is reflected in the cline by an increased width and a displaced, southern position. Mitochondrial DNA is more than nuclear loci prone to chance introgression due to its small effective population size and it is more prone to detection because it stays intact, due to its non-recombining nature (Toews and Brelsford, 2012). The hypothesis of hybrid zone movement can be tested by surveying a larger number of nDNA markers and search for further ‘genomic footprints of hybrid zone movement’ (Wielstra et al., 2017).

Marbled newt populations are infrequent in the Madrid area (García-París et al., 1989; Martínez-Solano, 2006). The two populations studied by Arntzen et al. (2014) are ca. 20 km apart, with no evidence for hybridization. It may here concern a residual hybrid zone with past but no present opportunities for hybridization and gene flow. Both species appear to suffer from regressing ranges in the east of the Iberian peninsula, as suggested by the isolated occurrences around Barcelona (T. marmoratus) and in Albacete province (T. pygmaeus). The southern fringe of

Table 6. Analysis of variance on morphological characters selected for species discrimination, applied to the groups Triturus marmoratus (1), admixed (2) and T. pygmaeus (3).

| Genetically classified groups | Mean character values # | Significance in Tamhane’s T2 post-hoc test |
|------------------------------|-------------------------|-------------------------------------------|
|                              | Sample size | SVI (mm) | Links | Groups compared | SVI |
| Intermediate group small     |             |          |       |                |     |
| 1 - T. marmoratus ($Q_p < 0.2$) | 393 | 69.1 | 71.8 | 1.49 | 12 | NS | NS | *** |
| 2 - admixed ($0.2 < Q_p < 0.8$) | 35 | 62.6 | 66.8 | 6.29 | 23 | NS | NS | NS |
| 3 - T. pygmaeus ($Q_p > 0.8$) | 466 | 58.2 | 62.9 | 7.97 | 13 | *** | *** | *** |
| Analysis of variance, F-value | | 453.5 | 194.6 | 150.9 | | *** | *** | *** |

Intermediate group large

| 1 - T. marmoratus ($Q_p < 0.1$) | 361 | 68.8 | 71.8 | 1.43 | 12 | *** | ** | *** |
| 2 - admixed ($0.1 < Q_p < 0.9$) | 80 | 62.9 | 67.7 | 5.61 | 23 | ** | *** | *** |
| 3 - T. pygmaeus ($Q_p > 0.9$) | 449 | 58.3 | 62.8 | 7.90 | 13 | *** | *** | *** |
| Analysis of variance, F-value | | 409.4 | 171.3 | 144.6 | | *** | *** | *** |

NS - not significant, ** - $P < 0.01$ and *** - $P < 0.001$

# To reduce the skewness of the character state distributions to marginally significant levels the data were transformed following the Box Cox procedure

Inferred for T. cristatus x T. marmoratus hybrids in France (Arntzen and Wallis, 1991).

The clines reconstructed for the transect in central Portugal are largely coincident (located at the same place) and congruent (of the same shape). Hybrid zones are frequently located in areas where populations are sparse and dispersal is impeded (i.e., ‘density troughs’, Barton and Hewitt, 1985), as may be the case here by the Tejo river. However, the course of the river is not exactly perpendicular to the transect and a more detailed survey over two spatial dimensions is in order to evaluate hybrid zone position as determined by the environment. The different profile of the mtDNA cline (Figure 7P) versus all other, nuclear markers is remarkable. With the knowledge that T. pygmaeus has been enlarging its range at the expense of T. marmoratus in the nearby coastal area of Portugal (Espregueira Themudo and Arntzen, 2007b) the most straightforward explanation is that T. marmoratus had a wider range in central Portugal too, was superseded by T. pygmaeus, but not without introgressive hybridization taking place (i.e. hybrid zone movement; Buggs, 2007). Seen like this, the southern mtDNA constitutes a ‘genetic footprint’ left behind by the receding species T. marmoratus. Modelling has shown that even limited hybridization can result in substantial introgression, when one species is competitively displacing the other (Currat et al., 2008). This genetic footprint is reflected in the cline by an increased width and a displaced, southern position. Mitochondrial DNA is more than nuclear loci prone to chance introgression due to its small effective population size and it is more prone to detection because it stays intact, due to its non-recombining nature (Toews and Brelsford, 2012). The hypothesis of hybrid zone movement can be tested by surveying a larger number of nDNA markers and search for further ‘genomic footprints of hybrid zone movement’ (Wielstra et al., 2017).

Marbled newt populations are infrequent in the Madrid area (García-París et al., 1989; Martínez-Solano, 2006). The two populations studied by Arntzen et al. (2014) are ca. 20 km apart, with no evidence for hybridization. It may here concern a residual hybrid zone with past but no present opportunities for hybridization and gene flow. Both species appear to suffer from regressing ranges in the east of the Iberian peninsula, as suggested by the isolated occurrences around Barcelona (T. marmoratus) and in Albacete province (T. pygmaeus). The southern fringe of
Figure 6. Geographical clines for morphological (left, panels A-E) and nuclear genetic data (right, F-H, K). Solid dots represent populations and the grey areas the 95% credibility interval. The parameters for formal cline descriptions are in Table 5. Note that the character transitions are mostly steep and located at the Tejo river. Clines are mostly coincident and congruent. The combination of characters (panels A, B and F) outperforms single characters (C-E and G, H, J, K) for species classification purposes. Morphological data - discriminant analysis (cf. Table 4) separates the species adequately, for individuals with species identity known (panel A), or unknown from genetic data (B). Note that in B the credibility interval is wider than in A and the $P_{\text{min}}$ and $P_{\text{max}}$ values are less differentiated. The character Links (panel E) outperforms SVI (C and D) in species diagnostics. In panels C and D note the preponderance of populations with intermediate SVI at the center of the transect, further analyzed in Table 6. Nuclear genetic data - the loci PepA and PepB outperform Ldh2, as they do in western Portugal (Espregueira Themudo and Arntzen, 2007). The locus PepD has a performance at par with PepA and PepB and accordingly discriminates species better in central than in western Portugal (see $\kappa$ values reported in Espregueira Themudo and Arntzen, 2007a). Population 100 is an outlier for the molecular data (panel F, see also Figure 3).
Castile and León remains to be studied. In the north of Extremadura the species contact is located at the lower altitudes of the Sierra de los Ángeles (south of Sierra de Gata), with *T. pygmaeus* at lower and *T. marmoratus* at higher altitudes. No obvious ecological correlate for hybrid zone position was found in eastern Portugal. I suggested that *T. marmoratus* and *T. pygmaeus* engage in a tension hybrid zone (sensu Barton and Hewitt, 1985) that locally follows the minimum spanning distance between fixed points at either side, these flanking positions being the Sierra de Gata in the east and the Tejo river in the west (Arntzen and Espregueira Themudo, 2008). The Tejo river as an approximate species border is followed over a length of at least 20 km. In the west of Portugal *T. marmoratus* and *T. pygmaeus* show more of a mosaic distribution, with no evidence for recent hybridization in the present data. The new morphological and molecular data confirm the presence of an isolated occurrence of *T. marmoratus* at the north of the Lisbon peninsula and show that the *T. pygmaeus* range follows the coastal zone up to Aveiro and the river Ave in the north, with local climate and soil type affecting the mutual species distribution (Espregueira Themudo and Arntzen, 2007ab; Arntzen and Espregueira Themudo, 2008). The diversity in environmental correlates to the joint species’ border illustrates the intricate and complicated relationship of hybrid zone position and the environment, the more so if the hybrid zone is, or has been moving northwards. Other congeneric Iberian amphibians and reptiles with to a large extent contiguous range borders show similar, but not coinciding, latitudinal bi- or tripartitions (eg. *Alytes* Wagler, 1829 - midwife toads; *Bulus* Wagler, 1830 – worm lizards) (Carretero et al., 2014). Several tripartitions along a longitudinal axis have recently come to light (*Pelodytes* Bonaparte, 1838 – parsley frogs; *Psammodromus* Fitzinger, 1826 – sand lizards). Yet other contiguous congenerics show a distribution mosaic with no particular axis dominating the distribution pattern (*Podaris* Wagler, 1830 – wall lizards). Given the observed disparity, spatio-temporal correlations between groups are unlikely to exist and evolutionary scenarios for the Iberian herpetofauna will be unique and taxon specific.

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**Online supplementary material**

*S1*. Allele frequencies at four nuclear loci over 56 populations of *Triturus marmoratus* and *T. pygmaeus* in France, Spain and Portugal. Data are in GenePop format.

*S2*. Marbled newt populations identified to the species with enzyme genetic data in .kml format, for visualization with e.g. Google Earth. Populations are identified as: large green dots - *Triturus marmoratus*, large red dots - *T. pygmaeus* and large grey dots - admixed. Data are as in Table 2.

*S3*. Marbled newt populations identified to the species with DNA sequence data in .kml format, for visualization with e.g. Google Earth. Populations are identifies as: small green dots - *Triturus marmoratus*, small red dots - *T. pygmaeus* and small grey dots - admixed. Data are as in Table 3.