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# Genetic pollution of a threatened native crested newt species through hybridization with an invasive congener in the Netherlands

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ABSTRACT

Genetic pollution of a native species through hybridization with an invasive species poses an insidious conservation threat. To expose genetic pollution, molecular methods employing multilocus data are required. We present a case study of genetic pollution via hybridization of a native crested newt species, *Triturus cristatus*, by the invasive *Triturus carnifex* on the Veluwe in the Netherlands. We sequenced 50 nuclear markers by next generation sequencing and one mitochondrial marker by Sanger sequencing for four populations from the native range of both parent species and eleven ponds on the Veluwe. We use three population genetic approaches (HIest, BAPS and Structure) to determine the genetic composition of the Veluwe newts based on all nuclear markers, a subset of 18 diagnostic markers and the complementary 32 non-diagnostic markers, with and without parental populations. BAPS underestimates genetic pollution, whereas Structure is comparatively accurate compared to HIest, although Structure's relative advantage decreases with the diagnosticity of the markers. Data simulation confirms these findings. Genetic composition of the Veluwe ponds ranges from completely native, via different degrees of genetic admixture, to completely invasive. The observed hybrid zone appears to be bimodal, suggesting negative selection against hybrids. A genetic footprint of the native species is present in invasive populations, evidencing that the invasive locally replaced the native species. Genetic pollution is currently confined to a small area, but the possibility of further expansion cannot be excluded. Removal of genetic pollution will not be easy. We emphasize the need for legal guidance to manage genetic pollution.
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## 1. Introduction

### 1.1. Invasive hybridizing species

Increased globalization enables species to reach locations previously inaccessible to them (Bright, 1999). Besides causing economic damage, invasive species pose an environmental threat: they can endanger populations of native species with extinction through competition, predation and transmission of diseases (Pimentel et al., 2001). A more insidious threat posed by invasive species is hybridization with a closely related native species (Allendorf et al., 2001; Crispo et al., 2011; Dubois, 2006; Largiadèr, 2007; Mooney and Cleland, 2001; Rhymer and Simberloff, 1996).

Hybrids usually have a higher mortality or infertility rate than parental species (Burke and Arnold, 2001; Dittrich-Reed and Fitzpatrick, 2013) and thus have a lower fitness than the parental species. For example, offspring between the invasive brook trout (*Salvelinus fontinalis*) hybridizing with the native bull trout (*Salvelinus confluentus*) are mostly infertile and few instances of backcrossing have been observed (Leary et al., 1993). However, hybridization could still accelerate *S. confluentus*' population decrease, as the act promotes a wasted reproduction effort.

Hybrids between an invasive and a threatened native species can occasionally be fertile and in these cases hybridization can have severe consequences to the genetic integrity of the native species. If hybridization is followed by backcrossing to one member of the species pair, the genetic material of the other will 'dilute' over the generations. However, some genetic material of the 'donor' species can be maintained, either by chance or because it has a selective advantage. This process is known as introgression

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(Mallet, 2005). Introgression resulting in the partial replacement of native genotypes by foreign ones has been labelled 'genetic pollution' (Butler, 1994; Goka, 1998). From a conservation point of view, genetic pollution is unwanted because it leads to a loss of biodiversity at the level of the gene and erases parts of local evolutionary history (Dubois, 2006; Petit, 2004).

Detecting genetic pollution generally is difficult because the hybridizing species involved and their genetically admixed offspring are often morphologically similar; the difficulty of identification is exacerbated over generations of backcrossing (Allendorf et al., 2001). Hence, to accurately identify genetic pollution, genetic data need to be consulted directly (Rhymer and Simberloff, 1996).

Mitochondrial DNA has been used to document genetic pollution from, for example the invasive mallard (*Anas platyrhynchos*) into the native grey duck (*Anas superciliosa superciliosa*) in New Zealand (Rhymer et al., 1994), the domestic dog (*Canis familiaris*) into the native coyote (*Canis latrans*) in North America (Adams et al., 2003), and the invasive ferret (*Mustela furo*) into the native polecat (*Mustela putorius*) in Britain (Davison et al., 1999). However, mitochondrial DNA is clonally inherited (Ballard and Whitlock, 2004) and relatively susceptible to introgress into the expanding species (Currat et al., 2008; Wielstra and Arntzen, 2012). Native mitochondrial DNA surfing the wave of expansion of an invasive hybridizing species would make genetically polluted individuals invisible from the mitochondrial DNA perspective. Hence, mitochondrial DNA provides a lower estimate of genetic pollution.

Microsatellites are popular in invasive hybridization studies and have for example been applied to the domestic cat (*Felis catus*) hybridizing with the native wildcat (*Felis silvestris*) in Europe (Beaumont et al., 2001; Oliveira et al., 2008; Randi et al., 2001) and the domestic dog (*C. familiaris*) hybridizing with the grey wolf (*Canis lupus*) in Europe (Vilà et al., 2003) and the Ethiopian wolf (*Canis simensis*) in Ethiopia (Gottelli et al., 1994). However, microsatellites have drawbacks. Mutations in primer binding sites can cause alleles not to amplify (i.e. null alleles) for genetically more diverged populations, e.g. across species, which would result in an underestimation of interspecific gene flow (Selkoe and Toonen, 2006). Moreover, convergent evolution could lead to misinterpreting the evolutionary origin of alleles, which would make gene flow at those loci undetectable (Selkoe and Toonen, 2006).

More recently single nucleotide polymorphisms (SNPs) have been used to illuminate invasive gene flow for, for example the introduced rainbow trout (*Oncorhynchus mykiss*) hybridizing with the native cutthroat trout (*Oncorhynchus clarkii lewisi*) in North America (Amish et al., 2012; Hohenlohe et al., 2011), the introduced eastern tiger salamander (*Ambystoma tigrinum*) hybridizing with the native Californian tiger salamander (*Ambystoma californiense*) (Fitzpatrick et al., 2010), domestic dogs (*C. familiaris*) hybridizing with wolfs (*C. lupus*) and coyotes (*C. latrans*) in northeast USA (Monzón et al., 2014), and introduced phylogenetically admixed red fox (*Vulpes vulpes*) hybridizing with the endemic red fox subspecies (*V. vulpes patwin*) in California (Sacks et al., 2011). The use of SNPs is susceptible to an ascertainment bias by the under-representation of rare alleles, in particular if a small group of individuals is used to characterize the SNPs (Rosenblum and Novembre, 2007; Schlötterer, 2004).

Consulting nuclear sequence data would give the highest possible resolution of genetic pollution (Schlötterer, 2004). Recent advancements in genetics, notably the development of next generation sequencing, allow the economic production of nuclear sequence data for a large number of nuclear markers for a large number of individuals (Twyford and Ennos, 2011). Here, we present a case study of two crested newt species (genus *Triturus*) that have started hybridizing in the Netherlands after human mediated contact. We analyze our dataset using three popular population genetics methods, while exploring the influence of the diagnosticity of the markers and the influence of including pure parental from throughout the native range as a reference. Finally, we discuss our findings and provide information that is pertinent to the management of the case.

### 1.2. Case study: crested newts in the Netherlands

This study involves two morphologically similar species of crested newt, the Northern crested newt (*Triturus cristatus*) and the Italian crested newt (*Triturus carnifex*). Together with their respective sister species they share a common ancestor c. 9 million years ago (Wielstra and Arntzen, 2011). Under natural circumstances *T. cristatus* and *T. carnifex* are parapatrically distributed, with a contact zone east of the Alps, in Austria and the Czech Republic (Arntzen, 2003; Fig. 1A). The two species hybridize in the laboratory (Callan and Spurway, 1951) and under natural conditions (Maletzky et al., 2008; Mikulíček et al., 2012). At localities where *T. carnifex* has been introduced inside the range of *T. cristatus*, populations appear to be stable and long lived (Arntzen and Thorpe, 1999; Brede et al., 2000; Maletzky et al., 2008). Moreover, asymmetric introgression of *T. cristatus* mitochondrial DNA into *T. carnifex* suggests displacement with hybridization of *T. cristatus* by *T. carnifex* in the Geneva basin in France and Switzerland (Arntzen et al., 2014).

*T. carnifex* was first observed on the Veluwe in the Netherlands in 1999, close to where native *T. cristatus* populations are established (Bogaerts, 2002). Initial introduction is presumed to have occurred in the late 1970s, when a garden center in Vaassen (black star; Fig. 1B–F) selling *T. carnifex* as 'garden ornaments' released livestock because a change in legislation prohibited sale (Bogaerts, 2002; Fig. A1). Recent surveys suggest a range expansion of *T. carnifex* from the original introduction site (Bosman and van Delft, 2011; van Hoogen and Crombaghs, 2012; Fig. A1). The presence of phenotypically intermediate individuals suggests that hybridization occurs with *T. cristatus* (Vleut and Bosman, 2005). Therefore, apart from direct competition, *T. carnifex* poses a potential threat to the native *T. cristatus* through genetic pollution. *T. cristatus* is listed as vulnerable on the Dutch Red List (van Delft et al., 2007) and on Appendix II and IV of the EU Habitats Directive and Appendix 2 of the Bern Convention. The Dutch government hence has an international responsibility to protect *T. cristatus*. For management considerations a detailed knowledge on the spread of genetic material derived from *T. carnifex* into *T. cristatus* is required.

We determine whether hybridization between the invasive *T. carnifex* and the threatened native *T. cristatus* occurs and, if so, to what extent *T. carnifex* genetic material has introgressed into *T. cristatus* populations. To this aim we sequence DNA for one mitochondrial and 50 nuclear markers. This case study provides one of a few examples where hybridization between invasive and native species is worked out in detail, and provides a benchmark for comparable conservation problems.

## 2. Material and methods

### 2.1. Study area and sampling

The study area is situated on the Veluwe, the Netherlands (Fig. 1). The Veluwe, part nature reserve, is an area with sandy soils, heathland, deciduous forest, evergreen forest and agricultural land. Natural water bodies are rare on the Veluwe. Most ponds are artificial, e.g. excavations created by loam mining, water troughs for cattle and game, and a concrete water system used for the water supply of a series of artificial ponds and lakes. We sampled 12 individuals from 11 ponds each ($n = 132$; Fig. 1; Table A1).

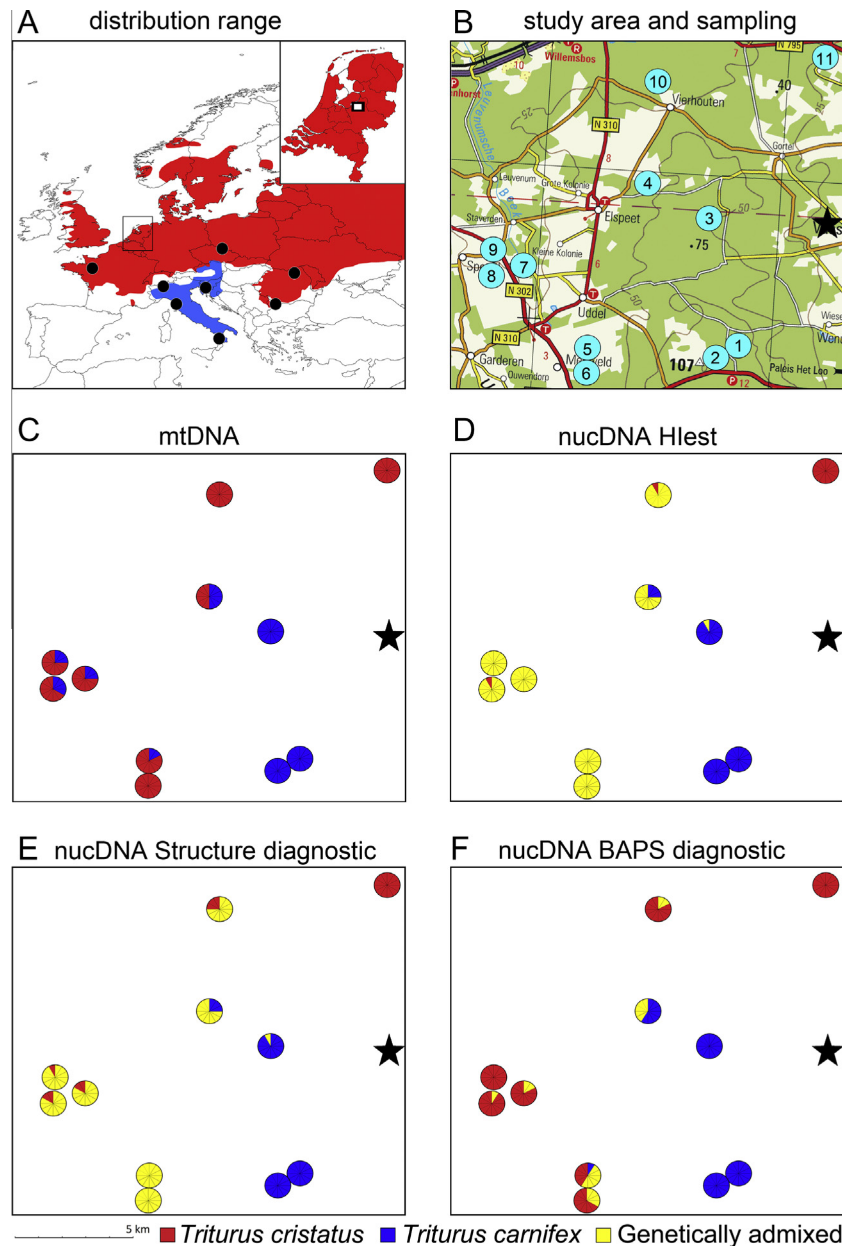


**Fig. 1.** Species distribution, study area and genetic composition of crested newt populations from the Veluwe, the Netherlands. Distribution of the two parental species, with *T. cristatus* in red and *T. carnifex* in blue and the reference populations marked with black dots, and the location of the study area shown in the cut out (A). The study area on the Veluwe in the Netherlands and the location of the studied ponds (B). The genetic composition of the 12 individuals per pond is represented in 11 pie charts for the following data: mitochondrial DNA (C); Hlest (D); Structure (subset of diagnostic markers) (E); and BAPS (subset of diagnostic markers) (F). Pie color indicates genetic identification with *T. cristatus* in red, *T. carnifex* in blue and genetically admixed individuals in yellow. The black star indicates the presumed initial release site of *T. carnifex*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Sampling included the area north, west and south of the putative introduction site; to the east the presence of the city Apeldoorn and the A50 highway were presumed to represent an insurmountable barrier. DNA was isolated from tail tips with the QIAGEN DNeasy Blood and Tissue Kit. We added three individuals, taken from four *T. cristatus* and four *T. carnifex* reference populations (i.e. $n = 24$ individuals, from Wielstra et al., 2014), distantly located from the natural contact zone of the species and hence presumed to be unaffected by interspecific gene flow (Table A1).

### 2.2. Mitochondrial DNA sequencing and analysis

We amplified the ND4 mitochondrial gene following the protocol described in Wielstra et al. (2013). Sanger sequencing was done commercially by BaseClear (Leiden). Sequences were checked by eye using Sequencher 4.10.1 (Gene Codes Corporation) and compared to a *Triturus* ND4 database (Wielstra et al., 2013) in MacClade 4.08 (Maddison and Maddison, 2005). We assigned newly identified haplotypes to species based on a Neighbor Joining phylogeny constructed with Mega 5.05 (Tamura et al., 2011).

### 2.3. Nuclear DNA sequencing

To obtain nuclear markers we applied the protocol of Wielstra et al. (2014). In brief, we sequenced 52 nuclear markers (140 ± 5 bp excluding primers) positioned in 3 prime untranslated regions. Markers were amplified using five multiplex PCRs (10–11 markers per multiplex). Multiplexes per individual were pooled

(5 µl of each) and unique tags were ligated to be able to recognize individuals. We sequenced amplicons on the Ion Torrent next generation sequencing platform. Output was processed with a bioinformatics pipeline that filtered out poor quality reads, identified alleles and converted data to a genotypic format in which each of the two alleles for each marker was represented by an integer. Two markers (*limch1* and *slc25*) failed to amplify for a large number of individuals and were omitted from further analysis (Tables A2–3).

### 2.4. Population genetics analysis

The reference individuals enabled us to determine which markers were diagnostic, i.e. contained only species-specific alleles (Table A2). For the Veluwe dataset we counted the number of *T. carnifex* and *T. cristatus* alleles per individual and summarized this in a hybrid index (Jiggins and Mallet, 2000). Alleles not found in the reference individuals were not assigned to either of the two parental species. We analyzed the diagnostic markers with the R package HIest, which uses likelihood to provide estimates for ancestry ($S$), the fraction of alleles derived from each parental species, and heterozygosity ($H_I$), the fraction of loci heterozygous for alleles from each parental species (Fitzpatrick, 2012). Each marker-individual combination was recoded according to the number of *T. carnifex* alleles present (i.e. 0, 1 or 2) or was scored as undetermined (NA) if the evolutionary origin of at least one of the alleles was unclear (i.e. not present in any of the reference individuals) or if data was missing.

Output from the bioinformatics pipeline was converted with CREATE (Coombs et al., 2008) into input files for BAPS v5.3 (Corander et al., 2008) and Structure 2.2 (Pritchard et al., 2000). These two programs use Bayesian statistics to assign individuals to groups probabilistically. We set the number of gene pools in BAPS and Structure to $K = 2$, because two species are involved. In BAPS we used ten replicates and tested for admixture between gene pools. In Structure we performed 5 runs with 100,000 MCMC replicates after a burn in of 50,000 and used the admixture model which recognizes that individuals may be of admixed origin, in combination with the correlated allele frequency model. We ran BAPS and Structure for the complete set of markers and the subsets of diagnostic and non-diagnostic markers. All datasets were analyzed with and without reference populations representing the parent species.

In the species assignment analyzes we used the lowest probability with which a reference individual was assigned to its respective species as a threshold. Veluwe individuals with a probability of belonging to a parental species above this threshold were regarded as having been identified as genetically pure.

### 2.5. Data simulation

To explore differences in the sensitivity of recording genetic pollution for the population genetic programs used we conducted two data simulation exercises. First, we simulated a dataset reflecting grades of genetic pollution. We included 18 diagnostic markers (the number present in our empirical dataset) with allele variants coded as 1 or 2. We created a dataset composed of 37 groups of 100 individuals in Microsoft Excel. The first group consisted entirely of alleles of the native species, each consecutive group contained one additional allele of the invasive species, until in the last group the invasive species was reconstituted. We randomized the position of foreign alleles within individuals in each groups using Kutools (www.extendoffice.com).

Next, we simulated a dataset that reflects backcrossing to a native species over multiple generations after an initial hybridization event with an invasive species. We used HybridLab (Nielsen et al., 2006), with the complete, diagnostic and non-diagnostic dataset. We created *T. cristatus* and *T. carnifex* populations *in silico* based on the empirical data of the reference individuals and crossed the two parental groups to create F1 individuals. The F1s and each consecutive group were crossed with pure parental *T. cristatus* to create 15 generations of backcrosses of 1000 individuals each.

## 3. Results

### 3.1. Mitochondrial DNA

Four different ND4 haplotypes were found on the Veluwe: one belonging to *T. carnifex* (Tcar03) and three to *T. cristatus* (Tcri01, Tcri42, Tcri43; Table A1). Tcar03 was previously reported from Sinac, Croatia and is nested in the genetically distinct Balkan *T. carnifex* clade (Fig. A2). Tcri01 is a haplotype that is widely distributed in the range of *T. cristatus*. Tcri42 and Tcri43 were newly identified and are closely related to Tcri01 (Fig. A2). In ponds 1–3 we found only *T. carnifex* mitochondrial DNA, while individuals from pond 6, 10 and 11 only possessed *T. cristatus* mitochondrial DNA; mitochondrial DNA of both species was found in ponds 4, 5 and 7–9 (Fig. 1C).

### 3.2. Nuclear DNA

The total number of aligned Ion Torrent reads after data filtering was 4,582,034 (564.8 reads ± 5.54 SE per marker per individual; Table A3). Eighteen markers were diagnostic for individuals on the Veluwe. All ponds except pond 11 contained alleles typical for *T. carnifex*. The hybrid index (Fig. 2A) revealed that individuals with alleles originating from both parental species in more or less equal amounts are rare. Individuals with a high heterozygosity ($H_I$; alleles originating from both species) as calculated by HIest were observed more often in the direction of *T. cristatus* ($S = 0$) than *T. carnifex* ($S = 1$; Fig. 2B), while F1 hybrids ($H_I$ & $S = 0.5$) were absent.

Threshold values were 0.964 (Structure) and 1.000 (BAPS) for the complete set of markers, 1.000 (HIest), 0.992 (Structure) and 1.000 (BAPS) for the diagnostic marker set and 0.704 (Structure) and 0.760 (BAPS) for the non-diagnostic marker set. Although results for the three programs and types of dataset analyzed deviate, they reveal a similar general pattern (Table 1, Table A1; Figs. 1D–F and 3). In three ponds pure parentals were observed exclusively. All individuals in pond 1 and 2 were identified as *T. carnifex*, while all individuals in pond 11 were assigned to *T cristatus*. Genetically admixed newts were found in pond 3–10, with HIest diagnostic observing the most (80), followed by Structure diagnostic (75), Structure complete (60), BAPS diagnostic (20), BAPS complete (18), BAPS non-diagnostic (11) and finally Structure non-diagnostic (9). Both in ponds 8 and 10 a single pure *T. cristatus* individual was observed (identified as such by all programs and confirmed not to contain any *T. carnifex* alleles). HIest identified all individuals that possessed *T. carnifex* alleles as such, followed by Structure diagnostic (5 misidentifications), Structure complete (18), BAPS diagnostic (54), BAPS complete (56), BAPS non-diagnostic (60) and finally Structure non-diagnostic (61).

Exclusion of reference populations for the two parental species had a small effect on Structure results. Three and one genetically admixed individuals were assigned to pure *T. cristatus* for the complete and diagnostic dataset, while results for the non-diagnostic dataset did not differ (Table 1). Exclusion of reference populations had a larger effect on BAPS: 24, 24 and one *T. cristatus* individuals were identified as genetically admixed for the complete, diagnostic and non-diagnostic dataset (Table 1).

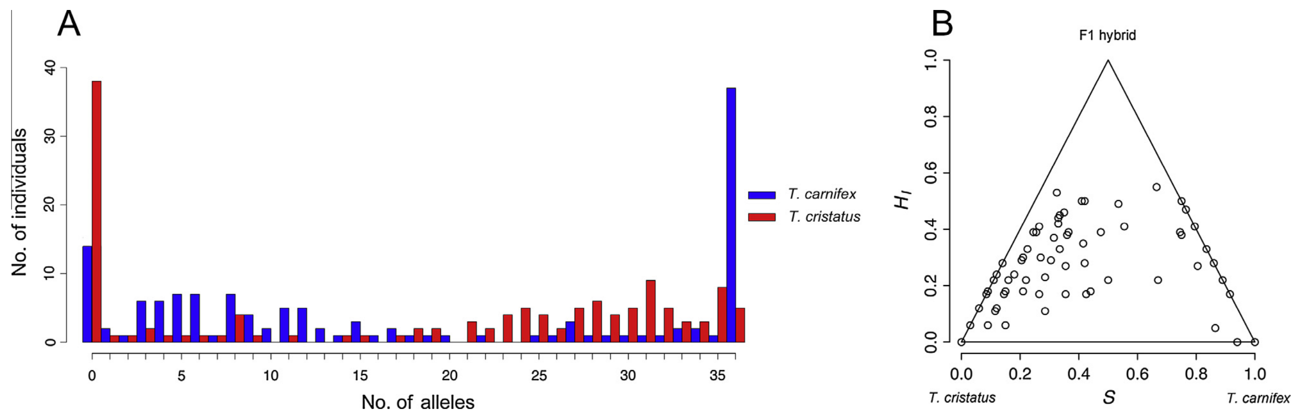


**Fig. 2.** The hybrid index and the ancestry and heterozygosity for crested newts from the Veluwe. Hybrid indices based on *T. cristatus* alleles (red) and *T. carnifex* alleles (blue) represented in a single bar plot (A). The estimation of Ancestry ($S$) and heterozygosity ($H_I$), determined with HIest, with $S = 0$ and $S = 1$ representing pure *T. cristatus* and pure *T. carnifex* and $H_I = 0$ and $H_I = 1$ representing complete homozygosity and complete heterozygosity (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

### 3.3. Data simulation

The simulated dataset reflecting grades of genetic pollution showed that HIest and Structure manage to pick up the minimal amount of genetic pollution possible (i.e. only a single invasive allele) whereas BAPS underestimates genetic pollution (Fig. A3). The simulated dataset reflecting backcrossing to a native species over multiple generations, after an initial hybridization event with a foreign species, showed that genetic pollution becomes invisible for BAPS sooner than it does for Structure for the complete dataset, while almost no difference between HIest, Structure and BAPS was observed for the diagnostic dataset. However, for the non-diagnostic dataset detection of foreign alleles declines faster for Structure than BAPS (Fig. A3).

### 3.4. Mitochondrial and nuclear DNA results compared

In pond 4 genetically admixed individuals with a large proportion of *T. carnifex* nuclear DNA possess *T. cristatus* mitochondrial DNA (Figs. 1 and 2). In ponds 7–9 some genetically admixed individuals with a large nuclear DNA proportion assigned to *T. cristatus* contain *T. carnifex* mitochondrial DNA.

## 4. Discussion

### 4.1. Hybridization on the Veluwe

We expose hybridization and backcrossing between two crested newt species in the Netherlands, the endangered native *T. cristatus* and the invasive *T. carnifex*. This result confirms that genetic pollution by *T. carnifex* should be considered in conservation efforts of *T. cristatus*. We observe populations of pure *T. carnifex* and *T. cristatus* individuals, and populations with different degrees of genetic admixture situated in between them, i.e. a hybrid zone (Hewitt, 1988). The hybrid index reveals a bimodal hybrid zone, which suggests that pre- or postzygotic isolating mechanisms are operating, reducing the probability that members of the two species mate and/or negatively influencing the fitness of their offspring (Jiggins and Mallet, 2000). The lack of F1 hybrids observed (Fig. 2; Table A1) could reflect strong selection against F1 hybrids. An alternative, but not mutually exclusive explanation is that because most individuals in the hybrid zone are themselves of genetically admixed ancestry their offspring are, by definition, not F1 hybrids (Fitzpatrick, 2012).

The subtlety of identifying genetic pollution is illustrated by the varying outcomes of the different population genetics analyzes used. BAPS overestimates individuals as genetically pure, meaning the program underestimates the geographical extent of *T. carnifex* genetic material, whereas Structure is mostly accurate (Fig. 3). Data simulation confirms our empirical finding that BAPS underestimates genetic pollution compared to Structure (Fig. A3). The higher power of Structure to detect genetic pollution compared to BAPS has been previously noted by Bohling et al. (2013). We took advantage of detailed background knowledge on the genetic make-up of the two parental species involved. This allowed us to identify diagnostic markers within our total dataset and map genetic pollution in detail (Fig. 1C–F). The population genetic programs Structure and BAPS can be applied without *a priori* knowledge on the parental species genetic make-up. We show that in particular Structure's success rate in terms of correctly identifying genetically polluted individuals is only marginally affected by the availability of reference populations. However, the power drops when, instead of using only the set of diagnostic markers, we analyze our complete dataset, and even more so when we analyze the set of non-diagnostic markers. Detection of introgression by BAPS slightly increases in the absence of reference populations. This could be related to the reference individuals representing a relatively high genetic variability compared to the parent populations from the Veluwe, considering that the introduced species represents a founder effect and the native species shows genetic depletion after post glacial recolonization (Wielstra et al., in press). Moreover, BAPS outperforms Structure when diagnostic markers are absent, although both programs perform poorly under these conditions.

### 4.2. Displacement of the native species

When an invading species partially displaces a native species and the two hybridize and backcross in the process, genetic traces of the native species are likely to remain (Currat et al., 2008). On the Veluwe we repeatedly found individuals containing only a few alleles of the native species *T. cristatus*, whereas the majority was derived from the invasive species *T. carnifex*. The fact that we find native alleles of multiple, presumably unlinked markers in predominantly invasive individuals is in favor of the explanation that displacement of *T. cristatus* by *T. carnifex* has occurred, as opposed to positive selection 'dragging' the *T. cristatus* alleles into the range of *T. carnifex* (Barton and Hewitt, 1985). Hence, the genetic data are in agreement with the documented situation of

**Table 1**
Assignment per pond of individuals to class by the different population genetics programs.

| Pond | Tcar alleles | HIest Diagnostic | | | | Structure Complete | | | | Structure Diagnostic | | | | Structure Non-diagnostic | | | | BAPS Complete | | | | BAPS Diagnostic | | | | BAPS Non-diagnostic | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Tcar | Adm | Tcri | Mis | Tcar | Adm | Tcri | Mis | Tcar | Adm | Tcri | Mis | Tcar | Adm | Tcri | Mis | Tcar | Adm | Tcri | Mis | Tcar | Adm | Tcri | Mis | Tcar | Adm | Tcri | Mis |
| *With reference:* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 | Yes | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 |
| 2 | Yes | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 |
| 3 | Yes | 11 | 1 | 0 | 0 | 11 | 1 | 0 | 0 | 11 | 1 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 |
| 4 | Yes | 3 | 9 | 0 | 0 | 4 | 8 | 0 | 0 | 3 | 9 | 0 | 0 | 11 | 1 | 0 | 0 | 7 | 5 | 0 | 0 | 7 | 5 | 0 | 0 | 11 | 1 | 0 | 0 |
| 5 | Yes | 0 | 12 | 0 | 0 | 0 | 11 | 1 | 1 | 0 | 12 | 0 | 0 | 1 | 5 | 6 | 6 | 1 | 6 | 5 | 5 | 1 | 6 | 5 | 5 | 1 | 5 | 6 | 6 |
| 6 | Yes | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 1 | 11 | 11 | 0 | 3 | 9 | 9 | 0 | 4 | 8 | 8 | 0 | 1 | 11 | 11 |
| 7 | Yes | 0 | 12 | 0 | 0 | 0 | 11 | 1 | 1 | 0 | 10 | 2 | 2 | 0 | 2 | 10 | 10 | 0 | 3 | 9 | 9 | 0 | 2 | 10 | 10 | 0 | 3 | 9 | 9 |
| 8 | Yes | 0 | 11 | 1 | 0 | 0 | 7 | 5 | 4 | 0 | 10 | 2 | 1 | 0 | 0 | 12 | 11 | 0 | 0 | 12 | 11 | 0 | 1 | 11 | 10 | 0 | 0 | 12 | 11 |
| 9 | Yes | 0 | 12 | 0 | 0 | 0 | 5 | 7 | 6 | 0 | 12 | 0 | 0 | 0 | 0 | 12 | 12 | 0 | 0 | 12 | 12 | 0 | 0 | 12 | 12 | 0 | 0 | 12 | 12 |
| 10 | Yes | 0 | 11 | 1 | 0 | 0 | 5 | 7 | 6 | 0 | 9 | 3 | 2 | 0 | 0 | 12 | 11 | 0 | 1 | 11 | 10 | 0 | 2 | 10 | 9 | 0 | 1 | 11 | 11 |
| 11 | No | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 |
| Total | | 38 | 80 | 14 | 0 | 39 | 60 | 33 | 18 | 38 | 75 | 19 | 5 | 48 | 9 | 75 | 61 | 44 | 18 | 70 | 56 | 44 | 20 | 68 | 54 | 48 | 11 | 73 | 60 |
| *Without reference:* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 | Yes | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 |
| 2 | Yes | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 |
| 3 | Yes | 11 | 1 | 0 | 0 | 11 | 1 | 0 | 0 | 11 | 1 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 11 | 1 | 0 | 0 | 12 | 0 | 0 | 0 |
| 4 | Yes | 3 | 9 | 0 | 0 | 4 | 8 | 0 | 0 | 3 | 9 | 0 | 0 | 11 | 1 | 0 | 0 | 5 | 7 | 0 | 0 | 3 | 9 | 0 | 0 | 11 | 1 | 0 | 0 |
| 5 | Yes | 0 | 12 | 0 | 0 | 0 | 11 | 1 | 1 | 0 | 12 | 0 | 0 | 1 | 5 | 6 | 5 | 1 | 10 | 1 | 1 | 1 | 10 | 1 | 1 | 1 | 6 | 5 | 5 |
| 6 | Yes | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 1 | 11 | 11 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 1 | 11 | 11 |
| 7 | Yes | 0 | 12 | 0 | 0 | 0 | 10 | 2 | 2 | 0 | 11 | 1 | 0 | 0 | 2 | 10 | 10 | 0 | 8 | 4 | 4 | 0 | 8 | 4 | 4 | 0 | 3 | 9 | 9 |
| 8 | Yes | 0 | 11 | 1 | 0 | 0 | 6 | 6 | 5 | 0 | 10 | 2 | 1 | 0 | 0 | 12 | 11 | 0 | 2 | 10 | 9 | 0 | 2 | 10 | 9 | 0 | 0 | 12 | 11 |
| 9 | Yes | 0 | 12 | 0 | 0 | 0 | 4 | 8 | 8 | 0 | 12 | 0 | 0 | 0 | 0 | 12 | 12 | 0 | 1 | 11 | 11 | 0 | 3 | 9 | 9 | 0 | 0 | 12 | 12 |
| 10 | Yes | 0 | 11 | 1 | 0 | 0 | 5 | 7 | 6 | 0 | 9 | 3 | 2 | 0 | 0 | 12 | 11 | 0 | 4 | 8 | 7 | 0 | 4 | 8 | 7 | 0 | 1 | 11 | 10 |
| 11 | No | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 | 0 |
| Total | | 38 | 80 | 14 | 0 | 39 | 57 | 36 | 22 | 38 | 76 | 18 | 3 | 48 | 9 | 75 | 60 | 42 | 44 | 46 | 32 | 39 | 49 | 44 | 30 | 48 | 12 | 72 | 58 |

Abbreviations: Tcar = *T. carnifex*; Adm = genetically admixed; Tcri = *T. cristatus*; and Mis = misidentified.

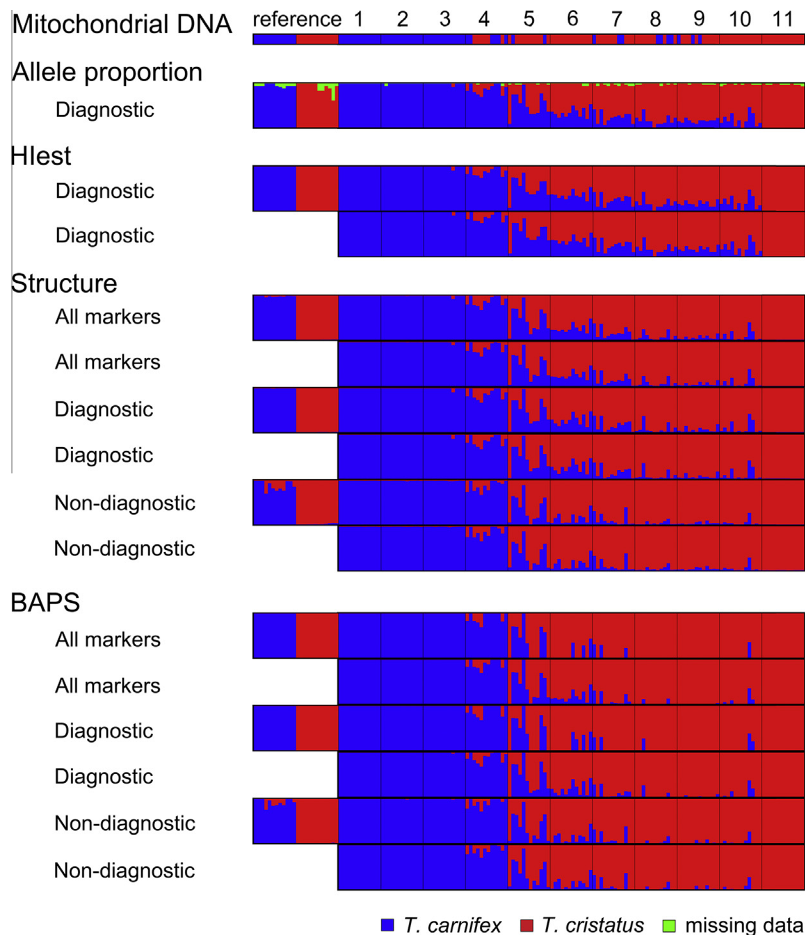


**Fig. 3.** Individual crested newts from the Veluwe, assigned to the two parental crested newt species. From top to bottom, genetic composition based on mitochondrial DNA, species specific allele proportion, ancestry *S* determined with HIest, Structure with complete, diagnostic, and non-diagnostic dataset, and BAPS with complete, diagnostic, and non-diagnostic dataset. Analyzes for Structure and BAPS were run with and without reference populations for the parental species. Bar plot boxes either represent reference populations or Veluwe populations (1–11). Bars represent the probability or proportion with which individuals belong to *T. cristatus* (red) and *T. carnifex* (blue); green (hybrid index only) represents missing data or alleles of unknown provenance. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

an expansion of the introduced *T. carnifex* at the expense of the native *T. cristatus*.

The mitochondrial DNA shows that the stock that gave rise to the Veluwe *T. carnifex* derives from the northern Balkans (Fig. A2; Canestrelli et al., 2012; Wielstra et al., 2013). Genetic pollution of *T. carnifex* reaches further than understood from morphological data (Bogaerts, 2002; Bosman and van Delft, 2011; van Hoogen and Crombaghs, 2012; Fig. A1) and is underestimated when relying on mitochondrial DNA alone (Fig. 3). The '*T. carnifex* core' appears to be positioned near Vaassen, the site where *T. carnifex* is thought to have been introduced in the late 1970s. The outer boundary of *T. carnifex* genetic material is situated approximately 10 km away (Fig. 1). Hence, the effective speed of dispersal of *T. carnifex* on the Veluwe is c. 250 m/year, which is roughly similar to the situation in the Basin of Geneva where introduced *T. carnifex* has been observed to disperse 14 km in 50 years at the expense of native *T. cristatus* (Arntzen, unpublished data).

Whether species displacement and hence hybrid zone movement is ongoing is unknown. Hybrid zones are known to stabilize along ecotones (Moore, 1977). Additionally, local density of the native species influences the process of species displacement and a high density native population would be harder to replace than a low density one, a phenomenon referred to as high density blocking (Waters et al., 2013). The dry and cultivated Veluwe area does not constitute typical *T. cristatus* habitat and the wider ecological amplitude of *T. carnifex* and its greater resistance to human disturbance of water bodies could have facilitated species displacement (Arntzen, 2003). Unfortunately, due to scarcity of field records for the study area from before the introduction of *T. carnifex*, we cannot make a clear statement on the former density of *T. cristatus* in the area of species displacement (Spitzen - van der Sluijs et al., 2007). However, genetic pollution has by now reached a core area of *T. cristatus* with habitat more typical for the native species. Follow-up studies are needed to test the stability of the hybrid zone. Nevertheless, even a stable hybrid zone would provide a window for genetic pollution via introgressive hybridization (Fitzpatrick and Shaffer, 2007).

### 4.3. Management of invasive hybridizers

With an elaborate methodology, such as used in this study, it is possible to identify morphologically similar species and their genetically admixed offspring. However, quantifying genetic pollution is only the first step, translating this information into management is the next one. Instead of focussing on fixed threshold values to label individuals as pure or contaminated, Allendorf et al. (2001) raised two factors to be considered in the management of invasive hybridizers. The first factor is whether pure populations of the native species are rare; if this is the case, hybrid populations have an increased conservation and restoration value. The red wolf

(*C. rufus*), genetically polluted by the jackal (*C. latrans*) (Allendorf et al., 2001) and the Californian tiger salamander (*A. californiense*), genetically polluted by the eastern tiger salamander (*A. tigrinum*) (Riley et al., 2003) are examples of rare species where genetic pollution has been so extensive that it affects most if not all of the populations. In such cases where cleansing the genetically polluted area of foreign alleles is no longer feasible it has been argued to restore the ecological authenticity of the original species (Fitzpatrick et al., 2010). The second factor is whether continued existence of hybrid populations poses a threat to unpolluted native populations via gene flow. In cases where such pollution was deemed a risk, culling of the invasive species and genetically admixed individuals has been conducted for e.g. the invasive ruddy duck (*Oxyura jamaicensis*) threatening the native white headed duck (*Oxyura leucocephala*) (Smith et al., 2005) and the invasive mallard (*A. platyrhynchos*) threatening the native Pacific black duck (*A. superciliosa*) (Tracey et al., 2008).

Given the (inter)national protected status of *T. cristatus* it is the Dutch government's responsibility to protect this species from the introduced *T. carnifex*. Hence, management involving removing the invasive *T. carnifex* might be set into place. Fortunately, the genetic swamping of the threatened, native *T. cristatus* by the invasive *T. carnifex* is currently geographically restricted. However, considering that genetically pollution on the Veluwe has now reached the core distribution area of the native species, the continued existence of hybrid populations poses a considerable threat to unpolluted native populations. Following the guidelines proposed by Allendorf et al. (2001), the invasive and hybrid populations have a low conservation value, while they pose a threat to the surrounding native populations. Hence, populations that contain invasive individuals beyond reasonable doubt could be targeted for direct eradication. Although this is a practical solution, we have observed individuals in such populations that were identified as genetically pure native *T. cristatus*. Hence, culling contaminated populations could cause casualties among pure native *T. cristatus* which, considering the protected status of the species, might have legal consequences. Currently the legal status of a genetically admixed individual is unclear and how to deal with populations containing such individuals is a legislative vacuum. Hence, policy on protecting the genetic authenticity of native species in the face of hybridizing invasive species is urgently needed and genetic screening is likely to play a key role in it.

## Data accessibility

Raw Ion Torrent reads in FASTQ format, scripts associated with the bioinformatics pipeline, BWA alignments in SAM format, Raw SNP reports in VCF format, filtered SNP report used to construct consensus sequences, FASTA files of reconstructed sequences, ND4 SPF file, input files for HIest, Structure and BAPS, input files for the data simulation exercises: Dryad Digital Repository entry http://dx.doi.org/10.5061/dryad.1fj75.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocon.2015.01.022.

## References


Adams, J., Leonard, J., Waits, L., 2003. Widespread occurrence of a domestic dog mitochondrial DNA haplotype in southeastern US coyotes. Mol. Ecol. 12, 541–546.

Allendorf, F.W., Leary, R.F., Spruell, P., Wenburg, J.K., 2001. The problems with hybrids: setting conservation guidelines. Trends Ecol. Evol. 16, 613–622.

Amish, S.J., Hohenlohe, P.A., Painter, S., Leary, R.F., Muhlfeld, C., Allendorf, F.W., Luikart, G., 2012. RAD sequencing yields a high success rate for westslope cutthroat and rainbow trout species-diagnostic SNP assays. Mol. Ecol. Resour. 12, 653–660.

Arntzen, J.W., 2003. *Triturus cristatus* superspecies – Kammmolch Artenkreis, including *T. cristatus* (Laurenti, 1768) – Northern crested newt, *T. carnifex* (Laurenti, 1768) – Italian crested newt, *T. dobrogicus* (Kiritzescu, 1903) – Danube crested newt and *T. karelinii* (Strach, 1870) – Southern crested newt. In: Grossenbacher, K., Thiesmeier, B., (Eds.), Handbuch der Reptilien und Amphibien Europas. Aula Verlag, Wiesbaden, pp. 421–514.

Arntzen, J.W., Thorpe, R.S., 1999. Italian crested newts (*Triturus carnifex*) in the basin of Geneva: distribution and genetic interactions with autochthonous species. Herpetologica, 423–433.

Arntzen, J.W., Wielstra, B., Wallis, G.P., 2014. The modality of nine *Triturus* newt hybrid zones assessed with nuclear, mitochondrial and morphological data. Biol. J. Linn. Soc. 113, 604–622.

Ballard, J.W.O., Whitlock, M.C., 2004. The incomplete natural history of mitochondria. Mol. Ecol. 13, 729–744.

Barton, N.H., Hewitt, G., 1985. Analysis of hybrid zones. Annu. Rev. Ecol. Syst., 113–148

Beaumont, M., Barratt, E., Gottelli, D., Kitchener, A., Daniels, M., Pritchard, J., Bruford, M.W., 2001. Genetic diversity and introgression in the Scottish wildcat. Mol. Ecol. 10, 319–336.

Bogaerts, S., 2002. Italian crested newts, *Triturus carnifex*, on the Veluwe, Netherlands. Z. Feldherpetologie 9, 217–221.

Bohling, J.H., Adams, J.R., Waits, L.P., 2013. Evaluating the ability of Bayesian clustering methods to detect hybridization and introgression using an empirical red wolf data set. Mol. Ecol. 22, 74–86.

Bosman, W., van Delft, J., 2011. Verspreiding van de Italiaanse kamsalamander in Nederland en mogelijkheden voor beheersing en eliminatie. Stichting RAVON, rapportnr 2011-008.

Brede, E.G., Thorpe, R.S., Arntzen, J.W., Langton, T.E.S., 2000. A morphometric study of a hybrid newt population (*Triturus cristatus/T. carnifex*): Beam Brook Nurseries, Surrey, UK. Biol. J. Linn. Soc. 70, 685–695.

Bright, C., 1999. Invasive species: pathogens of globalization. Foreign Policy, 50–64.

Burke, J.M., Arnold, M.L., 2001. Genetics and the fitness of hybrids. Annu. Rev. Genet. 35, 31–52.

Butler, D., 1994. Bid to protect wolves from genetic pollution. Nature 370, 497.

Callan, H., Spurway, H., 1951. A study of meiosis in interracial hybrids of the newt, *Triturus cristatus*. J. Genet. 50, 235–249.

Canestrelli, D., Salvi, D., Maura, M., Bologna, M.A., Nascetti, G., 2012. One species, three Pleistocene evolutionary histories: phylogeography of the Italian crested newt, *Triturus carnifex*. PloS One 7, e41754.

Coombs, J., Letcher, B., Nislow, K., 2008. CREATE: a software to create input files from diploid genotypic data for 52 genetic software programs. Mol. Ecol. Resour. 8, 578–580.

Corander, J., Marttinen, P., Siren, J., Tang, J., 2008. Enhanced Bayesian modelling in BAPS software for learning genetic structures of populations. BMC Bioinf. 9, 539.

Crispo, E., Moore, J.-S., Lee-Yaw, J.A., Gray, S.M., Haller, B.C., 2011. Broken barriers: human-induced changes to gene flow and introgression in animals. BioEssays 33, 508–518.

Currat, M., Ruedi, M., Petit, R.J., Excoffier, L., 2008. The hidden side of invasions: massive introgression by local genes. Evolution 62, 1908–1920.

Davison, A., Birks, J., Griffiths, H., Kitchener, A., Biggins, D., Butlin, R., 1999. Hybridization and the phylogenetic relationship between polecats and domestic ferrets in Britain. Biol. Conserv. 87, 155–161.

van Delft, J.J.C.W., Creemers, R.C.M., Spitzen-van der Sluijs, A.M., 2007. Basisrapport Rode Lijst Amfibieën en Reptielen volgens Nederlandse en IUCN-criteria. Stichting RAVON, Nijmegen.

Dittrich-Reed, D.R., Fitzpatrick, B.M., 2013. Transgressive hybrids as hopeful monsters. Evol. Biol., 1–6

Dubois, A., 2006. Species introductions and reintroductions, faunistic and genetic pollution: some provocative thoughts. Alytes 24, 147–164.

Fitzpatrick, B.M., 2012. Estimating ancestry and heterozygosity of hybrids using molecular markers. BMC Evol. Biol. 12, 131.

Fitzpatrick, B.M., Johnson, J.R., Kump, D.K., Smith, J.J., Voss, S.R., Shaffer, H.B., 2010. Rapid spread of invasive genes into a threatened native species. Proc. Natl. Acad. Sci. 107, 3606–3610.
Fitzpatrick, B.M., Shaffer, H.B., 2007. Hybrid vigor between native and introduced salamanders raises new challenges for conservation. Proc. Natl. Acad. Sci. 104, 15793–15798.
Goka, K., 1998. Influence of invasive species on native species—will the European bumble bee, *Bombus terrestris*, bring genetic pollution into the Japanese native species. Bull. Biograph. Soc. Jpn. 53, 91–101.
Gottelli, D., Sillero-Zubiri, C., Applebaum, G.D., Roy, M.S., Girman, D.J., Garcia-Moreno, J., Ostrander, E.A., Wayne, R.K., 1994. Molecular genetics of the most endangered canid: the Ethiopian wolf *Canis simensis*. Mol. Ecol. 3, 301–312.
Hewitt, G.M., 1988. Hybrid zones-natural laboratories for evolutionary studies. Trends Ecol. Evol. 3, 158–167.
Hohenlohe, P.A., Amish, S.J., Catchen, J.M., Allendorf, F.W., Luikart, G., 2011. Next-generation RAD sequencing identifies thousands of SNPs for assessing hybridization between rainbow and westslope cutthroat trout. Mol. Ecol. Resour. 11, 117–122.
van Hoogen, D., Crombaghs, B., 2012. Verspreidingsonderzoek Italiaanse kamsalamander, 2012. Natuurbalans - Limes Divergens BV, Nijmegen.
Jiggins, C.D., Mallet, J., 2000. Bimodal hybrid zones and speciation. Trends Ecol. Evol. 15, 250–255.
Largiadèr, C.R., 2007. Hybridization and introgression between native and alien species. Biological Invasions. Springer, pp. 275–292.
Leary, R.F., Allendorf, F.W., Forbes, S.H., 1993. Conservation genetics of bull trout in the Columbia and Klamath River drainages. Conserv. Biol. 7, 856–865.
Maddison, D.R., Maddison, W.P., 2005. MacClade 4: Analysis of Phylogeny and Character Evolution, Version 4.08. Sinauer Associates, Sunderland, Massachusetts.
Maletzky, A., Mikulíček, P., Franzen, M., Goldschmid, A., Gruber, H.-J., Horak, A., Kyek, M., 2008. Hybridization and introgression between two species of crested newts (*Triturus cristatus* and *T. carnifex*) along contact zones in Germany and Austria: morphological and molecular data. Herpetol. J. 18, 1–15.
Mallet, J., 2005. Hybridization as an invasion of the genome. Trends Ecol. Evol. 20, 229–237.
Mikulíček, P., Horák, A., Zavadil, V., Kautman, J., Piálek, J., 2012. Hybridization between three crested newt species (*Triturus cristatus* superspecies) in the Czech Republic and Slovakia: comparison of nuclear markers and mitochondrial DNA. Folia Zool. 61, 202–218.
Monzón, J., Kays, R., Dykhuizen, D., 2014. Assessment of coyote–wolf–dog admixture using ancestry-informative diagnostic SNPs. Mol. Ecol. 23, 182–197.
Mooney, H.A., Cleland, E.E., 2001. The evolutionary impact of invasive species. Proc. Natl. Acad. Sci. 98, 5446–5451.
Moore, W.S., 1977. An evaluation of narrow hybrid zones in vertebrates. Quart. Rev. Biol. 52, 263–277.
Nielsen, E.E., Bach, L.A., Kotlicki, P., 2006. Hybridlab (version 1.0): a program for generating simulated hybrids from population samples. Mol. Ecol. Notes 6, 971–973.
Oliveira, R., Godinho, R., Randi, E., Alves, P.C., 2008. Hybridization versus conservation: are domestic cats threatening the genetic integrity of wildcats (*Felis silvestris silvestris*) in Iberian Peninsula? Philos. Trans. Roy. Soc. B: Biol. Sci. 363, 2953–2961.
Petit, R.J., 2004. Biological invasions at the gene level. Divers. Distrib. 10, 159–165.
Pimentel, D., McNair, S., Janecka, J., Wightman, J., Simmonds, C., O'Connell, C., Wong, E., Russel, L., Zern, J., Aquino, T., Tsomondo, T., 2001. Economic and environmental threats of alien plant, animal, and microbe invasions. Agric. Ecosyst. Environ. 84, 1–20.
Pritchard, J.K., Stephens, M., Donnelly, P., 2000. Inference of population structure using multilocus genotype data. Genetics 155, 945–959.
Randi, E., Pierpaoli, M., Beaumont, M., Ragni, B., Sforzi, A., 2001. Genetic identification of wild and domestic cats (*Felis silvestris*) and their hybrids using Bayesian clustering methods. Mol. Biol. Evol. 18, 1679–1693.
Rhymer, J.M., Simberloff, D., 1996. Extinction by hybridization and introgression. Annu. Rev. Ecol. Syst. 27, 83–109.
Rhymer, J.M., Williams, M.J., Braun, M.J., 1994. Mitochondrial analysis of gene flow between New Zealand mallards (*Anas platyrhynchos*) and grey ducks (*A. superciliosa*). The Auk, 970–978.
Riley, S.P., Bradley Shaffer, H., Randal Voss, S., Fitzpatrick, B.M., 2003. Hybridization between a rare, native tiger salamander (*Ambystoma californiense*) and its introduced congener. Ecol. Appl. 13, 1263–1275.
Rosenblum, E.B., Novembre, J., 2007. Ascertainment bias in spatially structured populations: a case study in the eastern fence lizard. J. Hered. 98, 331–336.
Sacks, B.N., Moore, M., Statham, M.J., Wittmer, H.U., 2011. A restricted hybrid zone between native and introduced red fox (*Vulpes vulpes*) populations suggests reproductive barriers and competitive exclusion. Mol. Ecol. 20, 326–341.
Schlötterer, C., 2004. The evolution of molecular markers—just a matter of fashion? Nat. Rev. Genet. 5, 63–69.
Selkoe, K.A., Toonen, R.J., 2006. Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. Ecol. Lett. 9, 615–629.
Smith, G., Henderson, I., Robertson, P., 2005. A model of ruddy duck *Oxyura jamaicensis* eradication for the UK. J. Appl. Ecol. 42, 546–555.
Spitzen - van der Sluijs, A.M., Willink, G.W., Creemers, R., Ottburg, F.G.W.A., de Boer, R.J., Pfaff, P.M.L., de Wild, W.W., Stronks, D.J., Schröder, R.J.H., de Vos, M.T., Soes, D.M., Frigge, P., Struijk, R.P.J.H., 2007. Atlas reptielen en amfibieën in Gelderland 1985–2005. Stichting RAVON, Nijmegen.
Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739.
Tracey, J.P., Lukins, B.S., Haselden, C., 2008. Hybridisation between Mallard (*Anas platyrhynchos*) and Grey Duck (*A. superciliosa*) on Lord Howe Island and management options. Notornis 55, 1–7.
Twyford, A., Ennos, R., 2011. Next-generation hybridization and introgression. Heredity 108, 179–189.
Vilà, C., Walker, C., Sundqvist, A.-K., Flagstad, Ø., Andersone, Z., Casulli, A., Kojola, I., Valdmann, H., Halverson, J., Ellegren, H., 2003. Combined use of maternal, paternal and bi-parental genetic markers for the identification of wolf–dog hybrids. Heredity 90, 17–24.
Vleut, I.J.J., Bosman, W., 2005. Actuele verspreiding van de Italiaanse kamsalamander (*Triturus carnifex*) in Gelderland en een morfometrische vergelijking met de inheemse kamsalamander (*Triturus cristatus*). Rapport stichting RAVON, Nijmegen.
Waters, J.M., Fraser, C.I., Hewitt, G.M., 2013. Founder takes all: density-dependent processes structure biodiversity. Trends Ecol. Evol. 28, 78–85.
Wielstra, B., Arntzen, J.W., 2011. Unraveling the rapid radiation of crested newts (*Triturus cristatus* superspecies) using complete mitogenomic sequences. BMC Evol. Biol. 11, 162.
Wielstra, B., Arntzen, J.W., 2012. Postglacial species displacement in *Triturus* newts deduced from asymmetrically introgressed mitochondrial DNA and ecological niche models. BMC Evol. Biol. 12, 161.
Wielstra, B., Babik, W., Arntzen, J.W., 2015. The crested newt *Triturus cristatus* recolonized temperate Eurasia from an extra-Mediterranean glacial refugium. Biol. J. Linn. Soc. http://dx.doi.org/10.1111/bij.12446.
Wielstra, B., Crnobrnja-Isailovic, J., Litvinchuk, S.N., Reijnen, B.T., Skidmore, A.K., Sotiropoulos, K., Toxopeus, A.G., Tzankov, N., Vukov, T., Arntzen, J.W., 2013. Tracing glacial refugia of *Triturus* newts based on mitochondrial DNA phylogeography and species distribution modeling. Front. Zool. 10, 1–14.
Wielstra, B., Duijm, E., Lagler, P., Lammers, Y., Meilink, W.R.M., Ziermann, J.M., Arntzen, J.W., 2014. Parallel tagged amplicon sequencing of transcriptome-based genetic markers for *Triturus* newts with the Ion Torrent next-generation sequencing platform. Mol. Ecol. Resour. 14, 1080–1089.