Tracing species replacement in marbled newts

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Abstract

Secondary contact between closely related species can lead to the formation of hybrid zones, allowing for interspecific gene flow among taxa. Species replacement can take place if one of the species possesses a competitive advantage over the other, resulting in hybrid zone movement. This displacement may leave a genomic footprint across the landscape in the form of asymmetric introgression of selectively neutral alleles from the displaced to the advancing species. Hybrid zone movement has been suggested for marbled newts in the Iberian Peninsula, supported by the presence of a Triturus marmoratus stronghold surrounded by populations of the supposedly advancing T. pygmaeus in the northwest of the Lisbon Peninsula, i.e., an enclave. Moreover, a newly constructed two-species distribution model suggests that climate conditions following the Last Glacial Maximum may have favoured T. pygmaeus over T. marmoratus along the Atlantic coast. To test for the presence of a T. marmoratus genomic footprint in the area that may have witnessed species displacement, we developed and employed 54 nuclear SNPs and one mitochondrial DNA marker. We found no additional enclaves nor genetic traces of T. marmoratus in T. pygmaeus populations. Therefore, two main hypothesis arise in the absence of a genomic footprint: i) species replacement without hybridisation, either in allopatry or in sympatry under strong reproductive isolation; or ii) displacement with hybridisation where the footprint was eroded due to strong purifying selection. We predict testing for a genomic footprint north of the reported enclave could confirm that species replacement in the marbled newts occurred with hybridisation.

Introduction

Past population demographics shape present genetic structure (Avise, 2004). Population genetic methods unravelling current allelic patterns may thus provide insight into past and extant species distribution dynamics (Avise *et al.,* 1987; Crisci, Katinas, & Posadas, 2003). Hybrid zones, where genetically distinct populations meet and admix, are characterised by gene flow through time and space (Barton & Hewitt, 1985; Hewitt, 2001). These 'natural laboratories' are often established when recently diverged species meet, for example following secondary contact during range expansion of species from glacial refugia (Excoffier, Foll & Petit, 2009; Hewitt, 1988; Taberlet, Fumagalli, Wust-Saucy, & Cosson, 1998).

Spatio-temporal dynamics among hybridising taxa can result in the formation of enclaves (Buggs, 2007; Wielstra, Burke, Butlin, & Arntzen, 2017a). Enclaves form when the population of one species is surrounded by populations of a competing related species, becoming genetically isolated from the remainder of the receding species' range (Arntzen, 1978). Enclaves can therefore illustrate historical species replacement, particularly in ground-dwelling organisms with low dispersal capabilities. Moving hybrid zones may leave a specific spatial signature, in the form of a molecular genomic footprint (Scribner & Avise, 1993; Wielstra, 2019). As an advancing species spreads into a contact zone, neutral alleles may flow from the retreating to the invading taxon and introgression will be more pronounced in the advancing than in the receding species (Moran, 1981; van Riemsdijk, Butlin, Wielstra, & Arntzen, 2019). Asymmetric introgression may reflect neutral alleles left in the wake of the moving hybrid zone and eventually become geographically stable over time, as the genomic footprint is solely dependent on drift (Barton & Hewitt, 1985; Currat, Ruedi, Petit,

& Excoffier, 2008). Hence, introgression patterns of selectively neutral traits can be used to reconstruct the history of hybrid zones (Wielstra *et al.* , 2017b).

We previously documented an enclave and limited interspecific gene flow in the marbled newt *Triturus marmoratus* (Latreille, 1880) around Caldas da Rainha in the northwest of the Lisbon Peninsula, and suggested that the observation was best explained by the competitive advance of its sister-species, the pygmy marbled newt *T. pygmaeus*(Wolterstorff, 1905) (Espregueira Themudo & Arntzen, 2007; Wielstra, Sillero, Vörös, & Arntzen, 2014). Long-distance colonisation or human introduction seem unlikely to have originated the enclave, since the minimum distance to the contact zone exceeds the dispersal capacities of*Triturus* newts and there is no tradition of newt husbandry in Portugal (Espregueira Themudo & Arntzen, 2007). The potential for interspecific gene flow in these species allows exploring genomic signals and reconstructing hybrid zone movement (Arntzen, Wielstra, & Wallis, 2014). We here test for the presence of a genomic footprint of*T. marmoratus* in *T. pygmaeus* south of the enclave employing newly developed single-nucleotide polymorphism (SNP) markers with species diagnostic allele variants (Garvin, Saitoh, & Gharrett, 2010; Meilink, Arntzen, van Delft, & Wielstra, 2015).

Materials and methods

Sampling and DNA preparation

Fieldwork was carried out in the Lisbon Peninsula, where we searched for water bodies containing marbled newts. We specifically extended our search to include the Serra de Sintra mountains in the southwest of the peninsula, because of the observed presence of *T. marmoratus* at higher altitudes than *T. pygmaeus* in the northeastern section of the species' hybrid zone (Arntzen & Espregueira Themudo, 2008). Twenty-five populations were found in an area spanning over 2000 km^2, ranging from the Tejo River in the east and the south, the Atlantic Ocean in the south and the west, up to the city of Caldas da Rainha in the north, where our survey marginally overlapped with the area investigated by Espregueira Themudo *et al.* (2007). Adult and larval newts were captured by dip netting or with funnel traps. To reduce sampling bias e.g. towards siblings from particular breeding pairs (Goldberg & Waits, 2010) we made an effort to include all accessible sections of the water bodies. Tail tip tissue samples were collected and stored in 96% ethanol. The sampling was complemented with material from seven localities obtained earlier (Espregueira Themudo & Arntzen, 2007). DNA extraction of tissue samples followed the Kingfisher^{TM} (Thermo Scientific) and DNeasy extraction kit (Qiagen, Valencia, CA, USA) standard protocols.

SNP marker design

Species diagnostic SNPs were identified based on transcriptome data for one adult male *T. marmoratus* from San Pedro da Cova (coordinates 41.157 N, 8.496 W) and one adult male *T. pygmaeus* from Umbria, Serra de Monchique (coordinates 37.335 N, 8.506 W) that were sequenced at ZF-screens, Leiden on the Illumina HiSeq 2000 platform. The transcriptome libraries are available from Wielstra, McCartney-Melstad, Arntzen, Butlin, and Shaffer (2019), through the NCBI SRA at BioProject PRJNA498336 (*https://www.ncbi.nlm.nih.gov/bioproject/PRJNA498336*). Data filtering was carried out with Trimmomatic v0.36 (Bolger, Lohse, & Usadel, 2014) and the *de novo* transcriptome assembly with Trinity v2.5.1 (Grabherr *et al* ., 2011). SNP marker design followed the MIPs pipeline that encompasses advantages for targeted resequencing, including high specificity, a high level of multiplexing and no ascertainment bias (Hardenbol *et al.,* 2003; Niedzicka, Fijarczyk, Dudek, Stuglik, & Babik, 2016). The SNP design process followed van Riemsdijk, Butlin, Wielstra, and Arntzen (2019); for details, see Supplemental Information I. The *Xenopus tropicalis* (Gray, 1864) genome obtained from Biomart ENSEMBL (genome version JGI4.2) was selected as a reference. Both marbled newts' transcriptomes were blasted onto gene models of *X. tropicalis* in order to extract unambiguously mapping exon sequences for SNP discovery. SNP identification was performed in Mesquite v.3.40 after performing an alignment with Muscle v.3.8.31.

SNP detection and validation

SNP genotyping took place at the Institute of Biology Leiden / Naturalis SNP line facility using the Kompe-

titive Allele-Specific PCR (KASP) genotyping system (LGC genomics, UK). KASP is a fluorescence-based method determining the bi-allelic score of SNPs in uniplex assays. KASP is based on two allele-specific primers with a final base complementary to one of the two potential SNPs and unique tail sequence (Semagn, Babu, Hearne, & Olsen, 2014). The KASP master mix contains different fluorescent-labelled primers that become activated during PCR cycles, with the fluorescent signal increasing as more fluorescent primers are incorporated during the thermocycling of the PCR reaction. Primers were designed using the Kraken software and ordered from Integrated DNA Technologies (Wood & Salzberg, 2014).

For SNP validation, we used 120 *T. pygmaeus* and 118 *T. marmoratus* from 43 populations across the Iberian Peninsula, located outside the documented hybrid zone of these species (Arntzen, 2018; Figure 1 and Table S.1). The validation assay of 192 SNPs resulted in 147 markers being polymorphic, of which 81 were deemed species diagnostic for the reference samples. For the Lisbon Peninsula 354 individuals from 32 populations (25 new and 7 previously studied populations) were KASP genotyped for the 60 most promising nuclear SNPs. A further four primer sets were developed from the sequence information provided by Espregueira Themudo, Nieman, and Arntzen (2012), for the nuclear genes ß-Fibrinogen intron 7 (BF), Calreticulin intron C (CC) and Platelet-derived growth factor receptor α intron 11 (PDG) and for the mitochondrial gene NADH dehydrogenase subunit 4 (ND4).

Population genetics

Hardy-Weinberg equilibrium and genotypic disequilibrium among pairs of nuclear loci were tested with the R package GENEPOP v1.0.5, under the Benjamini-Hochberg correction (Benjamini & Hochberg, 1995; Rousset, 2008). Gene flow between genetically distinct populations produces admixture linkage disequilibrium among those loci that have different allele frequencies in the founding populations (Pfaff *et al.* , 2001). Admixture linkage disequilibrium was estimated following Barton & Gale (1993) and was based on 1,000 bootstrap replicates of the original dataset, using a script from van Riemsdijk, Butlin, Wielstra, and Arntzen (2019). The STRING v.10.5 protein-protein interaction network database (Szklarczyk *et al* ., 2015) was consulted to examine the functional linkage among the annotated nuclear markers, with reference to the *X. silurana* genome.

The SNP data were analysed with Structure software under the 'admixture model', given that neighbouring populations can interbreed (Pritchard, Stephens, & Donnelly, 2000; Falush, Stephens, & Pritchard, 2003). As the genotype pool could only be biallelic corresponding to the species to be analysed, the number of potentially differentiated gene pools was predetermined as K =2. The program was run for 100,000 generations after 100,000 generations of burn-in. Individuals were classified as pure *T. marmoratus* (Q $<$ 0.01), or pure *T. pygmaeus* ($Q > 0.99$) or admixed ($0.01 < Q < 0.99$).

Environmental modelling

Species distribution models were constructed by the comparison of presence-only data for both species under reference to contemporary climate conditions. The biological data employed were 108 *T. marmoratus* and *T. pygmaeus* records that were supported by molecular species identification (Arntzen, 2018; present paper). The records for three genetically admixed populations from Portugal and Spain and three *T. marmoratus* populations from France were excluded. Explanatory variables were the 19 climate parameters of WorldClim 2.0 (bio01-bio19; see Fick and Hijmans, 2017). To identify and subsequently reduce collinearity, we constructed a half-matrix of pairwise Spearman correlation coefficients (r_s). This matrix was subjected to clustering with UPGMA in PRIMER-e software (Clarke & Gorley, 2006). Parameters bio14 and bio15 were deemed unavailable for selection because of their regular high discrepancy between data sets (Varela, Lima-Ribeiro, & Terribile, 2015). Under the criterion of partial independence at r_s<0.7, the parameters retained were bio01, bio02, bio03, bio05, bio06, bio08, bio12 and bio17 (Supplementary Information II). Logistic regression analyses were performed with SPSS 20 (IBM SPSS, 2016) with 'species' as the dependent variable. Parameter selection was in the forward stepwise mode under the criteria of P_{in}=0.05 and P_{out}=0.10 under the likelihood ratio criterion. Model fit was assessed by the area under the curve (AUC) statistic. The resulting two-species distribution model was then applied to climate reconstructions for the mid-Holocene

and the Last Glacial Maximum (WorldClim version 1.4; Hijmans, Cameron, Parra, Jones, & Jarvis, 2005). In the absence of firm guidance of which climate reconstruction would be most appropriate to apply (Guevara, Morrone, & León-Paniagua, 2019), distribution models were derived for all of them (i.e. nine models for the mid-Holocene and three models for the Last Glacial Maximum). Distribution models were visualized with ILWIS (ILWIS, 2009).

Results

SNPs were considered species-diagnostic for 55 out of 64 markers, with missing data amounting to 2.7% (Table S.2). Significant deviations from Hardy-Weinberg equilibrium (heterozygote deficit) were found four times in four populations, involving the loci BF, mrpl41 and sostdc1. Per population deviations were significant three times in populations 10, 13 and 21. No significant pairwise linkage disequilibrium was detected per locus pair or population. Admixture linkage disequilibrium was significant for population 13. The water bodies involved in significant instances of heterozygote deficit and admixture linkage disequilibrium were highly isolated and had small dimensions (< 3 m^2).

Protein functions were described for the 47 markers that could be annotated (Table S.3). Interactions were uncovered among nine marker pairs; however, these markers do not appear to be co-expressed or involved in significant deviations from Hardy-Weinberg equilibrium. All markers were included in downstream analyses, as they appear to be physically and functionally unlinked.

Structure classified 20 individuals from two populations (nos. 1 and 2) as admixed and 334 individuals from 30 populations (nos. 3 - 32) as *T. pygmaeus* (Table S.4). For mtDNA, the SNP allele representing the *T. marmoratus* haplotype was found in populations 1 and 2, and the *T. pygmaeus* allele was found in all other populations. Allelic profiles consistent with enclave formation or genomic footprints were not observed (Table S.4). Accordingly, the genetic signature of *T. marmoratus* was restricted to the previously documented enclave and displayed notable levels of introgression with *T. pygmaeus*(Figure 1).

The selected two-species distribution model is represented by the logistic equation P_m=(1/(1+exp(-0.156*Bio17+7.767), in which P_m is the probability for the presence of *T. marmoratus* at the locality investigated, on a zero to unity scale and bio17 is 'precipitation of driest quarter'. Model fit is AUC=0.931+-0.025. The model describes more arid summer conditions for *T. pygmaeus* (mean precipitation over 60 localities = 36.4 mm) than for *Triturus marmoratus* (mean precipitation over 48 localities = 67.4 mm). The spatial interpretation of the model is shown in Figure 2A. Temporal extrapolations of the model (or 'hindcasts') are in Figure 2B for climate conditions of the Mid Holocene and in Figure 2C for the Last Glacial Maximum.

Discussion

We employed a large number of presumably unlinked neutral markers to test for species replacement of marbled newts in Portugal. Northwards hybrid zone movement has been suggested in this system, with the reported *T. marmoratus* enclave signalling the competitive advance of *T. pygmaeus* with incomplete replacement (Wielstra,Burke, Butlin, & Arntzen, 2017a). Species displacement might be driven by the desertification of the region, conferring *T. pygmaeus* a competitive advantage over its sister species. It is known *T. pygmaeus* thrives in ephemeral water bodies, and environmental modelling here suggests it is favoured by arid precipitation regimes, whereas *T. marmoratus* prefers smaller and more permanent breeding sites under more humid conditions (Espregueira Themudo & Arntzen, 2007; Harrison & Rand, 1989). In fact, climatic simulations also suggest the North Atlantic jet stream, associated with wetter conditions, was at its southernmost position during the Last Glacial Maximum, and it experienced a positive latitudinal shift after this period, parallel to *T. marmoratus* ' estimated northwards retreat (Beghin *et al.,* 2016). Unidirectional introgression of functionally and physically unlinked neutral markers was expected in the wake of a moving hybrid zone (Wielstra, Burke, Butlin, & Arntzen, 2017a). Yet, we found no evidence for an additional enclave or a genomic footprint consistent with enclave formation. Thus, two main biogeographic scenarios of enclave formation arise in the absence of a footprint: i) the species could have undergone negligible introgression or ii) displacement could have in fact occurred with introgressive hybridisation, but with the signal subsequently lost.

Species replacement in the absence of locally extensive hybridisation during *T. pygmaeus'* expansion could be responsible for enclave formation unaccompanied by a genomic footprint. Displacement might have occurred in allopatry, involving an initial recession of *T. marmoratus'* range during the Pleistocene glaciations, followed by a northwards postglacial expansion of *T. pygmaeus* . Under this scenario, the species would not have been in contact across their western ranges until meeting at the current hybrid zone. The *T. marmoratus* enclave would have locally persisted during the retreat of its main range, alike the fragmenting pattern of contracting species ranges described by Wilson, Thomas, Fox, Roy, and Kunin (2004), with *T. pygmaeus* later enveloping the pocket. However, it seems unlikely the marbled newts did not meet after the Last Glacial Maximum, given the similarity in suitable habitats predicted by the environmental modelling.

Alternatively, species replacement may have taken place sympatrically, with strong reproductive isolation preventing introgressive hybridisation. Prezygotic and postzygotic barriers could be limiting hybridisation among the species, resulting in reproductive isolation. Prezygotic effects, such as mating preference or genetic incompatibility, have not been reported in *Triturus* species, whereas postzygotic effects occur between crested and marbled newts, brought by the direction of the interspecific cross (Arntzen, Jehle, Bardakci, Burke, & Wallis, 2009). The northwards advance of the contiguous species ranges could therefore have been driven by species replacement without introgressive hybridisation, with the enclave likely persisting under *T. marmoratus'* presumed strong adaptation to local conditions.

The scenario of species replacement with hybrid zone movement is derived from previous findings supporting a moving contact zone between the marbled newts (Espregueira Themudo & Arntzen, 2007; Espregueira Themudo, Nieman, & Arntzen, 2012). Assuming widespread interspecific hybridisation, the erosion of *T. marmoratus'* molecular signal would explain the absence of a genomic footprint. The time passed since *T. marmoratus* inhabited the Lisbon Peninsula, environmental effects, and the competitive advance of *T. pygmaeus* might have led to the erosion of the footprint. Additionally, strong selection against hybrids, as suggested by the strong bimodality of the species' contact zone with few admixed individuals in Arntzen (2018), could have swiftly erased the footprint. Purifying selection could have operated to maintain the species' functional integrity, by eliminating deleterious sequences being pulled into the receding taxon (Oleksyk, Smith, & O'Brien, 2010). Under strong purifying selection, existing introgression of neutral variants might not have been detected despite the large number of markers here employed. In fact, the significant instances of heterozygote deficit and admixture linkage disequilibrium are likely due to a combination of incomplete lineage sorting or ancestral polymorphism and relatedness amongst the sampled larvae, resulting from the collection of siblings that can potentially bias the landscape genetic structure of populations (Goldberg & Waits, 2010).

The proposed biogeographic scenarios of species replacement are subject to the unknown past position of the species boundary. While environmental modelling supports *T. marmoratus* inhabiting the study area, it remains possible that this species did not previously inhabit the Lisbon Peninsula. Moreover, the absence of an additional enclave or disconnected footprints in mountainous areas, such as Serra de Sintra, does not clarify *T. marmoratus'* past range nor whether species replacement occurred with or without hybridisation. If in the past, the southernmost range of *T. marmoratus* included Caldas da Rainha, enclave formation could have occurred via incomplete species replacement. Thus, testing for a genomic footprint north of the enclave could potentially elucidate among the aforementioned biogeographic scenarios. We expect future studies on the area between the *T. marmoratus* enclave and its main distribution range to unravel a genomic footprint of species replacement with hybridisation, confirming the dynamic nature of the hybrid zone movement between the marbled newts.

Remarkably, the enclave in Caldas da Rainha showed notable levels of introgression, possibly signalling the beginning of its erosion, as predicted by Espregueira Themudo & Arntzen (2007), and further illustrating the dynamics of species replacement. The enclave is expected to eventually disappear under *T. pygmaeus'* competitive advance, leading to the loss of *T. marmoratus* gene variants over time. The expansion of *T. pygmaeus* is likely influenced by the loss of breeding sites in southern Spain and Portugal, an area with unique biodiversity patterns stricken by a decline in temporary ponds, driven by climate warming, desertification and

agricultural intensification (Arntzen *et al.* , 2004; Thomas, Franco, & Hill, 2006; van de Vliet *et al.* , 2014). Understanding shifts in species distributions, particularly when driven by climate change and anthropogenic activities, therefore becomes especially relevant in deciphering the dynamics of species replacement (Taylor, Larson, & Harrison, 2015).

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Figure legends

Figure 1: The Iberian Peninsula with records of *Triturus marmoratus* (solid round symbols) and *T. pygmaeus* (open round symbols). A – Localities sampled for the evaluation of SNP-marker diagnosticity. For details see Supplementary Information S1. The boxed area includes the Lisbon Peninsula and the Caldas da

Rainha area. B – Localities sampled in the Lisbon Peninsula, with symbols as above. For details see Supplementary Information S2. The continuous distribution with *T. marmoratus* in dark grey and *T. pygmaeus* in light grey is from Arntzen *et al.* (2009) and Arntzen (2018). Note the existence of a *T. marmoratus* enclave around Caldas da Rainha. Localities codes are C for Caldas da Rainha, L for Lisbon and S for the Serra de Sintra.

Figure 2: Two-species distribution models for the newts *Triturus marmoratus* and *T. pygmaeus* over the Iberian Peninsula, derived from the climatic variable 'precipitation of driest quarter'. A – present day. The colour legend shows the inferred probability for the presence of *T. marmoratus* (blue) and *T. pygmaeus* (P_m=0, red). Intermediate colours represent intermediate probabilities. The light shaded area falls outside the *Triturus* range (see Figure 1). B – distribution models over the western part of the Iberian Peninsula for the climate conditions of the Mid Holocene. Inferred species ranges are shown in grey (P_m>0.5) and in white (P_m<0.5). Model representation is binary and cumulative, so that the stepped grey scale represents the number of models supporting the presence of *T. marmoratus* , from zero to nine. C – as in B, for three climate reconstructions at the Last Glacial Maximum. Note that most models support the contiguous species border to be more or less stable, whereas one model supports a more southern species border during the Late Glacial Maximum.

Data accessibility

Supplemental text contains details of the SNP design and collinearity analysis:

EcolEvol_Supplementary_Information_Lopez-Delgado_2020.pdf

Supplemental tables are available in:

EcolEvol_Supplementary_Tables_Lopez-Delgado_2020.xlsx

Competing interests

The authors declare no competing interests.

Author contributions

JWA designed the study. JLD and JWA collected the material. JLD performed the bioinformatics analyses under supervision of IvR. JLD analysed the data and wrote the manuscript, with input from both co-authors.

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