



Extent of introgressive hybridization in the Hermann's tortoise (*Testudo hermanni hermanni*) from the south of France

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Abstract

The Western subspecies of Hermann's tortoise (*Testudo hermanni hermanni*: WT) is threatened by habitat loss, fragmentation, wildfires, illegal harvesting, and likely hybridization with *Testudo hermanni boettgeri* (ET), a subspecies introduced from Eastern Europe. To assess the prevalence of this hybridization, we used microsatellite markers and simulations to investigate the genetic status of 565 individuals of the Var district (France) in contrast to the genetic signature of 121 individuals sampled in the Balkans. The genetic differentiation between WT and ET indicated 18% of tortoises in the Var were hybrids between WT and ET (i.e. F1, F2, and F3). Although hybridization increases the genetic diversity within the genetically impoverished WT population, hybridization could also threaten WT genetic integrity. Identifying and removing all hybridized individuals (especially beyond F1) is logistically unfeasible. Instead, conservation actions should reinforce communication and education, notably towards pet owners, to limit further hybridization. Moreover, accurate genetic identification of captives is essential to programs that involve translocating individuals to fragile populations (e.g. those severely impacted by bush fires). Further studies should assess the extent that WT/ET hybridization is detrimental or beneficial to populations facing rapid global changes in the context of depressed genetic diversity.

Keywords Conservation · *Testudo hermanni* · Genetic introgression · Microsatellites · Reptile

Introduction

Understanding and monitoring hybridization is essential to conservation (Frankham et al. 2010), yet it is a particularly challenging, complex, and controversial problem (Gilman

and Behm 2011; Quilodran et al. 2018; Allendorf et al. 2001). Hybridization is defined as the interbreeding between individuals or populations that differ by one or more heritable characters (Harrison 1990; Grabenstein and Taylor 2018). Hybridization can cause genetic introgression and degrees of admixture that depends on hybrid fitness. Hybridization is also a factor of concern with respect to species conservation. Indeed, although the addition of new alleles may represent a raw material for adaptive evolutionary responses, these new alleles can also break the co-adapted genetic complexes, thus reducing local adaptations and representing a significant threat to populations (Tallmon et al. 2004; Frankham et al. 2010). Humans can cause hybridizations by (i) habitat disturbances that promote interactions between previously isolated taxa or (ii) via releases of non-native taxa. Before mitigating hybridization via removal or sterilization of hybrids, and reinforcing native populations through captive breeding programs, it is prudent to assess the prevalence and risk of hybridization.

The Eastern Hermann's tortoise (*Testudo hermanni boettgeri* [ET]; Mojsisovics 1889) largely occurs in the Balkan

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Peninsula, while the Western Hermann's tortoise (*T. h. hermanni* [WT]; Gmelin, 1789) is restricted to scattered and small continental populations (Italy, France, and Spain) and Mediterranean island populations (i.e. Mallorca, Minorca, Corsica, Sardinia, and Sicily). The subspecies' ranges contact in north-eastern Italy where they may hybridize naturally (Perez et al. 2014). *Testudo hermanni* is Near Threatened (Van Dijk et al. 2004), but WT is vulnerable in France due to population declines mainly caused by habitat destruction (agriculture, urbanization, wildfires) and overharvesting. In the Var district, continental populations (Fig. 1) are endangered (Livoreil 2009) and are geographically isolated from the nearest populations located in northern Italy about 300–400 km. Currently, the species benefits from a National Action Plan (PNA, 2018–2027) that aims to set up conservation measures (Celse et al. 2017).

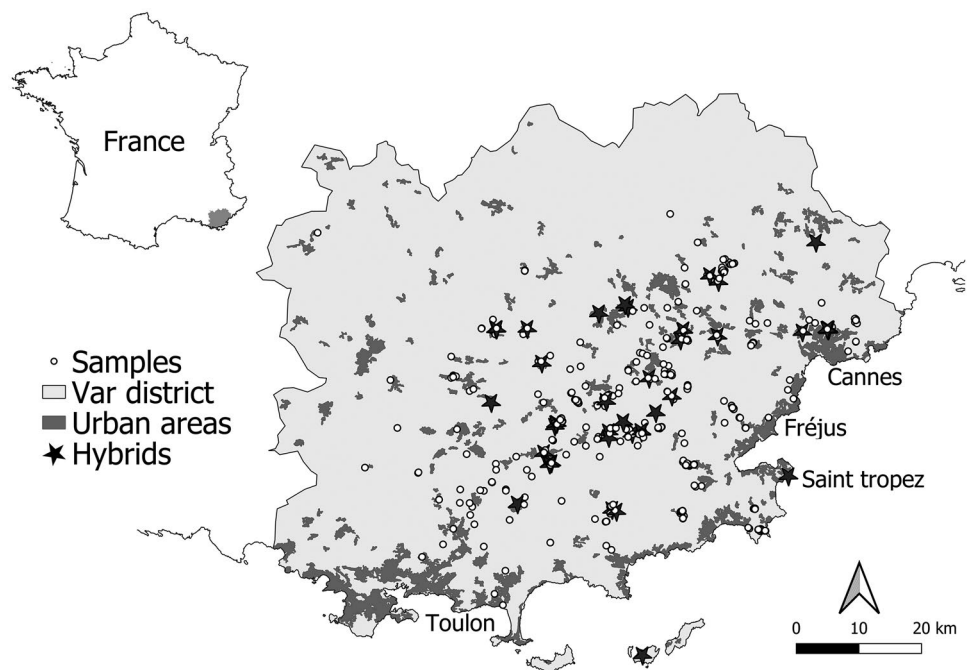
Since the middle of the nineteenth century, besides habitat destruction and over-harvesting, WT populations were faced to the introduction of ET individuals, particularly through massive imports of ET in Western Europe for pet trade. Moreover, in the 1990s, the SOPTOM released hundreds of tortoises in WT populations for conservation purposes, notably to restore populations (Devaux 1990). However, the origin of the released individuals was uncertain because it is very difficult to identify *Testudo hermanni* subspecies on clear morphological traits and no appropriate genetic tools were available at the time. (Bertolero et al. 2011). Currently, this uncontrolled releasing practice is banned, but many tortoises from undetermined origin continue to escape from private gardens (Ballouard et al. 2021). Indeed, pet trades has intensified considerably in recent decades (Moll and

Moll 2004; Zhou and Jiang 2008; Gong et al. 2009; Kraus 2008; Kopecký et al. 2013; Nikolić et al. 2018) and massive ET tortoises (mainly from Turkey and ex-Yugoslavia) were exported to Western countries (e.g. UK, Germany, France) during the 1970s (Lambert 1984; Ljubisavljević, 2011). As an official measure to prevent overexploitation and uncontrolled pet trade, *Testudo hermanni* has been listed by the Convention on international Trade in endangered Species of Wild fauna and Flora (CITES). However, imported tortoises were often released into gardens where they had high opportunities to reproduce and hybridize with local individuals. *T. hermanni* was prohibited for sale in pet-shops from the 1980s to 2006 (trade ban by the Washington convention and European Union), but sales are currently authorized in France under strict conditions (e.g. owning a breeding certificate). In practice, pet owners cannot determine the origin of the tortoises they possess. Furthermore, intensive illegal harvesting, breeding, and trading continue in southern France, causing multiple contacts between WT and ET (Guyot and Pritchard 1999; Celse et al. 2017).

To better understand the extent of hybridization, three studies, based on microsatellites molecular markers (Perez et al. 2014; Zenboudji et al. 2016; Biello et al. 2021), identified hybrids between the two sub-species, in continental populations of the Var district (France), the Ebro delta (Spain), and Northern and Central Italy. Particularly in France, much more information is needed to quantify hybridization threat to WT populations.

Here, we evaluate the extent of WT/ET hybridization in continental France, with large numbers of WT sampled in the field in the Var district and ET sampled in their natural

Fig. 1 Geographic locations of sampled individuals and urban areas in the Var district (the main cities are indicated on the map). Hybrids, inferred from NEWHYBRID software, are represented with black stars



range (North Macedonia), to improve the evaluation of hybridization. This study aimed to (i) estimate the proportion of WT/ET hybrids in the Var district, (ii) assess the level of hybridization using simulation of hybrids across generations (F1, F2, and F3), (iii) evaluate the genetic diversity of pure WT Var populations, and (iv) recommend management priorities of hybrids and French populations.

Materials and methods

Sampling

The main sites were in southeastern France's Var District, home to France's last mainland WT populations. Between 2012 and 2016, we sampled 565 wild tortoises in the remaining suitable patches. Moreover, in 2014–2015, we collected 22 captive bred individuals collected from 12 private farms/owners. Finally, we sampled 121 wild ET individuals in North Macedonia to obtain adequate genetic references (Bonnet et al. 2016a). The sampling was realized without any phenotype discrimination; that is, all captured tortoises were sampled. Blood samples were collected by puncture of the dorsal supra-carapacial cervical plexus (Bonnet et al. 2016a, b) and were then stored at -20°C . We analysed 708 individuals' samples. We used handheld GPS units to measure tortoise location (World Geodetic System 1984, WGS 84; Fig. 1).

Molecular markers

We extracted DNA from the 708 blood samples on a King-Fisher Flex purification system at the "GenSeq" platform of the LaBex CeMEB (Montpellier, France) using the *Nucleo-Mag® Blood* kit (Macherey–Nagel). We genotyped all DNA samples using 16 microsatellite molecular markers: THER4, THER20, THER23, THER36, THER40, THER48, THER51, THER69, THER73, THER94, THER101, THER106, THER110, THER112 (Zeboudji et al. 2018), and Test10 and Test56 (Forlani et al. 2005). Loci were amplified by polymerase chain reaction (PCR) and the QIAGEN multiplex kit according to the manufacturer standard microsatellite amplification protocol in a final volume of 10 μL and at 57°C for annealing temperature. PCR amplifications of microsatellite loci were performed in five multiplexes: THER106, THER110, THER51, and THER73 were grouped in the multiplex 1; THER23, THER101, and THER48 in the multiplex 2; Test 10, THER20, THER40, and THER69 in the multiplex 3; THER4, THER112, and THER36 in the multiplex 4; and Test56 and THER94 in the multiplex 5. We added 3 μL of PCR product to 15 μL of formamide and 0.5 μL of the LIZ600 standard (Life Technologies). Amplified products were detected on an ABI PRISM 3130 Genetic

analyzer at the "GenSeq" platform, and electrophoregrams were visualized using Genemapper version 3.7 (Applied Biosystems). We tested the presence of null alleles or scoring errors due to stuttering using the software Micro-Checker v 2.2.3 (Oosterhout et al. 2004). We assessed linkage disequilibria using exact tests (1200 permutations) implemented in Fstat v.2.9.3.2. (Goudet 2001). For all these analyses, we adjusted the level of significance for multiple testing using a Bonferroni correction.

Genetic analysis

Genetic polymorphism

We estimated genetic parameters over all loci using the GenAlEx software v 6.2 (Peakall and Smouse 2006): the allelic richness (AR), the expected heterozygosity (He), and the fixation index (Fis). From the Fis, we evaluated the departures from Hardy–Weinberg expectations (we adjusted the level of significance for multiple testing using a Bonferroni correction). AR and He were compared between different populations using Wilcoxon tests as implemented in the GraphPad InStat software v.3.10[®].

Genetic structure

At the individual scale, we assessed the global genetic structure using the Bayesian approach implemented in Structure v.2.0 (Pritchard et al. 2000). We choose the admixture model and the option of correlated allele frequencies among populations. This software allowed the assignment of individuals to a user-defined number of clusters (K) with K varying from 1 to 7. Each simulation was replicated 20 times as recommended (Evanno et al. 2005) with 10,000 burn-in iterations followed by 1,000,000 sample iterations. The number of independent genetic populations (K) was obtained using Structure Harvester software v.0.6.1 (Earl and vonHoldt 2012) which compared the likelihood of the data for the different values of K (Evanno et al. 2005).

Identification and distribution of hybrids

We estimated the level of genetic hybridization between both sub-species using simulations. From the previous Bayesian Structure analysis, the inferred proportions of ancestry were used to select, among all analysed individuals, those that revealed inferred proportions of ancestry > 0.97 for *T. h. hermanni* or *T. h. boetgeri*. Then, genotypes of selected individuals were implemented as "parent genotypes" in the software Hybridlab v1.0 (Nielsen et al. 2006) which simulated random mating and supplies in silico genotypes for artificial hybrids. Thus, 19 hybrid categories were created (F1, F2, F3, and all possible backcrosses; see supplementary file S1);

each of them composed of 40 hybrids. Hence, 760 artificial hybrids were generated in silico and combined to the 708 samples collected in the field. All samples (1468) were then analysed with the software NewHybrids (Anderson and Thompson 2002) to estimate the inference of each individual to five predefined different genotypic frequency categories: each pure sub-species (i.e. Pure_ *T.h.h.* and Pure_ *T.h.b.*) and hybrids (i.e. F1 hybrids, F2 hybrids, and F3 hybrids) (supplementary file S1). This procedure allowed us (i) to test the reliability of our approach by verifying the inference of our 760 known artificially generated hybrids to the suitable hybrid category and (ii) to infer the genotypic frequency categories for each individual collected in the field. An individual was assigned to a genotypic frequency category when its posterior probability was > 0.80 . NewHybrids was run with 10,000 burn-in iterations followed by 1,000,000 Markov chain Monte Carlo iterations. Analyses did not use prior allele frequency information but considered uniform prior distributions for Θ and p parameters (Anderson and Thompson 2002). Then, we used a statistical linear model (R packages lme4, Mass and nlme) to test if the proportion of hybrid was significantly different between populations.

Genetic structure of wild *T.h. hermanni* populations

To investigate the genetic structure of native *T.h. hermanni* populations (without the effect of the genetic signature of ET and WT/ET hybrids), we kept only the individuals previously inferred as pure_ *T.h.h.* in the results from NewHybrids software (Anderson and Thompson 2002). The longitude and latitude of these individuals allowed us to test the occurrence of isolation-by-distance (IBD) by regressing genetic distances against Euclidian distances using a simple Mantel test implemented in Allele In Space software (Miller 2005). We analyzed the population structure of pure WT in France with Structure v.2.0 (Pritchard et al. 2000) and using the same methodology and parameters as previously described for all samples.

Results

Molecular marker validation

For the 16 microsatellite loci, we detected no evidence for null allele (i.e. at a P -value threshold of $= 0.0001$ after Bonferroni correction) or for linkage disequilibrium (i.e. at a P -value threshold of $= 0.0004$ after Bonferroni correction). Only 24 tests out of 576 revealed significant departure from Hardy–Weinberg expectations (i.e. at a P -value threshold of $= 0.0001$ after Bonferroni correction). Significant tests were randomly distributed across loci and populations, suggesting that all markers were efficient. From this panel of 16 microsatellite loci, we obtained an allelic

richness (AR) varying from 1 to 11, expected heterozygosity (He) varying from 0 to 0.888 and a fixation index (Fis) varying from -0.712 to 0.997 (Table 1).

Genetic structure

Results obtained with Structure and Structure Harvester on the whole dataset ($n = 708$) inferred the highest posterior probability for two genetic clusters ($K = 2$) corresponding to the two subspecies (i.e. *T.h. hermanni* and *T.h. boetgeri*) (Fig. 2). French individuals collected in the field were mainly assigned to the genetic cluster 1 (mean \pm SD: 0.926 ± 0.085), while Balkan individuals were attributed mainly to the genetic cluster 2 (mean \pm SD: 0.993 ± 0.026). This significant genetic differentiation between both subspecies was also characterized by a high and significant F_{st} value $= 0.622$ (i.e. at a P -value threshold of $= 0.008$ after Bonferroni correction). Captive bred individuals fell mainly in genetic cluster 1 with a lower admixture proportion (mean \pm SD: 0.773 ± 0.024) as compared to wild individuals. They were genetically different from the Balkan populations (F_{st} value $= 0.45$; at a P -value threshold of $= 0.008$ after Bonferroni correction) and the French populations (F_{st} value $= 0.14$; at a P -value threshold of $= 0.008$ after Bonferroni correction) although the genetic difference was lower.

Detection and distribution of hybrids

Our approach was effective to detect hybrids as 98% (i.e. 746 out of 760) of the artificially generated hybrids were correctly assigned to one of the predefined hybrid genotypic categories. The 14 remaining individuals were falsely attributed to the genotypic categories of pure species (i.e. 6 into Pure_ *T. h. hermanni* and 8 into Pure_ *T. h. boetgeri*). Among the artificially generated F1-hybrids, 38 of 40 (i.e. 95%) were correctly identified as F1. The two remaining individuals, as well as artificially generated F2- and F3-hybrids, carry admixed genotypes and were not attributed to any predefined genotypic frequency category with a posterior probability > 0.8 . These individuals showed admixed genotypes resulting in posterior probabilities shared in the different predefined genotypic categories. From these results, it appeared that the genetic mixing between both sub-species was able to disturb the accuracy of inferences, mainly after the F1 generation.

Considering the distribution (Table 2) of the WT (565 samples) and ET (121) individuals collected in the field, the majority (i.e. 81% and 99%, respectively) were attributed to their corresponding subspecies, whereas the number of individuals not assigned is much higher for WT (i.e. 16.5% vs 1%, respectively). The results obtained for the WT samples align with those obtained from Structure analyses (Supplementary file S2). Finally, among the 22 captive bred individuals, the number attributed to Pure_ *T. h. hermanni* and not assigned to

Table 1 Summary of the genetic diversity parameters for each locus calculated for several tortoise groups (see text for definition). *N*: number of samples analysed; AR: allelic richness and He: expected heterozygosity. Fis (i.e. Fixation index) is represented in italics and bold when significantly deviating from Hardy–Weinberg expectations (i.e. significantly different at a *P*-value threshold of =0.0005 after Bonferroni correction)

Locus	Parameters	Captive bred	T.h. hermanni	Individuals	Individuals	Hybrids
		individuals	individuals	from France	from Balkans	
		<i>N</i> = 22	<i>N</i> = 461	<i>N</i> = 565	<i>N</i> = 121	<i>N</i> = 103
THER106	He	0.367	0.009	0.086	0.008	0.354
	AR	2.909	1.174	2.066	1.165	2.721
	Fis	-0.239	0.499	0.091	0	-0.188
THER11	He	0.44	0.451	0.442	0.008	0.388
	AR	2.909	2.202	2.171	1.165	2
	Fis	0.174	0.004	0.021	0	0.103
THER51	He	0.744	0.508	0.55	0.641	0.704
	AR	5.987	2.657	3.832	4.141	5.928
	Fis	-0.039	0.011	0.034	-0.005	0.057
THER73	He	0.409	0	0.079	0.049	0.356
	AR	2	1	1.821	1.666	2
	Fis	0.333	NA	0.185	-0.021	-0.011
THER23	He	0.324	0.203	0.208	0.366	0.228
	AR	3.903	2.242	2.626	2.167	3.504
	Fis	0.157	0.099	0.151	-0.071	0.35
THER101	He	0.331	0.009	0.065	0.487	0.274
	AR	2	1.165	1.75	2	2
	Fis	-0.235	-0.003	-0.034	-0.712	-0.19
THER48	He	0.888	0.769	0.788	0.629	0.856
	AR	11	6.299	7.757	7.114	10.394
	Fis	0.324	-0.002	0.024	0.044	0.099
Test10	He	0.56	0.096	0.166	0.755	0.445
	AR	6.974	2.713	3.854	7.856	6.991
	Fis	0.188	0.04	0.09	-0.002	0.044
THER20	He	0.505	0.096	0.187	0.643	0.518
	AR	4.818	2.555	3.607	4.869	5.829
	Fis	0.101	-0.032	0.038	-0.163	-0.07
THER40	He	0.426	0	0.074	0	0.338
	AR	2	1	1.797	1	2
	Fis	0.041	NA	-0.039	NA	-0.268
THER69	He	0.415	0.009	0.083	0.569	0.366
	AR	3.909	1.166	2.297	3.167	3.825
	Fis	-0.206	-0.003	0.081	-0.063	-0.028
THER4	He	0.359	0.011	0.09	0.008	0.366
	AR	2	1.205	2.063	1.165	2.676
	Fis	-0.012	0.799	0.204	0	-0.038
THER112	He	0.638	0.332	0.388	0.195	0.583
	AR	5	2.762	3.155	2.159	4.568
	Fis	-0.019	0.08	0.104	0.231	0.059
THER36	He	0	0	0.009	0.087	0.048
	AR	1	1	1.168	1.869	1.664
	Fis	NA	NA	-0.004	-0.043	-0.02
Test56	He	0.669	0.467	0.502	0.169	0.601
	AR	3.909	2.207	3.088	1.986	3.633
	Fis	-0.291	-0.01	-0.009	0.203	-0.134
THER94	He	0.779	0.604	0.621	0.604	0.686
	AR	8.72	3.685	4.454	4.447	6.522
	Fis	0.125	-0.017	-0.006	0.041	0.006

Table 1 (continued)

Locus	Parameters	Captive bred individuals	T.h. hermanni individuals	Individuals from France	Individuals from Balkans	Hybrids
		N = 22	N = 461	N = 565	N = 121	N = 103
All markers	He	0.491	0.223	0.271	0.326	0.444
	AR	4.315	2.190	2.969	2.996	4.141
	Fis	0.042	0.015	0.040	-0.075	-0.007

NA Not available

any category are equivalent (i.e. about 41% and 46%, respectively). The number of F1-hybrids for captive animals is also much higher as compared to ET but also to WT (13.6%, 0%, and 1.8%, respectively).

Herein, we considered that the absence of accurate assignation in some individuals likely resulted from their admixed genotypes between both subspecies, as it was previously the case for artificially generated F2- and F3-hybrids. Thus, even though we failed to really assign some individuals to F2 or F3 classes, we associated the individuals not assigned to any previous predefined categories to another class (non-assigned hybrids) likely including F2 and F3 individuals and potentially beyond (see also discussion). Considering all hybrid categories, our results identified 103 individuals as hybrids among the 565 French individuals sampled in the field (Table 2; Supplementary file S2). These hybrids occurred throughout the study area (Fig. 1).

Genetic diversity and structure of wild *T. h. hermanni* populations

After removing individuals previously identified as hybrids ($n = 103$) and *T. h. boetgeri* ($n = 1$), we kept 461 individuals likely belonging to *T. h. hermanni*. These remaining individuals showed a significant decrease of AR and He when

we removed the hybrids (Wilcoxon: $P < 0.0001$). This was expected since the hybrids harboured alleles both of *T. h. hermanni* and *T. h. boetgeri*. Moreover, these 461 *T. h. hermanni* individuals also revealed a lower AR (Wilcoxon: $P < 0.001$) than (i) their counterparts collected in the Balkans ($n = 121$) (quite significantly, $P = 0.055$), (ii) the hybrid individuals ($n = 103$), and (iii) the captive bred individuals ($n = 22$) which showed the highest genetic diversity (i.e. He and AR; Table 1). Furthermore, captive-bred individuals and hybrids revealed similar AR (Wilcoxon: $P = 0.376$) (supplementary file S3).

At the individual scale, results from Structure performed on the 461 *T. h. hermanni* did not converge toward a precise number of genetic clusters K suggesting a weak genetic structure for *T. h. hermanni* individuals (supplementary file S4). This result agreed with a weak isolation by distance (P value = 0.055) performed on the same individuals.

Discussion

Hybridization level in tortoise populations

We observed significant genetic difference between the two subspecies *T. h. hermanni* (WT) and *T. h. boetgeri* (ET)

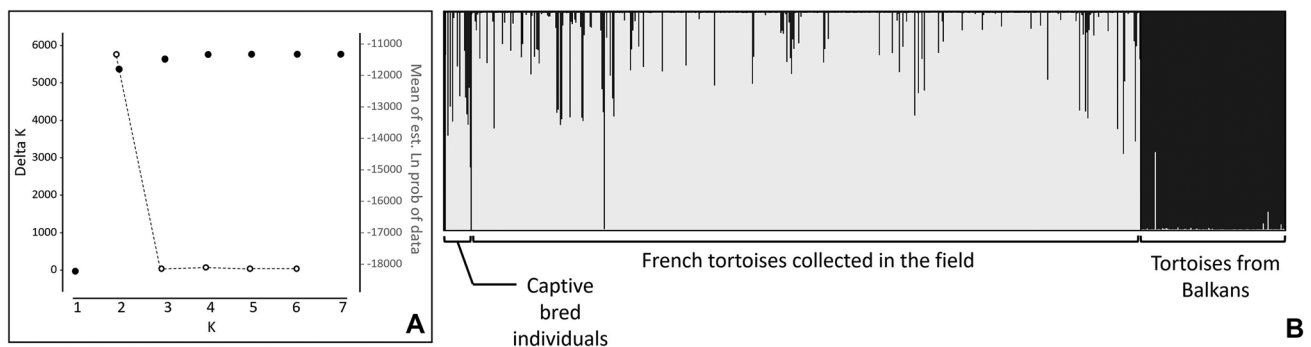


Fig. 2 Results from Structure and Structure harvester concerning population genetic structure of the whole sample ($n = 708$). **A** The mean log likelihood (Ln) of the data across the 20 replicates (white circles) with associated variance (however, the size of the variance is smaller than the size of the symbols) and Evanno's delta K statistic (black circles) for each simulated genetic cluster (K , from 1 to 7). The highest mean likelihood as well as delta K values indicated that the genetic structure of tortoise populations was characterized by

$K = 2$ genetic clusters. These figures have been realized using Structure Harvester software version 0.6.1 (Earl & vonHoldt 2012). **B** Results from Bayesian clustering method implemented in Structure software. Mean of proportions of ancestry for investigated individuals, inferred at $K = 2$. Each vertical bar represents an individual, each colour represents a different genetic cluster, and vertical black lines separate the different populations. We realized this barplot using Distruct (Rosenberg 2004)

Table 2 Results from NewHybrids software. Inferences for WT, ET, and captive individuals to the different predefined hybrid categories (in brackets the proportion of individuals for each hybrid category)

Samples analyzed (N)	Predefined hybrid genotypic categories in NewHybrids			
	Pure <i>T.h.h</i>	Pure <i>T.h.b</i>	F1 (<i>T.h.h</i> x <i>T.h.b</i>)	Non-assigned hybrids
WT individuals from the field (<i>n</i> = 565)	461 (81%)	1 (< 1%)	10 (1.7%)	93 (16.5%)
ET individuals (<i>n</i> = 121)	0 (0%)	119 (99%)	0 (0%)	2 (1%)
Captive animals (<i>n</i> = 22)	9 (40.9%)	0 (0%)	3 (13.6%)	10 (45.5%)

as already documented in previous studies including different populations covering almost the entire geographic range of both subspecies (Mazzotti 2004; Fritz et al. 2006; Pérez et al. 2014; Zenboudji et al. 2016; Biello et al. 2021). Moreover, hybridization between WT and ET was detected in continental France, Spain, and Italy, whereas Mediterranean Islands appear as rather hybrid-free (Pérez et al. 2014; Zenboudji et al. 2016; Biello et al. 2021). In this context, our study offers a more comprehensive information at a smaller scale in focusing on the Var district. Our large sample size provided a mean to quantify hybridization levels and genetic diversity in the threatened WT mainland French populations.

Our results revealed 103 hybrids (regardless of hybrid category) among 565 analysed individuals, leading to a global hybridization rate of 18.2%. This is a relatively high level of hybridization as compared to previous estimations in the Var that identified 1.7% of hybrids (2 out of 120 samples) in Perez et al. (2014) or 6.2% (5 out of 81) in Zenboudji et al. (2016). However, this number (18.2%) was not overestimated because assignment of artificially generated hybrids into the predefined hybrid genotypic categories (i.e. more than 98%) insured both effectiveness and accuracy of our analyses. Thus, our study outlines the importance of a fine-scale study involving numerous and geographically distributed samples in order to have a clear estimation of the level of hybridization in wild populations of *T. hermanni*.

Beside the 18.2% of hybrids, only one sample was attributed to pure ET, thus indicating that the ET inflow mainly results from hybrid input. Although our ET sampling is geographically limited, only one WT/ET hybrid was detected in Macedonia, thus confirming (Perez et al. 2014; Biello et al. 2021) the asymmetry of tortoise transfers between the sampled distribution areas (more introduction from ET to WT than the other way). In the Var district, natural contact between WT and ET cannot occur due to geographic distance (e.g. several hundred kilometres and impassable physical barriers; Perez et al. 2014). Consequently, the dispersal of non-native genes due to hybridization thus represents a form of cryptic invasion (hybrids are not morphologically identifiable) resulting from human-mediated translocations of alien ET.

Our study also indicated that hybrids are scattered throughout the whole study area and most of them seem to be hybrids > F1. Although we did not succeed in really

assigning the hybrids to F2 or F3 classes, these individuals are most likely representing hybrids > F1 since we accurately detected pure and F1 individuals. This inference is in agreement with the maturity of the tortoises which requires at least 8–12 years, a longevity of about 50 years (Bertolero et al. 2011), and the massive release (more than 4300 captive individuals, Devaux 1990) carried out notably in the 1990s by the SOPTOM in the Var district to reinforce declining populations. These releases most likely included unidentified hybrids, an hypothesis supported by the high proportion of hybrids we found among individuals bred in captivity (~59%) compared to natural populations (~18%). However, these releases were realized in specific zones, and although tortoises can travel long distances, the scattered distribution of hybrids throughout the whole study area suggests that another source was involved (supplementary file 5). According to Celse et al. (2017), pet tortoises that escape from gardens or intentionally released in the wild are another important source of the inflow. Pet owners use their intuition to select appropriate sites and finally release tortoises across the whole distribution creating multiple and scattered hybridization nuclei. For instance, we detected 47% and 41% of hybrids in two suitable habitat patches in the Var district where illegal releases by pet-owners frequently occurs (Sebastien Caron, personal communication). Thus, captive tortoises (estimated at about tens of thousands in the Var) represent a major reservoir of ET and WT/ET hybrids that likely contaminated wild WT over decades. Due to the longevity of the Hermann's tortoise (about 40 years), a long-term impact and widespread introgression are expected (hybridization type 5 in the classification of Allendorf et al. 2001), and consequently, conservation efforts should focus on maintaining pure WT populations (see “Recommendations for *T. h. hermanni* tortoise conservation”).

Management of hybrids

We need to assess the methods of limiting hybridization (culling, removal, sterilization, or de-introgression; Bohling 2016; Grabenstein and Taylor 2018) and their applicability to the human-induced hybridization of the Hermann's tortoise in the Var needs to be evaluated.

Culling raises ethical and ecological questions (Bohling 2016). The Hermann's tortoise is an emblematic species that

benefits from strong popularity, and killing individuals is not possible (Ballouard et al. 2019). Sterilization of hybrids would require invasive surgery and post-surgery monitoring by qualified personnel. In addition, the cost of the operation (about 500€ per individual) makes this option difficult to implement on a large number of individuals (likely several hundreds). Removal of hybrids from *natura* (spatial isolation) requires ad hoc refuges to host them over decades (tortoises are long-lived animals) which does not appear as a viable option in practice (see “[Recommendations for *T. h. hermanni* tortoise conservation](#)”) because refuges are currently overcrowded (Celse et al. 2017). Moreover, for these three options (culling, sterilization, removal), the main difficulty is the identification of hybrids (notably *in natura*) because most of them can be considered as cryptic hybrids. Indeed, although the two subspecies can be visually differentiated (Celse et al. 2017), this method is not infallible and inefficient with hybrids belonging to the categories F2 and beyond (Soler et al. 2012). Genetic methods are thus necessary to reliably identifying hybrids, but such tools are not easily accessible to naturalist centres, unless rapid genetic tests can be performed in the field.

De-introgression (Bohling 2016; Amador et al. 2014) is a method attempting to restore the genome of the admixed population (here WT) through successive controlled crossing between introgressed individuals. De-extinction has been successful to recover the original genetic background in some livestock (Amador et al. 2014). However, de-introgression cannot be easily implemented in wild populations because it necessitates extensive knowledge of the native genome through exclusive molecular markers (such as SNPs). Concerning the Hermann’s tortoise in the Var, such an approach is already applied based on morphological characters through the removal of tortoise with obvious ET morphological traits (see paragraph recommendations). If many hybrids are undoubtedly left in the field, the objective is to progressively remove ET phenotypes to allow the dominance of WT phenotype (and expectedly genotype).

Finally, the simpler option would be to leave hybrids in place. Indeed, possible detrimental effects of hybridization between WT and ET are not quantified. Several studies (Perez et al. 2014; Zenboudji et al. 2016; this study) point to lower genetic diversity in the Var compared to other continental or even insular (Corsica or Sicily) populations. Hybridization may thus contribute to restore or improve genetic diversity (Tallmon et al. 2004) although we do not know if hybridization benefits could outweigh potential risks (outbreeding depression). However, according to Bell et al. (2019), the risk of potential outbreeding depression should not dissuade genetic rescue attempts of endangered populations. Moreover, complete admixture between the two subspecies (type 6 of Allendorf et al. 2001) is unlikely as

long as ET individuals are a minority. Hence, the importance of limiting the ET influx as much as possible (Celse et al. 2017). Hermann’s tortoises in the Var are the last French continental populations and as such constitute an emblematic species of the Mediterranean ecosystem that deserve conservation recognition, even if partly admixed (Stronen and Paquet 2013).

Recommendations for *T. h. hermanni* tortoise conservation

From a legal perspective, *T. h. hermanni*, *T. h. boettgeri*, and their hybrids are protected (see <https://www.legifrance.gouv.fr/jorf/id/JORFTEXT000043113964>). In this context, we centred our recommendations on education and field actions with the objective to effectively reduce tortoise hybridization in the Var.

Educational outreach to private owners France likely hosts greater numbers of pet than free-ranging tortoises (Celse et al. 2017), and the popularity of pet tortoises means that this situation is not likely to change (Ballouard et al. 2019). It is imperative to reinforce outreach campaigns to raise concern and vigilance among pet owners (see Celse et al. 2017 for different actions).

Encouraging owners to not breed and release tortoises Most people are unaware that reproducing tortoises in captivity and releasing individuals in the field can threaten wild populations (Segura et al. 2020). Local information campaigns towards pet owners might be very efficient to prevent the illegal release of domestic turtles. The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) does not target pet owners and seems inefficient to prevent the overexploitation of live reptiles and to halt illegal trade (Auliya et al. 2016), especially because ET tortoises are legally purchased in French pet stores. Information regarding ecological issues should be clearly displayed in shop to inform customers. Eventually, sale and donation of Hermann’s tortoise should be prohibited.

Increasing hosting capacities of rescue centres Pet owners abandon around 1500 Hermann’s tortoises each year in France (Celse et al. 2017). State financial support is urgently needed to increase the size of external enclosures in rescue centres. Abandoned and rescued tortoises may, following health and genetic assessments, help restore populations severely impacted by fires (Lecq et al. 2014; Pille et al. 2018) or restock areas in the South of France from which the Hermann’s tortoise disappeared (Celse et al. 2017). Moreover, rescue centres also contribute to the maintenance of a reservoir of pure WT populations.

Removal of individuals Alien tortoises that are sometimes found in the Var, such as the Greek tortoise (*Testudo graeca*) or pure *T. h. boettgeri*, are currently removed from *natura* and maintained at the SOPTOM/Tortoise Village Center. Indeed, beyond the risk of genetic introgression for WT population, ET tortoises carry many pathogens, especially those release or escaped from captivity (Ballouard et al. 2021). Before a convenient genetic tool is available, selective removal of obvious WT/ET hybrids could be practiced to promote de-introgression. To minimize the risk of removing pure WT, a detailed analysis of morphological traits should be conducted to better identify native WT.

Future directions Long-term monitoring of *Testudo hermanni* in *natura* is essential to guide field actions, especially with respect to the forthcoming impacts of global changes or to establish conservation priorities (e.g. habitat protection versus genetic integrity).

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Declarations

Ethical statement We sacrificed no animals. To limit harm to tortoises, we collected small volumes (<200 µl) of blood by a puncture of the dorsal supra-carapacial cervical plexus using 1 ml syringes and small needles (27 G and 30 G; Bonnet et al. 2016b). As recommended in Beaupre et al. (2004), we minimized handling time by not anesthetising tortoises, and cleaned the puncture wound with an antiseptic solution before releasing the tortoise.

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