

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/329339051>

Who's who in the western Hermann's tortoise conservation: a STR toolkit and a reference database for wildlife forensic genetic analyses

Preprint · November 2018

DOI: 10.1101/484030

CITATIONS

0

READS

20

15 authors, including:



Roberto Biello
University of Ferrara

3 PUBLICATIONS 0 CITATIONS

[SEE PROFILE](#)



Claudia Corti
University of Florence

163 PUBLICATIONS 1,653 CITATIONS

[SEE PROFILE](#)



Marta Biaggini
University of Florence

34 PUBLICATIONS 158 CITATIONS

[SEE PROFILE](#)



Luciano Di Tizio
Societas Herpetologica Italica

14 PUBLICATIONS 17 CITATIONS

[SEE PROFILE](#)

Some of the authors of this publication are also working on these related projects:



Improving the knowledge of Sardinian grass snake (*Natrix natrix cetti*) [View project](#)



Use of phytogenic feed additives in sheep diets [View project](#)

1 **Who's who in the western Hermann's tortoise conservation: a STR toolkit and**
2 **reference database for wildlife forensic genetic analyses**

3
4
5

6 Biello Roberto^{1§}, Zampiglia Mauro^{2§}, Corti Claudia³, Deli Gianluca⁴, Biaggini Marta³, Delaugerre Michel⁵, Di Tizio
7 Luciano⁶, Leonetti Francesco Luigi⁷, Olivieri Oliviero⁴, Pellegrino Francesco², Romano Antonio^{8,9}, Sperone Emilio⁷,
8 Trabalza-Marinucci Massimo⁴, Bertorelle Giorgio^{1*}, Canestrelli Daniele².

9
10
11
12

13 ¹ Dipartimento di Scienze della Vita e Biotecnologie, Università di Ferrara, Via Luigi Borsari 46, 44121 Ferrara,
14 Italy.

15 ² Dipartimento di Scienze Ecologiche e Biologiche, Università della Tuscia, Largo dell'Università s.n.c., 01100
16 Viterbo, Italy.

17 ³ Museo di Storia Naturale dell'Università di Firenze, Sezione di Zoologia "La Specola", Via Romana 17, 50125
18 Firenze, Italy.

19 ⁴ Dipartimento di Medicina Veterinaria, Università di Perugia, Via San Costanzo 4, 06126 Perugia, Italy.

20 ⁵ Conservatoire du littoral, Résidence St Marc, 2, rue Juge Falcone, 20200 Bastia, France.

21 ⁶ Societas Herpetologica Italica, Sezione Abruzzo-Molise, Via Federico Salomone 112, 66100 Chieti, Italy.

22 ⁷ DiBEST, Università della Calabria, via P. Bucci, 87036, Rende (CS), Italy.

23 ⁸ MUSE: Museo delle Scienze, Sezione di Zoologia dei Vertebrati, corso del Lavoro e della Scienza 3, 38122
24 Trento, Italy.

25 ⁹ CNR-ISAFOM: Consiglio Nazionale delle Ricerche, Istituto per i sistemi agricoli e forestali del Mediterraneo, Via
26 Patacca 85, 80056 Ercolano (NA), Italy.

27
28

29 [§] These authors contributed equally to this work

30

31 * Corresponding author: ggb@unife.it

32 **ABSTRACT**

33 Illegal trade is threatening tortoise populations worldwide since decades. Nowadays, however,
34 DNA typing and forensic genetic approaches allow to investigate geographic origin of
35 confiscated animals and to relocate them into the wild, provided that suitable molecular tools and
36 reference data are available. Here we assess the suitability of a small panel of microsatellite
37 markers to investigate patterns of illegal translocations and to assist forensic genetic applications
38 in the endangered Mediterranean land tortoise *Testudo hermanni hermanni*. We used the
39 microsatellite panel to (i) increase the understanding of the population genetic structure in wild
40 populations with new data from previously unsampled geographic areas (overall 461 wild
41 individuals from 28 sampling sites); (ii) detect the presence of non-native individuals in wild
42 populations; and (iii) identify the most likely geographic area of origin of 458 confiscated
43 individuals hosted in Italian seizure and recovery centers. Our analysis initially identified six
44 major genetic clusters corresponding to different geographic macro-areas along the
45 Mediterranean range. Long-distance migrants among wild populations, due to translocations,
46 were found and removed from the reference database. Assignment tests allowed us to allocate
47 approximately 70% of confiscated individuals of unknown origin to one of the six Mediterranean
48 macro-areas. Most of the assigned tortoises belonged to the genetic cluster corresponding to the
49 area where the respective captivity center was located. However, we also found evidence of
50 long-distance origin of confiscated individuals, especially in centers along the Adriatic coast and
51 facing the Balkan regions, a well-known source of illegally traded individuals. Our results
52 clearly show the role for reintroduction projects of the microsatellite panel, which was useful to
53 re-assign most of the confiscated individuals to the respective macro-area of origin. At the same

54 time, the microsatellite panel can assist future forensic genetic applications to detect illegal trade
55 and possess of *Testudo hermanni* individuals.

56

57

58 **KEYWORDS**

59 Wildlife forensic genetics, Pet trade, Illegal animal translocation, Assignment tests, STR toolkit,
60 Mediterranean tortoises, *Testudo hermanni*.

61 **INTRODUCTION**

62 Over-collection and illegal trade of wildlife species for consumption or pet market are among the
63 main threats to biodiversity [1], and reptiles currently represent the second most affected
64 vertebrate class, after birds [2,3]. According to [4], the European Union (EU) is the top global
65 importer of live reptiles for the pet trade (valued at €7 million in 2005). Because of this practice,
66 a significant number of reptile populations have already been severely decimated (e.g., [5–8]).
67 Intentional harvest is considered the second largest threat to the survival of many reptile species
68 [9] and, as a consequence, reptiles' pet trade is strongly restrained by CITES.

69 Relocating confiscated individuals implies the identification of their natural source areas,
70 which has long been a challenging task in the absence of clear morphological differences among
71 natural populations and the consequent lack of simple diagnostic traits [1]. However, DNA
72 typing and forensic genetic tools are providing straightforward and increasingly appreciated
73 approaches for this purpose, allowing also the identification of hybrids. Noteworthy, the use of
74 these wildlife forensic genetic tools implies the gathering of multiple population genetics
75 information in a single analytic framework, such as the assessment of the genetic variation and
76 its deep population structure at the geographical level.

77 Aside obvious consequences on the consistency and genetic diversity of natural
78 populations, when followed by release of individuals in the non-native range, pet trade can
79 trigger several processes posing additional threats to wildlife: i) hybridization between native and
80 translocated individuals [10,11]; ii) introduction of exotic parasites and pathogens [12]; iii)
81 ecosystem imbalance [13,14]; iv) new biological invasions [15,16]. Therefore, limiting collection
82 within the areas of origin, and correctly relocating confiscated individuals are activities of the
83 utmost importance [17].

84 The Mediterranean land tortoises are known to be largely threatened by pet trade,
85 especially in the Balkans [18–22], where the former Yugoslavia had an important role in tortoise
86 exports during the past century [23–25]. According to the Federal Statistical Office, a total of
87 2,615 tons of tortoises were exported from the former Yugoslavia within a 41-year period during
88 the 20th Century, approximating 2 million traded individuals [23]. The Hermann’s tortoise
89 (*Testudo hermanni* Gmelin, 1789) has been particularly affected by this trade [26]. This species
90 has its natural range spanning from Spain to the Balkans, mainly along the Mediterranean coastal
91 regions, and in various Mediterranean islands. Two subspecies with clear genetic differences are
92 commonly recognized (the eastern *T. h. boettgeri* and the western *T. h. hermanni*), and the
93 geographic structure of the genetic variation in both subspecies, although with some under-
94 sampled areas, has been assessed [27, 28]. Intensive harvesting for pet trade, especially before
95 the 1980s when it was not banned yet [23], and releases of non-native individuals into local
96 populations, are long-recognized threats for this species [26], along with habitat reduction [29].
97 As a consequence, *T. hermanni* is included in the list of the strictly protected species by the *Bern*
98 *Convention on the Conservation of European Wildlife and Natural Habitat*, and the western
99 subspecies *T. h. hermanni* is classified as “Endangered” by the Italian IUCN Red List of
100 Vertebrates [30]. However, source and fate of illegally translocated individuals are still poorly
101 assessed in vast portions of the species’ range.

102 In this paper, we test a small panel of microsatellite markers to investigate patterns of
103 illegal translocations of *T. hermanni hermanni* among a large sample of individuals hosted in
104 Italian seizure and recovery centers. To this end, we began by complementing previous
105 assessments of population genetic structure of wild populations [28], with new data from
106 previously unsampled geographic areas. Subsequently, we used information gathered from the

107 Bayesian genetic clustering exercises to assign confiscated individuals to the most probable
108 geographic area of origin.

109

110

111 **MATERIALS AND METHODS**

112 **Sampling and laboratory methods**

113 We collected 154 blood samples from wild *Testudo hermanni* individuals throughout mainland
114 Italy, Sicily, Sardinia, Corsica and Lampedusa and 458 blood samples from confiscated tortoises
115 kept in captivity by local authorities (e.g., the Carabinieri Corps) or animal conservation NGOs.
116 Sampling sites of wild individuals and location of recovery centres are shown in Fig 1 and 2,
117 respectively. Blood samples were taken from nape or coccygeal vein and about 75 µl were
118 spotted on FTA® Classic Cards (Whatman™, GE Healthcare) and stored at room temperature.
119 Alternatively, whole blood (100 µl – 1 ml) was treated with K3-EDTA and stored at -20° C.
120 DNA was extracted from both FTA-Cards and whole blood using a solution of 5% Chelex® 100
121 Resin (Bio-Rad, [31], see Supplementary Material). Initially, all individuals were genotyped at 9
122 microsatellite loci (Test10, Test56, Test71, Test76, Test88, Gal136, Gal75, Gal73, and Gal263)
123 as in [28] (see also [32, 33]). However, two loci (Test88 and Gal73) yielded inconsistent
124 reactions and were discarded from downstream analyses. Detailed protocols are provided as
125 Supplementary Material. In order to combine our dataset with the dataset from Perez et al. [28]
126 avoiding mislabelling of alleles, we re-genotyped selected samples from [28], and we
127 recalibrated binning set and allele nomenclature to match their dataset. Fragment analysis of
128 PCR products was performed by MacroGen Inc. on an ABI 3730xl Genetic Analyser (Applied
129 Biosystems) with a 400HD size standard. Allele calling was performed with GENEMAPPER®

130 4.1 checking electropherograms by eye. All electropherograms were scored by two persons and
131 only concordant multilocus genotypes were retained for subsequent analyses.

132

133 **Genetic structure and reference database**

134 As the first step to assess the area of origin of confiscated individuals, we carried out a
135 population structure analysis of individuals that can be confidently considered as belonging to
136 natural populations (hereon 'wild'), in order to define possible source populations and to compile
137 a reference database of individuals genuinely belonging to each identified population. The
138 multilocus genotypes of the 154 newly collected wild tortoises were joined to the dataset from
139 [28], excluding from the latter all the individuals that were reported to be migrant or 1st and 2nd
140 generation hybrids, and the admixed population of Bosco Nordio. The joint wild dataset
141 consisted of 461 individuals (Fig 1). We performed the cluster analyses on the wild dataset using
142 the Bayesian method implemented in STRUCTURE 2.3.4 [34]. Analyses were conducted
143 choosing a model with admixture, uncorrelated allele frequencies, and a non-uniform ancestry
144 prior ALPHA among clusters, as suggested by Wang [35] for uneven samplings. We run 20
145 replicates for each value of K from K=1 to K=12 (K is the number of inferred genetic groups),
146 with 750000 MCMC after a burnin of 500000. Structure results were summarized and visualized
147 with the web server CLUMPAK [36]. We used STRUCTURE HARVESTER [37] to infer the
148 best value of K, based on both the probability of the data given K [34] and the Evanno approach
149 [38].

150 The reference database of wild individuals was then prepared based on the following
151 three-step analysis. First, we added to each wild individual the prior information about the
152 genetic cluster most represented in the geographic area from which they were sampled. Second,

153 we re-run STRUCTURE to identify migrants and hybrids, using the same parameters as above
154 but fixing K at its optimal value (see results), and activating the USEPOPINFO option. Finally,
155 all individuals that resulted as ‘non-pure’ in their respective geographic area (i.e. those
156 individuals with less than 50% posterior probability to belong to their prior assigned cluster)
157 were excluded from the reference database.

158

159 **Assignment of individuals of unknown origin**

160 According to Manel and colleagues [39] fully Bayesian methods of assignment, as implemented
161 in STRUCTURE, outperform partially Bayesian methods [40] with higher assignment rates and
162 lower assignment error. However, this method considers all populations simultaneously with the
163 drawback of assigning individuals to reference population even if the true population of origin is
164 actually unsampled [39]. To overcome this problem, Manel and colleagues [39] suggests
165 performing both fully Bayesian assignment tests and exclusion tests.

166 We performed assignment tests on 458 confiscated individuals with STRUCTURE using
167 the POPFLAG for individuals in the reference database and activating the “update allele
168 frequencies using only individuals with POPFLAG=1” option under a USEPOPINFO without
169 admixture model. Other run parameters were the same as in the USEPOPINFO run described
170 above. We assigned individuals to a source population when the probability of an individual to
171 belong to that population was above 80%.

172 Exclusion tests were performed with the partially Bayesian exclusion method [41]
173 implemented in GENECLASS2 [42]. We compared observed genotypes of confiscated
174 individuals with an expected likelihood distribution of genotypes generated for each reference
175 population by simulating 1000000 individuals with Monte Carlo resampling [43]. We excluded

176 reference populations as the likely source of an individual when likelihood values were below
177 0.01.

178

179

180 **RESULTS**

181 The analysis of the complete wild dataset indicated that K=2 and K=6 were the most supported
182 numbers of clusters. The log probability of data increased sharply from K=1 to K=2 and then
183 more slowly from K=3 to K=6 where it reached a plateau (see Supplementary Material Fig 1).

184 The delta K analysis [38] provided two modes at K=2 and K=6, respectively. The first and most
185 evident partition discriminated eastern and western subspecies (see Supplementary Material Fig
186 2), whereas the second mode at K=6 suggested a subdivision of *T. h. hermanni* in 4 groups and
187 of *T. h. boettgeri* in 2 groups (Fig 1). The *T. h. hermanni* groups were Italian Peninsula (ITP) (all
188 the populations from central and southern Italian Peninsula, except samples from central and
189 southern Calabria), mainland France (FRA), Calabria (CAL) and Mediterranean islands (Sicily,
190 Sardinia, Corsica, Pantelleria) joined with Spain (ISS). The *T. h. boettgeri* groups were Greece
191 (GRE) and Bosco Mesola with Croatia and Macedonia (MCM). These results agree with the
192 groups previously obtained by Perez et al. [27, 28, 44, 45], but with the additional CAL cluster,
193 emerging from an area that was previously unsampled.

194 The analyses carried out in STRUCTURE using the prior population information allowed
195 us to detect the presence of one hybrid and six migrant individuals among wild populations (Tab
196 1). While the hybrid was from an admixture area between two geographically contiguous clusters
197 and one of the migrants was from the same subspecies, the other five migrants were from the

198 other subspecies (four of them from spatially very distant clusters). Genotypes from these seven
199 individuals were excluded from the reference database.

200 In order to assign the 458 confiscated individuals to the most probable geographic area of
201 provenance, we used $K=6$ as the optimal K value, and $q > 0.8$ as the assignment threshold. Using
202 these parameter values we were able to assign more than 90% of samples to one of the six
203 clusters. When assigned individuals were downgraded to unassigned by the exclusion test (area
204 of origin excluded with $P < 0.01$), 38.7% of the confiscate tortoises were assigned to the ITP
205 cluster, 14.8% to MCM, 6.5% to GRE, 5.7% to the ISS, 3.1% to CAL and 0.2% FRA, while 31%
206 of the individuals were not assigned to any predefined cluster (NA). Decreasing the significance
207 level of the exclusion test to 0.001 to avoid false positives in multiple testing decreased the
208 fraction of unassigned individuals to 22%.

209 Most of the assigned tortoises belonged to the genetic cluster corresponding to the area
210 where the captivity center was located (see Figure 2). However, we also found evidence of long
211 distance translocations of individuals, especially in the centers along the Adriatic coast and
212 facing the Balkan regions, known to be a source of illegal trades. In Apulia, for example, only
213 one of the 14 assigned individuals belonged to the local ITP genetic cluster, whereas eleven of
214 them were classified as MCM. In the Emilia-Romagna center, 60% of the assigned individuals
215 were probably local or imported from Balkan areas genetically very similar (*Testudo hermanni*
216 *boettgeri* MCM cluster), but about 25% and 14% and of them were classified as imported from
217 Greece (GRE) or classified within the *Testudo hermanni hermanni* ITP cluster, respectively. In
218 the Umbria centers, more than 20% of the assigned individuals had a Greek origin. On the other
219 hand, when the small fraction of unassigned samples was excluded, more than 90% of the

220 captive individuals from the Western and most Southern areas (Basilicata, Calabria, Sicily, and
221 Sardinia) belonged to the local cluster.

222

223

224 **DISCUSSION**

225 The main purpose of this work was to test a small panel of microsatellite markers potentially
226 useful as a tool to identify the most probable geographic origin of *T. hermanni* tortoises, and to
227 apply it to individuals of unknown origin confiscated because illegally owned or imported and
228 currently hosted in Italian seizure and recovery centres. We found that this tool is able to assign a
229 large fraction of individuals to specific macro-regions, thus contributing to forensic analysis
230 and/or to projects of release in the wild of confiscated animals.

231 Results from the overall assignment tests showed that most of the assigned individuals
232 were native of the Italian Peninsula (clusters ITP and CAL) or from clusters at least partially
233 falling within national borders (clusters ISS and MCM). A significant 6.5% of genetically
234 assigned tortoises hosted in Italian centres turned out to be of Greek origin, with evidence of
235 long distance translocations. Only one individual was assigned to the French genetic cluster.
236 These fractions, however, vary widely across seizure centres, with some of them hosting
237 significant numbers of non-local individuals.

238 We found an overall 31% (22% using a more stringent criteria to exclude the source
239 population identified by the assignment method) of captive individuals that were unassigned.
240 This could be explained in the light of three main considerations. First, source populations of
241 unassigned individuals may have remained unsampled. Our sampling scheme of the wild
242 populations increased the coverage of the species range within the Italian borders (*T. h.*

243 *hermanni*) [45] compared to previous studies [27, 28, 44, 46]. However, areas from outside this
244 range remain poorly sampled, especially along the Balkan Peninsula, so it is possible that
245 additional samples will improve the assignment performance in the future. Alternatively, an
246 assignment approach combined with even more strict criteria to exclude populations may be used
247 to assign additional individuals to populations which are genetically very similar, though distinct,
248 from the source population. Second, unassigned individuals could be hybrids, due to mating
249 occurred in captivity or in the wild (where introduced animals can be found) between individuals
250 with different origin. In this case, assignment algorithms fail of course to assign individuals with
251 high probability to a specific cluster. Third, different populations share relevant fractions of
252 genetic variation, and therefore only more microsatellite markers could increase the
253 discriminatory power of this assignment tool.

254 Our assessment of the genetic structure of wild populations confirmed the overall pattern
255 found by Perez et al. [28], but also revealed further structure. Despite we used 7 microsatellite
256 markers instead of the 9 used by Perez et al. [28], our results are fully consistent with theirs,
257 showing 2 main genetic pools corresponding to the 2 recognized subspecies, and further structure
258 within them. The increased sampling effort along the Italian Peninsula allowed us to recognize a
259 further cluster in Calabria, a region recognized as glacial refugium and hotspot of genetic
260 diversity for many temperate species [47–51]. The increased sampling effort on some
261 Mediterranean islands (i.e., Sardinia and Lampedusa) confirmed the presence of a single insular
262 genetic cluster.

263 The preliminary analysis performed on wild populations revealed the presence of six
264 migrants and one hybrid among wild populations. While the hybrid individual found in the
265 northern area of Calabria can reasonably be considered as a consequence of a natural admixture

266 zone between Italian peninsular and Calabrian clusters, the presence of the migrants from far
267 distant areas of origin could be explained by human-driven translocations. In particular, the
268 presence of *T. h. boettgeri* individuals from Greece in wild populations along the Italian
269 Peninsula and Sicily could be a consequence of the wide pet trade affecting this species, with
270 hundreds of thousands tortoises collected mostly in south-eastern Europe between the 1960s and
271 the 1980s and shipped to western Europe [18,52] or even of more ancient translocations [53].
272 This evidence clearly indicates that the escape or the release of non-endemic individuals among
273 wild endemic populations is not so rare, with potential genetic and epidemiological implications.

274 A priority concern that motivated this study and requires urgent solutions is the
275 management of the tortoises kept in captivity in seizure/recovery centres. These animals, usually
276 confiscated from local authorities or found by private citizens far from natural populations and
277 likely escaped from domestic contexts, cannot be released in nature without knowledge of their
278 origin. Their number is increasing, with increasing problems related to their management and
279 health condition. The assembly of a genetic reference database, and the assessment of the most
280 probable geographic origin of captive tortoises, are fundamental steps towards the development
281 of plans of reintroduction in the wild, which will not only reduce the problems and the costs
282 associated with the captive animals, but also re-create wild populations in areas where this
283 species was present in the past but is now extinct. In addition, the reference database represents a
284 useful forensic tool to investigate the genotype of individuals when their declared origin is
285 legally disputed.

286 Future efforts should be devoted to achieve higher geographic resolution of genetic
287 population structure analyses, and to reduce the fraction of unassigned individuals. These goals
288 could be achieved with one or both of the following strategies. First, to sample still poorly

289 covered areas, in order to get a complete representation of the genetic variation in the whole
290 species' range. Second, to increase the number of informative genetic markers, possibly
291 decreasing the costs. Next Generation Sequencing (NGS) technologies could help in this
292 direction, allowing to develop a panel of diagnostic SNPs to be assessed with the increasingly
293 cheap genotyping methods [1, 54].

294

295

296 **ACKNOWLEDGMENTS**

297 Animal handling and sample collection were allowed by the Ministero dell'Ambiente e della
298 Tutela del Territorio e del Mare (0044068 - 4/12/2012-PNM-II; 0001805/PNM - 4/2/2015;
299 ISPRA 68754/T-A31 – 28/11/2016) and the Regione Autonoma della Sardegna Prot.4749,
300 Rep.N.73 07/03/2017. We would like to thank all the colleagues and friends who helped during
301 fieldwork: Carabinieri per la Tutela dell'Ambiente; Centro Regionale di Recupero degli animali
302 selvatici di Bonassai, Centro Recupero Fauna Selvatica Bosco di Ficuzza; Centro Recupero
303 Animali Selvatici Formichella; Centro Recupero Fauna Selvatica "Stretto di Messina"; Centro
304 Recupero Animali Selvatici Provinciale di Policoro; Parco Nazionale dell'Asinara - Area Marina
305 Protetta "Isola dell'Asinara"; Parco Nazionale dell'Aspromonte, Parco Nazionale del Circeo,
306 Riserva Naturale Regionale Lecce di Torino di Sangro; Riserva Naturale Bosco della Mesola -
307 Parco Delta del Po; Oasi WWF "Lago di Conza. Finally, a special thank to Federica Baldo and
308 Giulia Fabbri for their help and assistance, and Giovanni Nobili and all the Carabinieri Forestali
309 at Punta Marina (Ravenna) and Bosco Mesola (Ferrara) for the continuous support and help.
310 Funding was also provided by the University of Ferrara and the Ufficio Territoriale Carabinieri
311 per la Biodiversità (Punta Marina).
312

313 **REFERENCES**

- 314 [1] R. Ogden, A. Linacre, Wildlife forensic science: A review of genetic geographic origin
315 assignment, *Forensic Sci. Int. Genet.* 18 (2015) 152–159. doi:10.1016/j.fsigen.2015.02.008.
316 [2] E.R. Bush, S.E. Baker, D.W. Macdonald, Global Trade in Exotic Pets 2006–2012, *Conserv.*
317 *Biol.* 28 (2014) 663–676. doi:10.1111/cobi.12240.
318 [3] M. Auliya, S. Altherr, D. Ariano-Sanchez, E.H. Baard, C. Brown, R.M. Brown, et al., Trade
319 in live reptiles, its impact on wild populations, and the role of the European market, *Biol.*
320 *Conserv.* 204 (2016) 103–119. doi:10.1016/j.biocon.2016.05.017.
321 [4] M. Engler, R. Parry-Jones, Opportunity or threat: The role of the European Union in global
322 wildlife trade, (2007). <http://agris.fao.org/agris-search/search.do?recordID=XF2015017467>.

- 323 [5] M.W. Klemens, D. Moll, An assessment of the effects of commercial exploitation on the
324 pancake tortoise, *Malacochersus tornieri*, *Tanzan Chelon Cons Biol.* 1 (1995) 197-206.
- 325 [6] D. Ariano-Sánchez, M. Torres-Almazán, Rediscovery of *Abronia campbelli* (Sauria:
326 Anguillidae) from a pine-oak forest in southeastern Guatemala: habitat characterization,
327 natural history, and conservation status, *Herpetol. Rev.* 41 (2010) 290.
- 328 [7] B.D. Horne, C.M. Poole, A.D. Walde, Conservation of Asian tortoises and freshwater
329 turtles: setting priorities for the next ten years, *Conclus. Workshop Singap.* (2011).
- 330 [8] M. Flecks, F. Weinsheimer, W. Böhme, J. Chenga, S. Lötters, D. Rödder, Watching
331 extinction happen: the dramatic population decline of the critically endangered Tanzanian
332 Turquoise Dwarf Gecko, *Lygodactylus williamsi*, *Salamandra.* 48 (2012) 12–20.
- 333 [9] M. Böhm, B. Collen, J.E.M. Baillie, P. Bowles, J. Chanson, N. Cox, et al., The
334 conservation status of the world's reptiles, *Biol. Conserv.* 157 (2013) 372–385.
335 doi:10.1016/j.biocon.2012.07.015.
- 336 [10] F.W. Allendorf, R.F. Leary, P. Spruell, J.K. Wenburg, The problems with hybrids: setting
337 conservation guidelines, *Trends Ecol. Evol.* 16 (2001) 613–622. doi:10.1016/S0169-
338 5347(01)02290-X.
- 339 [11] J.J. Fong, T.-H. Chen, DNA evidence for the hybridization of wild turtles in Taiwan:
340 possible genetic pollution from trade animals, *Conserv. Genet.* 11 (2010) 2061–2066.
341 doi:10.1007/s10592-010-0066-z.
- 342 [12] H.S. Young, I.M. Parker, G.S. Gilbert, A. Sofia Guerra, C.L. Nunn, Introduced Species,
343 Disease Ecology, and Biodiversity–Disease Relationships, *Trends Ecol. Evol.* 32 (2017)
344 41–54. doi:10.1016/j.tree.2016.09.008.
- 345 [13] S.Y. Strauss, J.A. Lau, S.P. Carroll, Evolutionary responses of natives to introduced
346 species: what do introductions tell us about natural communities?, *Ecol. Lett.* 9 (2006) 357–
347 374. doi:10.1111/j.1461-0248.2005.00874.x.
- 348 [14] M. Vilà, C. Basnou, P. Pyšek, M. Josefsson, P. Genovesi, S. Gollasch, et al., How well do
349 we understand the impacts of alien species on ecosystem services? A pan-European, cross-
350 taxa assessment, *Front. Ecol. Environ.* 8 (2010) 135–144. doi:10.1890/080083.
- 351 [15] S. Masin, A. Bonardi, E. Padoa-Schioppa, L. Bottoni, G.F. Ficetola, Risk of invasion by
352 frequently traded freshwater turtles, *Biol. Invasions.* 16 (2014) 217–231.
353 doi:10.1007/s10530-013-0515-y.
- 354 [16] P.E. Hulme, Invasion pathways at a crossroad: policy and research challenges for managing
355 alien species introductions, *J. Appl. Ecol.* 52 (2015) 1418–1424. doi:10.1111/1365-
356 2664.12470.
- 357 [17] IUCN, IUCN guidelines for the placement of confiscated animals, Switzerland IUCN,
358 2000.
- 359 [18] M.R. Lambert, Trade and the Mediterranean tortoises, *Oryx.* 15 (1979) 81–82.
- 360 [19] M. Auliya, Hot trade in cool creatures: a review of the live reptile trade in the European
361 Union in the 1990s with a focus on Germany, *TRAFFIC Eur. Bruss. Belg.* (2003).
- 362 [20] O. Türkozan, A. Özdemir, F. Kiremit, International testudo trade, *Chelonian Conserv. Biol.*
363 7 (2008) 269–274.
- 364 [21] N.A. Cox, H.J. Temple, European red list of reptiles, IUCN, Gland (Suiza), 2009.
- 365 [22] I. Pérez, A. Giménez, J.A. Sánchez-Zapata, J.D. Anadón, M. Martínez, M.Á. Esteve, Non-
366 commercial collection of spur-thighed tortoises (*Testudo graeca graeca*): a cultural
367 problem in southeast Spain, *Biol. Conserv.* 118 (2004) 175–181.
368 doi:10.1016/j.biocon.2003.07.019.

- 369 [23] K. Ljubisavljević, G. Džukić, M.L. Kalezić, The commercial export of the land tortoises
370 (*Testudo* spp.) from the territory of the former Yugoslavia: a historical review and the
371 impact of overharvesting on wild populations, North-West. J. Zool. 2 (2011) 250–260.
- 372 [24] R. Honegger, The reptile trade, Int. Zoo Yearb. 14 (1974) 47–52.
- 373 [25] M.R. Lambert, Threats to Mediterranean (West Palaearctic) tortoises and their effects on
374 wild populations: an overview, Amphib.-Reptil. 5 (1984) 5–15.
- 375 [26] [26] A. Bertolero, M. Cheylan, A. Hailey, B. Livoreil, R.E. Willemsen. *Testudo hermanni*
376 (Gmelin 1789) – Hermann’s Tortoise. In: A.G.J. Rhodin, P.C.H. Pritchard, P.P. van Dijk,
377 R.A. Saumure, K.A. Buhlmann, J.B. Iverson, R.A. Mittermeier (Eds.), Conservation
378 Biology of Freshwater Turtles and Tortoises: A Compilation Project of the IUCN/SSC
379 Tortoise and Freshwater Turtle Specialist Group, Chelonian Research Monographs No. 5
380 (2011), pp. 059.1-059, 20, doi:10.3854/crm.5.059.hermann.v1.2011, [http://www.iucn-](http://www.iucn-tftsg.org/cbftt/)
381 [tftsg.org/cbftt/](http://www.iucn-tftsg.org/cbftt/).
- 382 [27] U. Fritz, M. Auer, A. Bertolero, M. Cheylan, T. Fattizzo, A. K. Hundsdörfer, M. Martín
383 Sampayo, J. L. Pretus, P. Siroky, M. Wink. A rangewide phylogeography of Hermann’s
384 tortoise, *Testudo hermanni* (Reptilia: Testudines: Testudinidae): implications for taxonomy.
385 *Zoologica Scripta*, 35 (2006) 531–543.
- 386 [28] M. Perez, B. Livoreil, S. Mantovani, M.-C. Boisselier, B. Crestanello, J. Abdelkrim, et al.,
387 Genetic variation and population structure in the endangered Hermann’s tortoise: the roles
388 of geography and human-mediated processes, *J Hered.* 105 (2014) 70–81.
389 doi:10.1093/jhered/est071.
- 390 [29] D. Stubbs, I.R. Swingland, A. Hailey, E. Pulford, The ecology of the mediterranean tortoise
391 *Testudo hermanni* in northern Greece (the effects of a catastrophe on population structure
392 and density), *Biol Conserv.* 31 (1985) 125–152. doi:10.1016/0006-3207(85)90045-X.
- 393 [30] C. Rondinini, A. Battistoni, V. Peronace, C. Teofili (eds.). (2013) *Lista Rossa IUCN dei*
394 *Vertebrati Italiani*. Comitato Italiano IUCN e Ministero dell’Ambiente e della Tutela del
395 Territorio e del Mare, Roma.
- 396 [31] P.S. Walsh, D.A. Metzger, R. Higuchi, Chelex 100 as a medium for simple extraction of
397 DNA for PCR-based typing from forensic material., *BioTechniques.* 10 (1991) 506–513.
- 398 [32] C. Ciofi, M.C. Milinkovitch, J.P. Gibbs, A. Caccone, J.R. Powell, Microsatellite analysis of
399 genetic divergence among populations of giant Galápagos tortoises, *Mol. Ecol.* 11 (2002)
400 2265–2283.
- 401 [33] A. Forlani, B. Crestanello, S. Mantovani, B. Livoreil, L. Zane, G. Bertorelle, L. Congiu,
402 Identification and characterization of microsatellite markers in Hermann’s tortoise (*Testudo*
403 *hermanni*, Testudinidae), *Mol. Ecol. Notes.* 5 (2005) 228–230.
- 404 [34] J.K. Pritchard, M. Stephens, P. Donnelly, Inference of Population Structure Using
405 Multilocus Genotype Data, *Genetics.* 155 (2000) 945–959.
- 406 [35] J. Wang, The computer program structure for assigning individuals to populations: easy to
407 use but easier to misuse, *Mol Ecol Resour.* 17 (2017) 981–990. doi:10.1111/1755-
408 0998.12650.
- 409 [36] N.M. Kopelman, J. Mayzel, M. Jakobsson, N.A. Rosenberg, I. Mayrose, Clumpak: a
410 program for identifying clustering modes and packaging population structure inferences
411 across K, *Mol Ecol Resour.* 15 (2015) 1179–1191. doi:10.1111/1755-0998.12387.
- 412 [37] D.A. Earl, B.M. vonHoldt, STRUCTURE HARVESTER: a website and program for
413 visualizing STRUCTURE output and implementing the Evanno method, *Conserv. Genet.*
414 *Resour.* 4 (2012) 359–361. doi:10.1007/s12686-011-9548-7.

- 415 [38] G. Evanno, S. Regnaut, J. Goudet, Detecting the number of clusters of individuals using the
416 software structure: a simulation study, *Mol. Ecol.* 14 (2005) 2611–2620.
417 doi:10.1111/j.1365-294X.2005.02553.x.
- 418 [39] S. Manel, P. Berthier, G. Luikart, Detecting wildlife poaching: Identifying the origin of
419 individuals with Bayesian assignment tests and multilocus genotypes, *Conserv. Biol.* 16
420 (2002) 650–659. doi:10.1046/j.1523-1739.2002.00576.x.
- 421 [40] J.M. Cornuet, S. Piry, G. Luikart, A. Estoup, M. Solignac, New methods employing
422 multilocus genotypes to select or exclude populations as origins of individuals, *Genetics.*
423 153 (1999) 1989–2000.
- 424 [41] B. Rannala, J.L. Mountain, Detecting immigration by using multilocus genotypes, *Proc*
425 *Natl Acad Sci U A.* 94 (1997) 9197–9201.
- 426 [42] S. Piry, A. Alapetite, J.-M. Cornuet, D. Paetkau, L. Baudouin, A. Estoup, GENECLASS2: a
427 software for genetic assignment and first-generation migrant detection, *J Hered.* 95 (2004)
428 536–539. doi:10.1093/jhered/esh074.
- 429 [43] D. Paetkau, R. Slade, M. Burden, A. Estoup, Genetic assignment methods for the direct,
430 real time estimation of migration rate: a simulation based exploration of accuracy and
431 power, *Mol. Ecol.* (2004) 55–65. doi:10.1046/j.1365-294X.2003.02008.x.
- 432 [44] S. Zenboudji, M. Cheylan, V. Arnal, A. Bertolero, R. Leblois, G. G. Astruc, et al.,
433 Conservation of the endangered Mediterranean tortoise *Testudo hermanni hermanni*: The
434 contribution of population genetics and historical demography. *Biological Conservation,*
435 195 (2016) 279–291. doi:10.1016/j.biocon.2016.01.007.
- 436 [45] C. Corti, L. Bassu, M. Biaggini, N. Bressi, M. Capula, A.R. Di Cerbo, et al., Updated
437 distribution of *Testudo hermanni hermanni* in Italy. In: Caron, S. (ed.), Proceedings of the
438 International workshop on the management and restoration of Hermann’s tortoise habitats
439 and populations. Atelier international sur la gestion et la restauration des populations et
440 habitats de la Tortue d’Hermann International workshop on the management and restoration
441 of Hermann’s tortoise populations and habitats. Soptom, Gonfaron, France (2013).
442 Download at: www.tortue-hermann.eu Copyright: © 2014 SOPTOM-CRCC.
- 443 [46] G. Giacalone, M. Lo Valvo, U. Fritz. Phylogeographic link between Sicilian and Corso-
444 Sardinian *Testudo h. hermanni* confirmed. *Acta Herpetologica*, 4(2) (2009) 119-123.
- 445 [47] D. Canestrelli, F. Sacco, G. Nascetti, On glacial refugia, genetic diversity, and
446 microevolutionary processes: deep phylogeographical structure in the endemic newt
447 *Lissotriton italicus*, *Biol. J. Linn. Soc.* 105 (2012) 42–55. doi:10.1111/j.1095-
448 8312.2011.01767.x.
- 449 [48] D. Canestrelli, R. Cimmaruta, G. Nascetti, Population genetic structure and diversity of the
450 Apennine endemic stream frog, *Rana italica*– insights on the Pleistocene evolutionary
451 history of the Italian peninsular biota, *Mol. Ecol.* 17 (2008) 3856–3872.
452 doi:10.1111/j.1365-294X.2008.03870.x.
- 453 [49] A. Chiochio, R. Bisconti, M. Zampiglia, G. Nascetti, D. Canestrelli, Quaternary history,
454 population genetic structure and diversity of the cold-adapted Alpine newt *Ichthyosaura*
455 *alpestris* in peninsular Italy, *Sci. Rep.* 7 (2017) 2955. doi:10.1038/s41598-017-03116-x.
- 456 [50] D. Canestrelli, R. Cimmaruta, V. Costantini, G. Nascetti, Genetic diversity and
457 phylogeography of the Apennine yellow-bellied toad *Bombina pachypus*, with implications
458 for conservation, *Mol. Ecol.* 15 (2006) 3741–3754. doi:10.1111/j.1365-294X.2006.03055.x.

459 [51] D. Canestrelli, G. Aloise, S. Cecchetti, G. Nascetti, Birth of a hotspot of intraspecific
460 genetic diversity: notes from the underground, *Mol. Ecol.* 19 (2010) 5432–5451.
461 doi:10.1111/j.1365-294X.2010.04900.x.

462 [52] I.R. Swingland, M.W. Klemens, *The conservation biology of tortoises*, IUCN, 1989.

463 [53] M. Masseti, *I Rettili e l'uomo: elementi di archeozoologia ed etnozoologia* (p. 67-94). In
464 Corti C., Capula M., Luiselli L., Razzetti E. & Sindaco R. (eds). *Fauna d'Italia. Reptilia.*
465 Edizioni Calderini de Il Sole 24 Ore Editoria Specializzata S.r.l.», Bologna, (2010), 874 pp.

466 [54] J.E. Seeb, G. Carvalho, L. Hauser, K. Naish, S. Roberts, L.W. Seeb, Single-nucleotide
467 polymorphism (SNP) discovery and applications of SNP genotyping in nonmodel
468 organisms, *Mol. Ecol. Resour.* 11 (2011) 1–8. doi:10.1111/j.1755-0998.2010.02979.x.

469

470

471

472

473

474

475

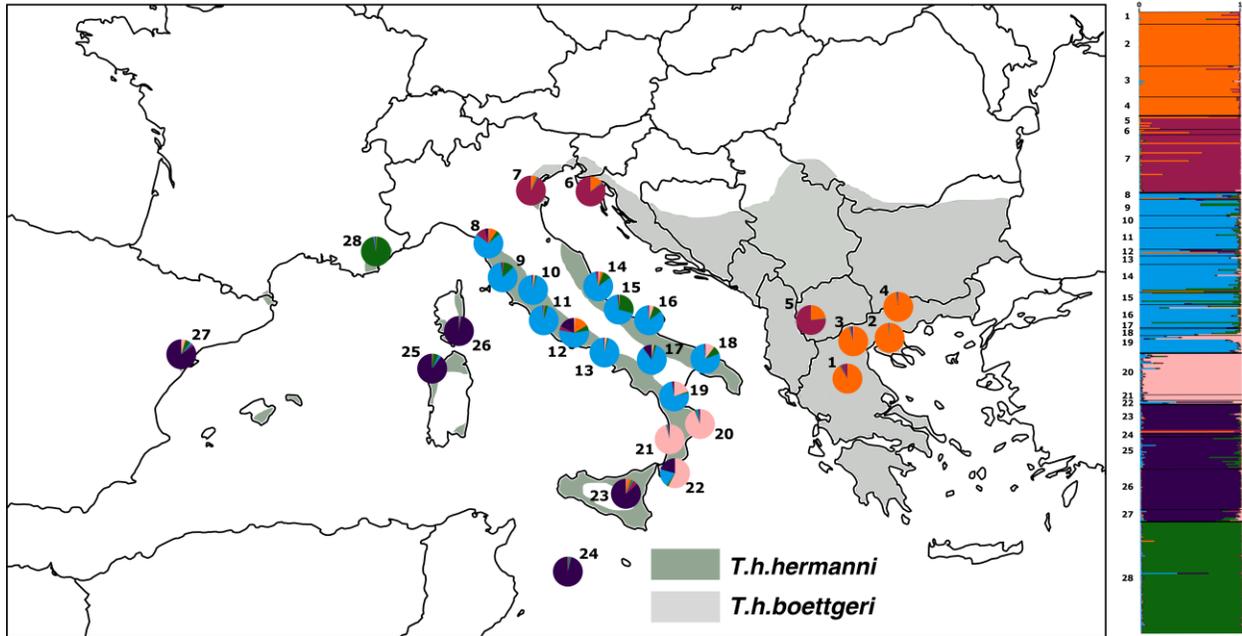
476

477

478

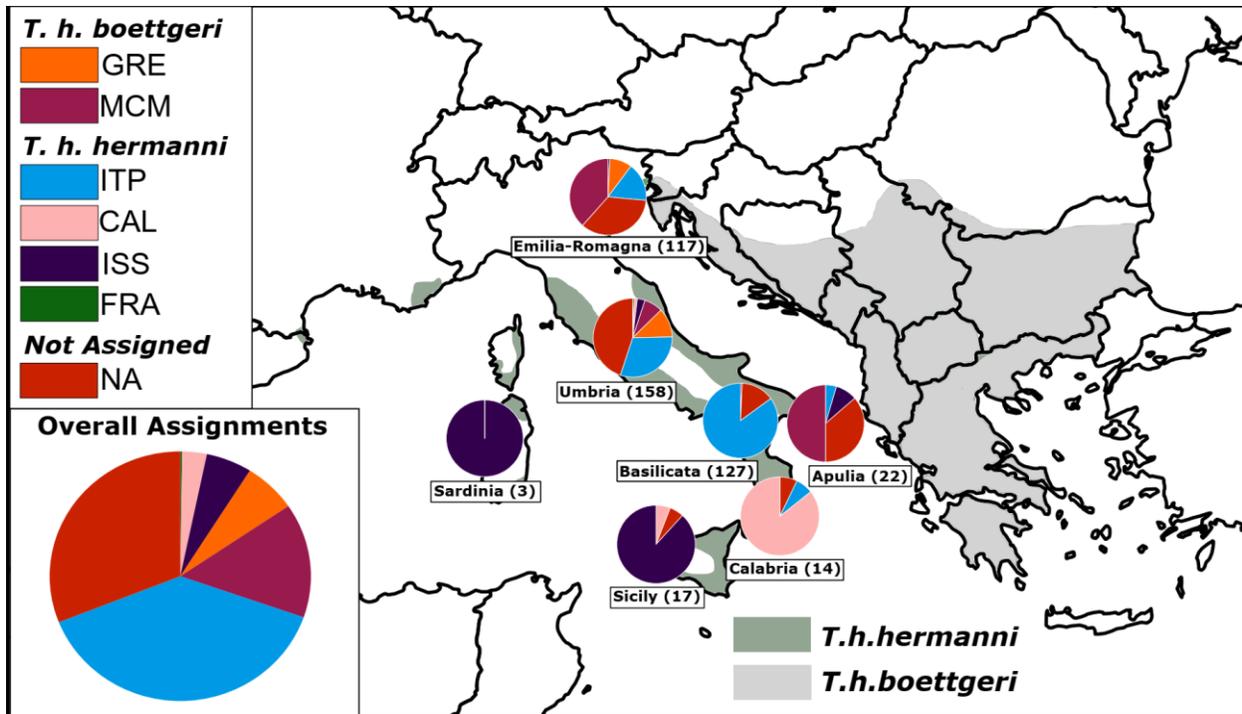
479

480



481 **Figure 1.** Genetic structure of wild *Testudo hermanni* populations estimated using
482 STRUCTURE. The wild dataset resulted from the integration of samples collected in this study
483 and individuals from [28]. Sampling locations and number of individuals sampled per sites (N)
484 of the integrated dataset are: 1-Meteore [Greece] (N=9), 2-Epanomi [Greece] (N=31), 3-Aliki
485 [Greece] (N=23), 4-Kerkini [Greece] (N=14), 5-Prespa Lake [Macedonia] (N=10), 6-Vodnjan
486 [Croatia] (N=4), 7-Emilia-Romagna (N=43), 8-Tuscany North (N=5), 9-Tuscany South (N=12),
487 10-Lazio North (N=9), 11-Lazio Center (N=16), 12-Lazio South (N=5), 13-Campania North
488 (N=6), 14-Abruzzo (N=21), 15-Molise (N=9), 16-Puglia North (N=17), 17-Campania Center
489 (N=1), 18-Puglia South (N=5), 19-Calabria North (N=13), 20-Calabria Center-North (N=31),
490 21-Calabria Center-South (N=4), 22-Calabria South (N=3), 23-Sicily (N=22), 24-Lampedusa
491 (N=2), 25-Sardinia (N=24), 26-Corsica (N=30), 27-Ebro [Spain] (N=9), 28-Var [France]
492 (N=83). In italic are shown new sampling sites from this study and locations whose sampling
493 was increased from [28].

494



495

496 **Figure 2.** Geographic assignment of 458 confiscated samples from seven Italian seizure and
 497 recovery centres. Overall assignments are showed in the pie chart in the lower left corner. Local
 498 assignments for each recovery centre are showed in the pie charts on the map (in brackets the
 499 samples size). GRE = Greece; MCM = Bosco Mesola, Croatia and Macedonia; ITP = Italian
 500 Peninsula; CAL = Calabria; ISS = Mediterranean Islands and Spain; FRA = France; NA = not
 501 assigned samples.

502

503

504

505

506

Individual ID	Sampling Location (Prior Cluster)	Most probable result
77	Site 7 (MCM)	Migrant from GRE
CA5	Site 8 (ITP)	Migrant from MCM
SAB3	Site 12 (ITP)	Migrant from GRE
6TS	Site 14 (ITP)	Migrant from GRE
RG1	Site 19 (ITP)	Hybrid ITPxCAL
RI1	Site 23 (ISS)	Migrant from MCM
RI2	Site 23 (ISS)	Migrant from GRE

507

508 **Table 1.** List of hybrid and migrant samples detected among wild populations. Sites are referred

509 to the sampling locations (see fig. 1).