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Original research article

Genetic pollution risk in newts at the far end of the speciation continuum

Anagnostis Theodoropoulos[a,b,1,*], Aziz Avcı[c,2], Daniel Fernández-Guiberteau[d,3], Albert Ferran[e], Kurtuluş Olgun[c,4], Nazan Üzüm[c,5], Salvador Carranza[f,6], Ben Wielstra[a,b,7]

[a] Institute of Biology Leiden, Leiden University, P.O. Box 9505, Leiden 2300 RA, the Netherlands
[b] Naturalis Biodiversity Center, P.O. Box 9517, Leiden 2300 RA, the Netherlands
[c] Department of Biology, Faculty of Science, Aydın Adnan Menderes University, Efeler, Aydın 09010, Türkiye
[d] Centre de Recerca i Educació Ambiental de Calafell (CREAC-GRENP-Ajuntament de Calafell), Tarragona, Spain
[e] Servei de Gestió de Parcs Naturals. Diputació de Barcelona, Barcelona, Spain
[f] Institute of Evolutionary Biology (CSIC-Universitat Pompeu Fabra), Barcelona 08003, Spain

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ABSTRACT

Invasive species can erase native biodiversity through genetic pollution: the diminishing of native genetic variation via anthropogenic hybridization. We investigated hybridization between the invasive crested newt *Triturus anatolicus* and the native marbled newt *T. marmoratus* in Montnegre i el Corredor Natural Park (Catalonia, Spain). Using genome-wide target sequence capture (NewtCap) and mitochondrial DNA barcoding, we determine hybrid viability, hybrid class composition, and hybridization directionality, and compared invasive genotypes to reference populations across the Turkish range to infer provenance. We detect numerous F1 hybrids and a relatively high frequency of backcrosses compared to the natural marbled and crested newt hybrid zone in France. All F1 hybrids carry *T. anatolicus* mitochondrial DNA, indicating asymmetric hybridization with *T. anatolicus* mothers. Later-generation hybrids demonstrate hybrid fertility. All later-generation hybrids are products of advanced crosses with *T. marmoratus*. Nuclear and mitochondrial data trace the invasive population to Lake Abant in northwestern Türkiye. The observed backcrossing toward the native species indicates that invasive alleles can seep into the native gene pool, meaning the prerequisites for genetic pollution are in place. Our study underscores the importance of timely genome-wide monitoring when a closely related species is introduced into the range of a native species, to inform conservation management in order to prevent the erosion of native genetic diversity.

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* Corresponding author at: Institute of Biology Leiden, Leiden University, P.O. Box 9505, Leiden 2300 RA, the Netherlands.
E-mail address: a.theodoropoulos@biology.leidenuniv.nl (A. Theodoropoulos).

[1] 0009-0003-0272-6358
[2] 0000-0001-8017-9714
[3] 0009-0003-8138-5917
[4] 0000-0002-9782-5492
[5] 0000-0003-4421-4302
[6] 0000-0002-5378-3008
[7] 0000-0002-7112-5965

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1. Introduction

Invasive species are widely considered to be one of the main drivers of global biodiversity loss (IPBES, 2023; Seebens et al., 2025). The negative impacts of invasive species are well documented and occur across all levels of biological organization, contributing to the extinction of native biodiversity and promoting long-term global homogenization of biodiversity (Bellard et al., 2016; Pyšek et al., 2020). However, the consequences of hybridization with invasive species on native species' gene pools remain relatively understudied compared to impacts at the ecological and ecosystem level (Draper et al., 2021; Petit, 2004; Rhymer and Simberloff, 1996; Theodoropoulos et al., 2025).

Invasive species are regularly able to hybridize with their native congeners in a process called anthropogenic hybridization (Huxel, 1999; Mcfarlane and Pemberton, 2019). Anthropogenic hybridization may result in hybrid offspring with reduced fitness, thereby wasting native reproductive effort and imposing demographic and genetic costs (Barton and Hewitt, 1989; Kleindorfer et al., 2014; Mayr, 1963; Ottenburghs, 2021). However, accumulating evidence shows that hybrid offspring could also exhibit fitness comparable to, or even exceeding, that of their parental species (Arnold and Hodges, 1995; Mallet, 2005; Porretta and Canestrelli, 2023). Under these conditions, hybridization can result in hybrid swarms (mixed populations, dominated by interbreeding hybrids), and, in extreme cases, genetic swamping (replacement of native genomes by invasive ones through repeated backcrossing), causing the loss of genetic integrity of the native species (Abbott et al., 2013; Barton, 2001, 1989; Cockayne and Allan, 1926; Mallet, 2007; Payseur and Rieseberg, 2016; Porretta and Canestrelli, 2023; Tensen and Fischer, 2024; Todesco et al., 2016).

Anthropogenic hybridization can permanently alter the genetic composition of native populations in a process often referred to as genetic pollution (Asztalos et al., 2021; Dubois and Morere, 1980; Dufresnes et al., 2016; Meilink et al., 2015; Rhymer and Simberloff, 1996; Theodoropoulos et al., 2025; Wielstra et al., 2016). In this process, native alleles are overwritten by invasive alleles, altering the genetic integrity of native populations (Rhymer and Simberloff, 1996; Todesco et al., 2016). Through repeated backcrossing and introgression, invasive alleles can spread within the affected gene pool, reshaping population genetic makeup over time (Barton, 2020; Seehausen et al., 2014). Ultimately, genetic pollution is biodiversity loss at the level of the gene, a form of cryptic extinction that leads to the erasure of native, locally adapted alleles (Huxel, 1999; Petit, 2004).

A series of amphibian introductions documented in Catalonia, Spain, provide excellent case studies of the negative impacts of invasive species. These introductions involve multiple non-native newt taxa, including Anatolian crested newts (*Triturus anatolicus*), alpine newts (*Mesotriton alpestris* and *M. apuanus*), Bosca's newt (*Lissotriton boscai*) and banded newts (*Ommatotriton ophryticus* × *O.*

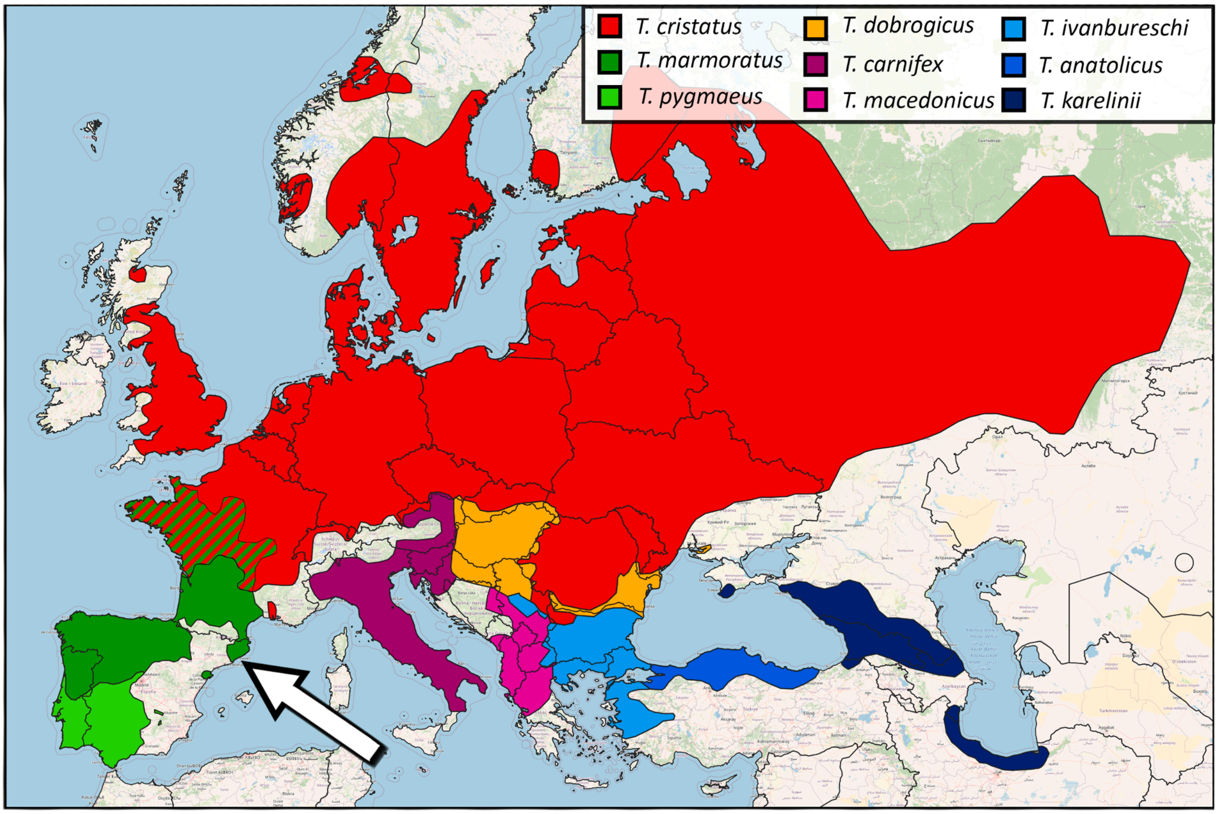

Fig. 1.. The distribution of the marbled and crested newt genus *Triturus*. The species in green are marbled newts and the remaining ones are crested newts. Note the partially overlapping ranges of the crested newt *T. cristatus* and the marbled newt *T. marmoratus* where hybridization occurs. The location of the study area is indicated with a white arrow.

nesterovi hybrids) (Carranza and Fernandez Guiberteau, 2018; Fibla et al., 2015; Fontelles et al., 2011; Martel et al., 2020; Martínez-Martínez, 2017; van Riemsdijk et al., 2018). The *Triturus* case in particular raises the conservation concern of genetic pollution, because the introduced crested newt (*T. anatolicus*) can be expected to hybridize with a native congener, the marbled newt (*T. marmoratus*).

The presence of non-native *Triturus* within Montnegre i el Corredor Natural Park was first reported in February 2017 from the pond of Can Maresme (Sant Iscle de Vallalta), based on an individual photographed by a private citizen. Subsequent surveys of nearby ponds revealed additional individuals, with the largest numbers detected in the pond of Ca l'Oller, and smaller numbers in nearby sites such as Coll Senís. At the Ca l'Oller pond, a further conservation concern emerged: putative hybrid individuals between the introduced *T. anatolicus* and the native marbled newt *T. marmoratus* were detected, based on intermediate morphology (Carranza and Fernandez Guiberteau, 2018; Martínez-Martínez, 2017).

Approximately ten species of marbled and crested newts are currently recognized (Arntzen, 2024b; Fahrbach and Gerlach, 2018; Wielstra et al., 2021) (Fig. 1). Marbled newts and crested newts belong to the same genus and form two deeply divergent lineages, with their most recent common ancestor being dated to approximately 24–30 million years ago (Steinfartz et al., 2007; Stewart and Wiens, 2025; Wielstra et al., 2019). These two lineages meet in a well-characterized natural hybrid zone in France, involving the northern crested newt, *T. cristatus,* and *T. marmoratus* (Fig. 1). Both *T. anatolicus* and *T. cristatus* belong to the crested newt lineage and are equally distantly related to the marbled newt, *T. marmoratus* (Wielstra et al., 2019). Therefore, the outcome of anthropogenic hybridization in the Catalonian newt case can be directly compared to the natural crested and marbled newt hybrid zone.

The natural hybrid zone is bimodal, indicating strong selection against hybrids. Hybrids form only a fraction of the adult population (~3–4%) and consist mostly of F1 individuals (Arntzen et al., 2021, 2009, 2026). Hybridization is highly asymmetric, with most F1 hybrids (~90%) being crested-mothered (Arntzen et al., 2021, 2026). F1 hybrids also exhibit skewed sex ratios: crested-mothered hybrids are predominantly female, consistent with Haldane's rule (Haldane, 1922), whereas marbled-mothered hybrids are almost exclusively male, presumably due to cytonuclear incompatibilities between the *T. cristatus* X chromosome and *T. marmoratus* cytoplasm (Arntzen et al., 2009). A small proportion (~4%) of F1 hybrids are triploids (Arntzen et al., 2026). Deeper-generation hybrids are rare, comprising only ~2–3% of hybrids overall, concerning backcrosses toward both *T. marmoratus* and *T. cristatus* (Arntzen et al., 2021, 2026). Interspecific gene flow is extremely limited and mainly occurs toward *T. cristatus* (Arntzen et al., 2021; Arntzen, 2024a).

Anthropogenic secondary contact between highly divergent species provides an excellent opportunity to study the potential of genetic pollution at the far end of the speciation continuum. We use genome-wide target capture data and mtDNA barcoding to resolve the extent, dynamics and conservation implications of hybridization between the invasive *T. anatolicus* and native *T. marmoratus* in Montnegre i el Corredor Natural Park and compare the outcome of this anthropogenic hybridization with that observed in the natural crested and marbled newt hybrid zone in France. Furthermore, we trace the source population of the *T. anatolicus* introduced into the park within the native Turkish range. Our study shows how genome-wide data can provide an early warning of genetic pollution, helping to detect the preconditions of interspecific gene flow before it impacts native biodiversity.

2. Materials and methods

2.1. Sampling methodology, DNA extraction and concentration measurement

We examined 45 individuals from Montnegre i el Corredor Natural Park (Barcelona, Spain), collected between 2017 and 2022 (Table S1). Additionally, DNA samples from 65 *T. anatolicus* individuals, from 27 localities throughout the natural distribution range, were obtained from a previous study (Wielstra et al., 2017). DNA extractions were performed using a salt-based extraction protocol (Sambrook and Russell, 2001) with the Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA. The DNA yield and purity were measured with a Lunatic UV/Vis Absorbance Spectrophotometer (Unchained Labs, Pleasanton, CA, USA). Samples with concentrations lower than minimum of 150 ng/μl were concentrated using a Concentrator Plus (Eppendorf, Hamburg, Germany).

2.2. Library preparation and target enrichment

We followed the NewtCap protocol (de Visser et al., 2025a) and prepared libraries at quarter volume using the NEBNext Ultra™ II FS DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA). Unique i5 and i7 primers (Integrated DNA Technologies, Leuven, Belgium) were employed for indexing. NucleoMag™ magnetic separation beads (Macherey-Nagel, Düren, Germany) were utilized for 300 bp size selection. Library concentration and fragment size were measured using the 5300 Fragment Analyzer System (Agilent Technologies, Santa Clara, CA, USA).

Libraries were pooled into batches of 16 samples with a total mass of 4000 ng of DNA (250 ng per sample) and target captured using the MyBaits v5.0 kit (Arbor Bioscience, Ann Arbor, MI, USA) with RNA probes designed to target 7139 exon sequences (product Ref: # 170210–32; Wielstra et al., 2019). The concentrations and fragment sizes of the enriched pools were assessed using the Agilent 4150 TapeStation (Agilent Technologies, Santa Clara, CA, USA). Pools were subjected to 150 bp paired-end sequencing (1 GB per sample) on an Illumina NovaSeq 6000 platform (Illumina Inc., San Diego, CA, USA) at BaseClear B.V. (Leiden, Netherlands).

2.3. Post processing and filtering

Sequence data were processed following the NewtCap pipeline (de Visser et al., 2025a), which includes the following steps: clipping reads to a length of 150 bp using BBDuk (BBMap -Bushnell B. - https://sourceforge.net/projects/bbmap/), removing low-quality reads and adapters with Trimmomatic (Bolger et al., 2014), conducting quality checks before and after trimming using FastQC (Andrews, 2010), mapping reads to a reference set of 7139 *T. dobrogicus* sequences using BWA_MEM (Li, 2013), deduplicating reads using Picard v.2.25. (http://broadinstitute.github.io/picard/), calling variants using HaplotypeCaller and creating multisample (ms)gVCF files using CombineGVCFs and GenotypeGVCFs in GATK v.4.1.3.0 (van der Auwera and O'Connor, 2020). Two msgVCF files were created, a file that comprised all individuals collected in Spain, called the “Catalan set”, and a file that comprised all the pure *T. anatolicus* individuals from Spain and the native *T. anatolicus* individuals from Türkiye, called the “Provenance set”.

The resulting msgVCFs files were filtered for potential paralogous targets by removing variant sites with heterozygote excess ($p < 0.05$) using BCFtools v.1.15.1 (Danecek et al., 2021). We filtered for INDELs, depth quality, mapping quality, Fisher’s strand bias, systematic sequencing bias, systematic artifacts, variant quality, genotype quality, minor allele frequency and percentage of missing data. The “Provenance set” dataset was filtered to discard sites with over 50% missing data, whereas the “Catalan set” was filtered to discard all sites with missing data and randomly maintain a single of the remaining SNPs per marker. Summary statistics were calculated using a custom Bash pipeline integrating SAMtools v.1.18 (Li et al., 2009), VCFtools v.0.1.16 (Danecek et al., 2011), and R v.4.4.1 (R Core Team, 2024).

2.4. Assessing ancestry, hybrid class and ploidy for the “Catalan set”

The “Catalan set” was converted to GDS format using the snpgdsVCF2GDS function, and a PCA was performed with default settings using the SNPRelate package (Zheng et al., 2012) in R v.4.4.1 (R Core Team, 2024). A total of 5895 SNPs were included in the analysis. The maximum likelihood of individual ancestries from the same dataset were estimated using ADMIXTURE v1.3.0 (Alexander et al., 2009). The analysis was run for 25 replicates, assuming two ancestral genetic populations (K=2), i.e., the two parental species. The output was summarized using CLUMPAK (Kopelman et al., 2015) and visualized with the ggplot2 R package (Wickham, 2016).

To assign individuals to hybrid classes, we used the *vcfr* (Knaus and Grünwald, 2017) and *triangulaR* packages (Wiens and Colella, 2024) in R v4.4.1 (R Core Team, 2024). From the total of 5895 SNPs, the R package identified 3023 SNPs that are reciprocally fixed between the parental species. Hybrid indices and interclass heterozygosity were calculated and visualized results using a triangle plot. Hybrid classes were further evaluated using simulated crosses generated from parental individuals with the recom-sim hybridization tool (Nielsen et al., 2006; https://github.com/salanova-elliott/recom-sim). In the simulations, POP1 corresponds to *T. anatolicus* and POP2 corresponds to *T. marmoratus*. Cross notation follows the structure implemented in recom-sim: for example, B2POP1 denotes an F1 × POP1 cross, and B3POP1 denotes B2POP1 × POP1. More complex classes (e.g., (B2POP2 × POP1) × POP2) represent explicit crosses between previously simulated hybrid classes and parental populations. The number of possible crosses increases exponentially and quickly becomes intractable. All unique possible crosses were simulated up to the fourth generation. For the fifth, sixth, and seventh generations, crosses were simulated selectively, to ensure broad coverage of the hybrid index–heterozygosity space, with emphasis on combinations that would fit the empirical data (Table S2). For most crosses, ten individuals were simulated, whereas crosses of those that closely fitted the empirical data were simulated with 100 individuals.

To confirm that hybrid ancestry of individuals highlighted as putative backcrosses was not misinterpreted due to polyploidy (van Veldhuijzen et al., 2025), we used nQuire (Weiß et al., 2018), a statistical framework that can distinguish diploid from triploid and tetraploid individuals. The BAM files from the “Catalan set” were processed to generate.bin files using the nQuire create function, followed by noise reduction with nQuire denoise. The ploidy model likelihoods were then inferred using the lrdmodel command across both raw and denoised datasets. Histograms of allele frequency distributions were extracted and plotted for visual inspection in R v.4.4.1 (R Core Team, 2024).

2.5. Sex genotyping

We performed a multiplex PCR to determine the sex of the “Catalan set”. Specifically, we amplified a Y-linked marker using the ‘TiY−384959-short’ primer pair (France et al., 2025a) and included the autosomal CDK−17 marker (Meilink et al., 2025) as a control. PCR amplification using the QIAGEN Multiplex PCR Master Mix (QIAGEN B.V, Venlo, Netherlands) and gel electrophoresis were performed according to France et al. (2025a).

Sex ratios were evaluated separately for the parental species (*T. anatolicus* and *T. marmoratus*) and hybrids. For the hybrids, individuals were further separated into F1 and backcross classes (see Results). For each group, we tested whether the observed sex ratio deviated from the expected 1:1 ratio under the null hypothesis of equal male and female probabilities ($p = 0.5$), using exact binomial tests in R v.4.4.1 (R Core Team, 2024).

2.6. MtDNA analysis

The KARF4-KAR1 primer pair was used to amplify a 658 bp fragment of the NADH dehydrogenase 4 (ND4) mtDNA (Wielstra et al., 2013). PCR amplification was performed using the QIAGEN Multiplex PCR Master Mix (QIAGEN B.V, Venlo, Netherlands), with an annealing temperature of 55°C according to de Brouwer et al. (2022). Sanger sequencing was outsourced to BaseClear B.V. (Leiden, Netherlands) and sequences were edited and trimmed using Geneious Prime 2023.2.1 (https://www.geneious.com). The ND4 sequences were matched against a large database of all known *Triturus* ND4 haplotypes compiled from previous studies (de Brouwer et al., 2022), using ‘DNA to haplotype collapser and converter’ function in FaBox (Villesen, 2007). A Maximum Likelihood phylogenetic tree was constructed using the RAxML plugin in Geneious Prime 2023.2.1 (Stamatakis, 2006). We partitioned sequences by codon position, applied the GTR + GAMMA substitution model and conducted 1000 bootstrap replicates.

To test whether hybridization between parental species occurred symmetrically, mitochondrial haplotype frequencies were analyzed for F1 and backcross hybrids separately. Within each hybrid class, the observed number of individuals carrying *T. anatolicus* versus *T. marmoratus* mtDNA was compared against a null expectation of equal maternal contribution (1:1 ratio), using exact binomial goodness-of-fit tests in R v.4.4.1 (R Core Team, 2024).

2.7. *Provenance of invasive species*

To investigate the genetic relationships between introduced and native *T. anatolicus* individuals included in the “Provenance set,” we performed both a principal component analysis (PCA) and a hierarchical clustering analysis. The PCA followed the same approach described for the “Catalan set”. For the hierarchical clustering analysis, identity-by-state distances were calculated using the SNPRelate package (Zheng et al., 2012) in R v4.4.1. Pairwise IBS values were computed from the GDS-formatted SNP dataset, and a dissimilarity matrix was generated using the snpgdsIBS function. Clustering was then performed with snpgdsHCluster and refined using snpgdsCutTree, resulting in a dendrogram used to visualize individual clustering patterns. Both analyses were based on 76,604 SNPs.

3. Results

3.1. *Target capture performance*

Sequencing produced an average of 10.1 million read pairs per sample (SD = 5.8 million), of which 47% (SD = 9%) successfully mapped to the reference set of targeted loci. Among the mapped reads, 44% (SD = 21%) were identified as PCR duplicates and filtered out. After deduplication, the median coverage across 100 bp windows ranged from 14 × to 188 × , with a mean of 91 × (SD = 38.6 ×) (Table S1).

3.2. *Hybridity of Catalan samples*

The PCA separates the two parental species along the first eigenvector (Fig. 2). Most hybrid individuals are positioned right in between the parentals, consistent with them being first generation hybrids, but four are positioned towards *T. marmoratus*. The second eigenvector separates the four ‘outlier’ hybrid individuals into two distinct groups (7349, 7287 and 7348, 7288). These individuals are interpreted as later-generation hybrids.

The ADMIXTURE analysis reveals 12 genetically pure *T. anatolicus* individuals, seven genetically pure *T. marmoratus* individuals,

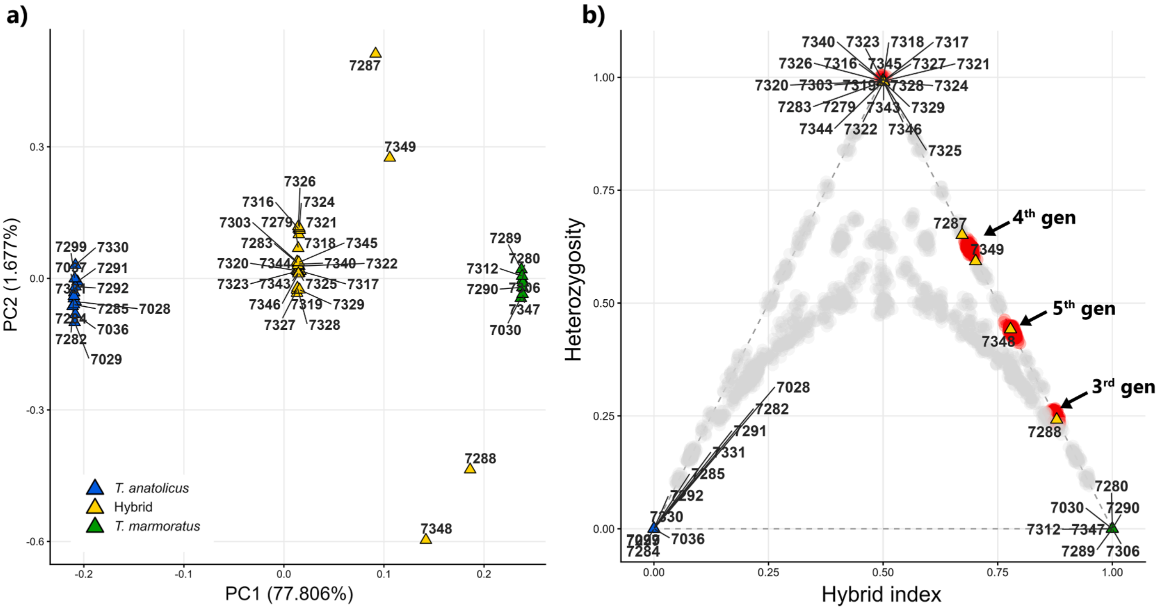

Fig. 2.. Principal Component Analysis and Heterozygosity/Hybridity analysis of *Triturus* newts sampled in Catalonia. (a) PCA plot displaying PC1 vs. PC2 (b) The hybrid index (the proportion of alleles inherited from the two parental species) ranges from 0 to 1, i.e., from pure *T. anatolicus* to pure *T. marmoratus* ancestry and the interclass heterozygosity (the proportion of loci heterozygous for parental alleles) ranges from 0 to 1; an F1 hybrid is therefore expected at the top center of the triangle. Each triangle corresponds to a single individual, colored according to species identity and labelled with its individual ID (Table S1). Simulated hybrid individuals are shown in grey, with the simulated classes fitting the F1 hybrids and the four outlier hybrid individuals highlighted in red.

and 22 F1 hybrids with approximately equal ancestry proportions from both parental species (Q-score ≈ 0.5) (Fig. 3a). Additionally, the four individuals identified as outliers in the PCA (Fig. 2) exhibit ancestry proportions that deviate from the F1 hybrid expectation, with ancestry leaning towards *T. marmoratus* (Q-scores between 0.67 and 0.88), in line with them being later-generation hybrids (Fig. 3a).

In the interclass heterozygosity versus hybrid index analysis, the two parental species cluster in the opposite bottom corners and the inferred F1 hybrids in the top corner (Fig. 2b). F1 hybrids are positioned slightly below the top corner, presumably reflecting the several dozens of markers that are situated on non-homologous 1 A and 1B regions of the *Triturus* genome (de Visser et al., 2025b; France et al., 2025b), which effectively behave as hemizygous markers (Meilink et al., 2025).

The four outlier individuals are positioned along the *T. marmoratus* side of the triangle (Fig. 2b). Their combinations of hybrid index and interclass heterozygosity point to more advanced backcross classes. Individual 7288 best fits a third-generation backcross class (B3POP2), with a hybrid index of 0.879 and heterozygosity of 0.242. Individual 7348 best fits a fifth-generation hybrid class ((B3POP1xPOP2) x POP2), with a hybrid index of 0.778 and heterozygosity of 0.442. Individuals 7287 and 7349 fit a fourth-generation hybrid class ((B2POP2xPOP1) x POP2), with hybrid indices of 0.672 and 0.702 and heterozygosities of 0.651 and 0.593; their displacement from the simulated class centroid presumably reflects stochastic variation resulting from recombination. In all cases, the assigned classes represent the minimum number of generations that would be required to produce the observed genomic composition.

The nQuire analysis confirms that all hybrid individuals in the study are diploid (Figure S1).

3.3. Sex genotyping

Sex determination by multiplex PCR (Fig. 3b) reveals six males out of 12 *T. anatolicus* (50.0%) and four males out of seven *T. marmoratus* (57.1%), with no significant deviation from a 1:1 sex ratio (exact binomial tests, $p = 1.00$ and $p = 1.00$). Among hybrids, F1 individuals show 11 males out of 22 (50.0%) with no significant deviation from a 1:1 sex ratio ($p = 1.0$). Although, backcross hybrids were all female (0 males out of four; 0.0%), given the small sample size this deviation was not statistically significant ($p = 0.125$).

3.4. Maternal versus paternal species analysis

All 22 F1 hybrid individuals carry *T. anatolicus* mtDNA, a significant deviation from an expected 1:1 ratio of equal maternal contribution (exact binomial test, $p < 0.000001$). For the later-generation hybrids, three individuals (7287, 7248 and 7249) carry *T. anatolicus* mtDNA and the fourth individual (7288) carries *T. marmoratus* mtDNA (Fig. 3c), which does not differ significantly from the null ratio ($p = 0.625$).

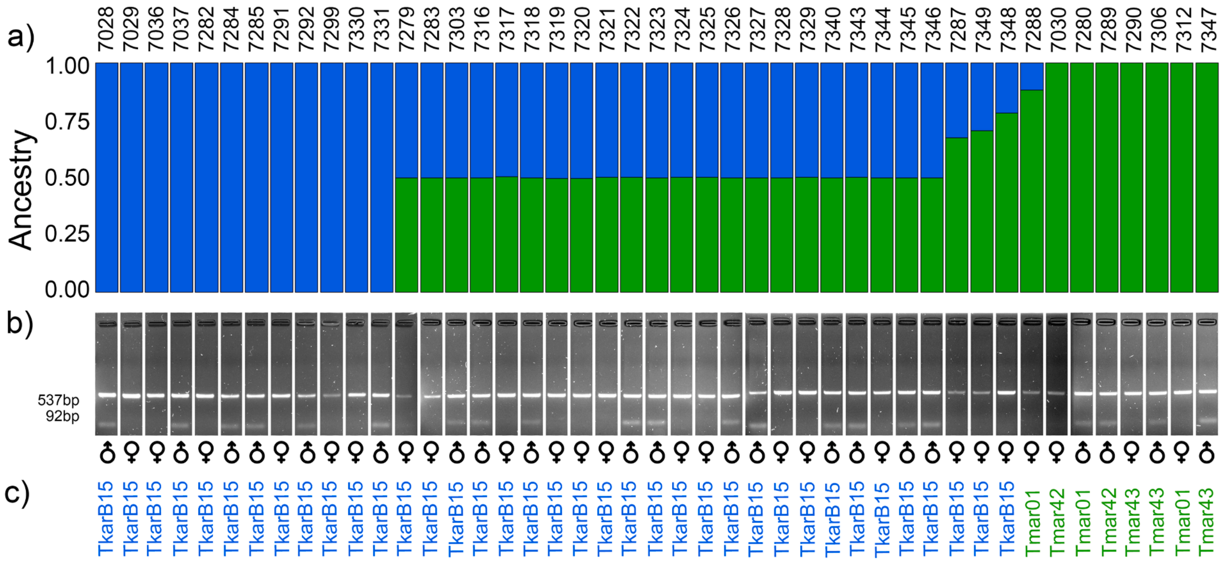

Fig. 3.. Ancestry and sex determination of *Triturus* newts sampled in Catalonia. (a) ADMIXTURE ancestry plot for K= 2 genetic clusters. Each vertical bar represents an individual, with colors indicating ancestry of either *T. anatolicus* (blue) or *T. marmoratus* (green). Individuals are ordered by ancestry proportion (Q-score) and individual IDs are shown on top (Table S1). (b) Electrophoresis results from sex genotyping. The upper control band corresponds to an autosomal marker and the lower band corresponds to a Y-linked marker (i.e., only present in males). The inferred sex for each individual is indicated below the lanes (c) ND4 mtDNA haplotypes for each individual (*T. anatolicus* in blue and *T. marmoratus* in green).

3.5. Origins of invasive *T. anatolicus*

A single *T. anatolicus* mtDNA haplotype, TkarB15, was identified in Catalonia (Figure 3c and S2). Within the native range, TkarB15 is restricted to a small region in northwestern Türkiye: locality 16 (Abant Gölü) and locality 17 (Seben), in Fig. 4a. In the individual-based hierarchical clustering analysis using nuclear DNA (Fig. 4b), all invasive individuals group into a clade and their closest native relatives are found in localities 16 and 17. The PCA shows the invasive individuals forming a tight and distinct cluster (Figure S3). The closest native cluster again comprises individuals from localities 16 and 17.

4. Discussion

We use genome-wide target-capture data and mtDNA barcoding to investigate hybridization dynamics between invasive *Triturus anatolicus* and native *T. marmoratus* in Montnegre i el Corredor Natural Park, Catalonia (Spain). We find that F1s are fertile and capable of backcrossing at an unexpectedly high rate. This process could have ramifications for the genetic integrity of native populations through genetic pollution.

4.1. Anthropogenic hybridization differs from natural conditions

Genome-wide data reveal that a significant proportion of our samples are F1 individuals. The natural hybrids between crested and marbled newts in France (*T. cristatus* × *T. marmoratus*) make up only ~3–4% of the adult population (Arntzen et al., 2021, 2009, 2026; Arntzen and Wallis, 1991). In contrast, in the anthropogenic hybrid zone in Catalonia, over a third of individuals appear to be hybrids (Carranza, personal observation). This may reflect the recent establishment of secondary contact, only a few generations ago (Martel et al., 2020). Practically no time has been available for natural selection against hybridization (i.e. reinforcement), compared to in the natural hybrid zone, which is considered to have been established several millennia ago (Visser et al., 2017). Although reinforcement might eventually strengthen reproductive barriers in the anthropogenic hybrid zone, in the foreseeable future hybridization will

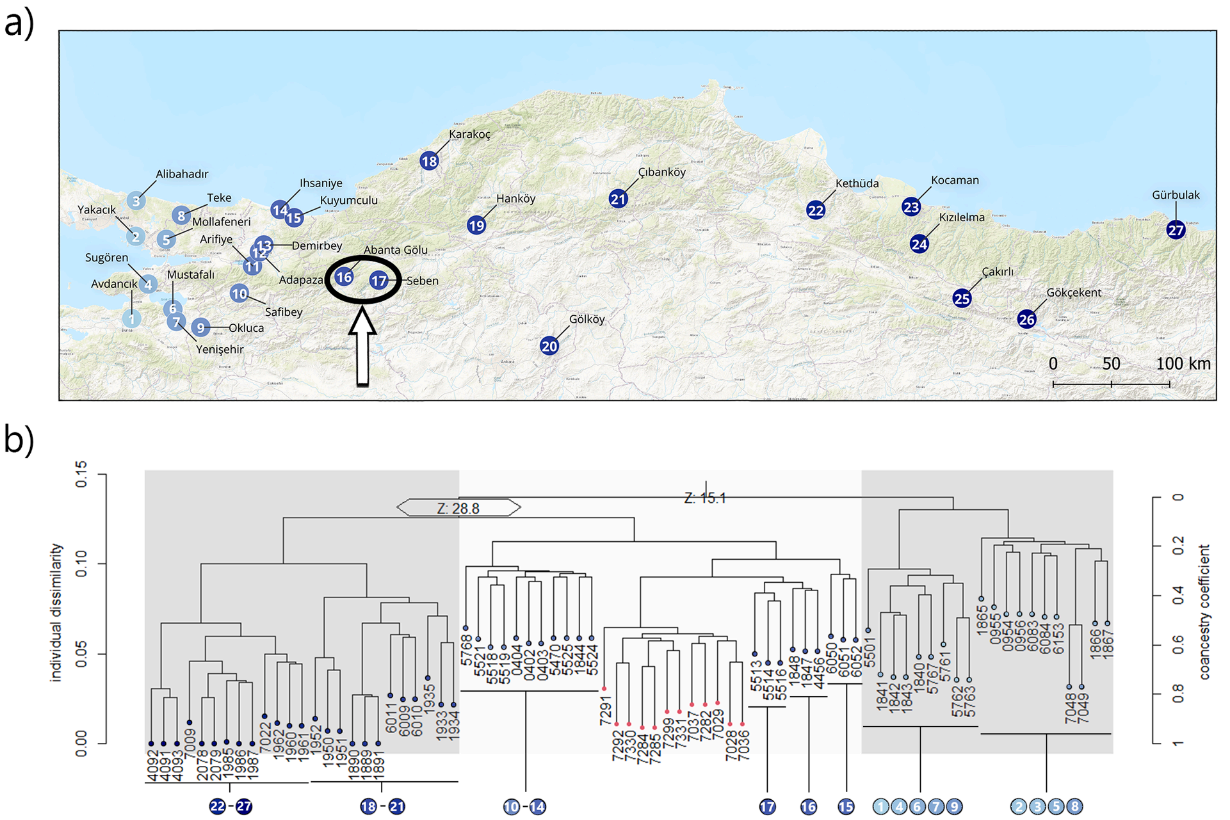

Fig. 4.. Provenance of the invasive *Triturus anatolicus*. (a) Map showing the sampling locations of the *T. anatolicus*; population numbers correspond to those listed in Table S1. The white arrow indicates the populations that share the same mtDNA haplotype as the invasive individuals detected in Catalonia. (b) Hierarchical clustering dendrogram of the analyzed samples. Sample IDs are shown at the branch tips, with the corresponding population numbers indicated below the dendrogram. Catalan *T. anatolicus* individuals are highlighted in red and cluster with populations 16 and 17 (indicated by white arrows in panel a). Z-scores displayed above the branches represent the relative strength of genetic differentiation among clusters after distance standardization.

remain rampant.

Hybridization is strongly asymmetrical. All F1 individuals carry *T. anatolicus* mitochondrial DNA (Fig. 2c), indicating that successful hybridization occurs exclusively via crosses between *T. anatolicus* females and *T. marmoratus* males. Such asymmetry can be explained by the Hubbs' principle, a.k.a. the desperation hypothesis, where a species is more likely to mate with members of another species when it is rare (Hubbs, 1955). In newts, females are the choosy sex (Arntzen, 2003; Fahrbach and Gerlach, 2018), so a small source population of *T. anatolicus* females may be forced to mate with relatively abundant *T. marmoratus* males. However, the observed bias towards *T. anatolicus* mothers also mirrors the natural *T. cristatus* × *T. marmoratus* hybrid zone, where ~90% of hybrids carry crested mtDNA, even though the ratio of the parental species is not particularly skewed (Arntzen et al., 2009). This suggests the observed bias towards *T. anatolicus* mothers may reflect genetic incompatibilities (Brandvain et al., 2014).

Unlike the natural hybrid zone, which shows a strongly female-biased sex ratio among *T. cristatus* mothered F1 hybrids, we do not observe a sex bias in the anthropogenic hybrid population (Fig. 2b) (Arntzen et al., 2021). The excess of females in the natural hybrid zone has been proposed to reflect the Haldane effect, according to which a sex-bias in hybrids is predicted to disfavor the heterogametic sex, i.e. the male in *Triturus* newts (Arntzen, 2024a; Haldane, 1922; Schilthuizen et al., 2011). Evidently, this explanation does not apply to the anthropogenic hybrid population. In the natural hybrid zone, the rarity of male *T. marmoratus*-mothered F1s is suggested to reflect an incompatibility between the crested newt X chromosome and marbled newt cytoplasm (Arntzen et al., 2021, 2009). Because we did not detect *T. marmoratus*-mothered F1s in our dataset – which could reflect rarity, rather than true absence– we cannot assess sex-ratio patterns for this cross.

While a small proportion (~4%) of F1 hybrids in the natural hybrid zone are triploids (Arntzen et al., 2026), we only observe diploid hybrid individuals. This might reflect our small sample size.

4.2. *Fertility of hybrids provides the preconditions for genetic pollution*

We detect later-generation hybrids, which proves that the F1 hybrids in the anthropogenic hybrid population are capable of producing viable offspring. We find four genetically confirmed later-generation hybrids (Figs. 2a, 2b, 3a), among 26 hybrids in total (i.e., 15%); a pattern consistent with reduced fitness of F1 hybrids relative to the parental species, as expected under postzygotic isolation. Yet, the frequency of later-generation hybrids is surprisingly high. In the natural hybrid zone, later-generation hybrids have been identified in five out of 121 genetically screened hybrids (Arntzen et al., 2021). In the anthropogenic hybrid population, the later-generation hybrids were exclusively female, whereas in the natural hybrid zone only males have been reported. This could be a chance effect, given the small numbers. Although our sample size is small, the occurrence of later-generation hybrids in the anthropogenic hybrid population appears to be considerably higher than in the natural hybrid zone, suggesting an increased potential for interspecific gene flow.

Unlike the natural hybrid zone, where backcrosses toward both parental species have been observed (Arntzen et al., 2021), we only identified later-generation hybrids located on the marbled newt side of the heterozygosity/hybridity triangle (Fig. 2b). However, our data simulation shows that, to produce the hybrid class of individual 7348, backcrossing toward *T. anatolicus* is required (Fig. 2b). Our results indicate that at least five generations are required to explain the presence of all observed hybrid classes, suggesting that the introduction must have taken place earlier than the first record of *T. anatolicus* in Montnegre i el Corredor Natural Park in 2016 (Martel et al., 2020). In the natural hybrid zone, low frequency introgression mostly flows from *T. marmoratus* into *T. cristatus* (Arntzen et al., 2021; Arntzen, 2024a). While the anthropogenic hybrid population is still too young for introgression to have taken place, the observed pattern reveals that backcrossing mainly occurs toward the native *T. marmoratus*.

The unexpectedly high frequency of later-generation hybrids and the observed backcrossing toward the native species should be considered an early warning sign: the conditions required for genetic pollution are in place. The risk of genetic pollution may be intensified by a highly lethal outbreak of *Batrachochytrium salamandrivorans* (*Bsal*) that is ongoing in the region since 2018 (Bosch et al., 2021; Fitzpatrick et al., 2018; Martel et al., 2013, 2020). The outbreak has been causing severe mortality in *T. marmoratus*, while it has been previously suggested that *T. anatolicus* is less susceptible to the disease (Bosch et al., 2021; Martel et al., 2020). Pathogen-driven selection could shift species and hybrid frequencies, potentially accelerating population replacement and introgressive hybridization (Edelman and Mallet, 2021; Porretta and Canestrelli, 2023; Valencia-Montoya et al., 2020).

Our study shows that genetic pollution of native *T. marmoratus* in Montnegre i el Corredor Natural Park represents a credible risk. Fortunately, we detected the process at a relatively early stage and removal actions are already ongoing. In this case, management authorities decided to remove the entire *Triturus* population from the affected ponds, regardless of the genetic purity of individual newts. While this is the most reliable way to prevent genetic pollution, such an extreme measure might not always be feasible or even legal, e.g. if the native species is highly threatened (Allendorf et al., 2001). In practice, the genetic profile of individuals might first need to be determined before deciding if they should be retained or removed (Wielstra et al., 2016).

4.3. *Origin of invasive T. anatolicus*

MtDNA barcoding of the Catalonian *T. anatolicus* population identifies a haplotype that is naturally found in a small region in northwestern Türkiye (see also Carranza and Fernandez Guiberteau (2018)). While mtDNA provides useful information on matrilineal ancestry, it reflects just one component of an individual's evolutionary history. Genome-wide data can provide a more accurate reconstruction of the invasion pathway. This is exemplified by *Ommatotriton* newts introduced in a nearby national park (Fontelles et al., 2011): while they all carry mtDNA of a single species, nuclear DNA has revealed them to be interspecific hybrids (van Riemsdijk et al., 2022). For *T. anatolicus*, genome-wide nuclear data suggest that a single species is involved and pinpoints the same geographical origin as suggested by mtDNA (Fig. 4a). The lack of genetic differentiation among invasive individuals (Figure S2) is consistent with a founder event from a single source population.

5. Conclusions

This study highlights how high-resolution genomic approaches can uncover invasion pathways, characterize hybridization and guide conservation measures aimed at protecting native biodiversity from genetic pollution. We demonstrate that the invasive *T. anatolicus*, originating from northwestern Türkiye, has been introduced to Catalonia and now hybridizes there with native *T. marmoratus*, producing not only F1 hybrids, but also later-generation hybrids. The observed backcrossing towards native marbled newts underscores that genetic pollution should be considered a genuine risk. Therefore, we propose that *T. anatolicus* is formally recognized as a high-risk invasive species in Spain.

Ethical approval

All fieldwork, capture, and handling of animals were conducted under permits issued by the competent environmental authorities of the Generalitat de Catalunya (Departament d'Acció Climàtica, Alimentació i Agenda Rural; SF/0036/2019, SF/0197/21, SF/0193/22, SF/0243/23, SF/0056/24). These permits authorize the capture and handling of wild fauna for scientific purposes and confirm the relevance of the work for conservation and management. All procedures complied with Spanish Law 42/2007 on Natural Heritage and Biodiversity and relevant regional regulations. Animal handling followed permit protocols to minimize stress and harm, with strict biosecurity measures to prevent disease transmission in amphibians. No experimental procedures were performed; the work was limited to field sampling of wild individuals. Native individuals were released after sampling, while non-native (invasive) individuals were removed in accordance with current legislation. Therefore, under national regulations, no additional approval from an institutional animal ethics committee was required.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used Claude (Anthropic) to assist in developing and refining bioinformatics scripts and ChatGPT (OpenAI) to improve English grammar and readability. After using these tools, the authors reviewed and edited all content as needed and take full responsibility for the content of the published article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.gecco.2026.e04308.

Data availability

Illumina sequencing reads are available in the NCBI Sequence Read Archive under BioProject PRJNA1449913. All scripts and workflows are uploaded to Zenodo (https://zenodo.org/records/19352830).

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