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# 1 Harbor porpoise losing its edge: genetic time series suggests a rapid 2 population decline in Iberian waters over the last 30 years

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38

## Abstract

39 Impact of climate change is expected to be especially noticeable at the edges of a species'  
40 distribution, where they meet sub-optimal habitat conditions. In Mauritania and Iberia, two  
41 genetically differentiated populations of harbor porpoises (*Phocoena phocoena*) form an  
42 ecotype adapted to local upwelling conditions and distinct from other ecotypes further north  
43 on the NE Atlantic continental shelf and in the Black Sea. By analyzing the evolution of  
44 mitochondrial genetic variation in the Iberian population between two temporal cohorts (1990-  
45 2002 vs. 2012-2015), we report a substantial decrease in genetic diversity. Phylogenetic  
46 analyses including neighboring populations identified two porpoises in southern Iberia  
47 carrying a divergent haplotype closely related to those from the Mauritanian population, yet  
48 forming a distinct lineage. This suggests that Iberian porpoises may not be as isolated as  
49 previously thought, indicating possible dispersion from Mauritania or an unknown population  
50 in between, but none from the northern ecotype. Demo-genetic scenario testing by approximate  
51 Bayesian computation showed that the rapid decline in the Iberian mitochondrial diversity was  
52 not simply due to the genetic drift of a small population, but models support instead a  
53 substantial decline in effective population size, possibly resulting from environmental  
54 stochasticity, prey depletion, or acute fishery bycatches. These results illustrate the value of  
55 genetics time series to inform demographic trends and emphasize the urgent need for  
56 conservation measures to ensure the viability of this small harbor porpoise population in  
57 Iberian waters.

58

59 **KEYWORDS:** cetacean conservation genetics, genetic time series, genetic diversity,  
60 bycatch, approximate Bayesian computation, population genetic modelling

## 61 **1. Introduction**

62 The impact of climate change on species is expected to be especially noticeable at their  
63 distribution edges, where species meet the limits of their ecological tolerance. Marginal  
64 populations of temperate species at the rear (warm) distribution edge are often considered more  
65 susceptible to warming than central populations because of the warmer ambient temperatures  
66 they experience, but this overlooks the potential for local adaptation (Bennett et al., 2015; Vilà-  
67 Cabrera et al., 2019).

68 Cetaceans are particularly vulnerable to climate change (MacLeod, 2009) due to their  
69 crucial function as apex predators in marine ecosystems (Bowen, 1997). Climate change  
70 influences the availability of their prey and modifies the habitat, thereby impacting several (if  
71 not all) cetacean species and may even put some of them at a high risk of extinction (Learmonth  
72 et al., 2006). As a response, several cetacean species are shifting their distribution ranges to  
73 track suitable habitats or adapt locally by switching to different food resources (Lambert et al.,  
74 2011; Learmonth et al., 2006; MacLeod, 2009; Williamson et al., 2021). For example,  
75 temperate and subpolar small cetaceans like the harbor porpoises (*Phocoena phocoena*) are  
76 likely to show a poleward shift (Heide-Jørgensen et al., 2011; MacLeod, 2009). Due to an  
77 elevated metabolic rate and relatively short generation time, this small cetacean is heavily  
78 dependent on a continuous food supply (Hoekendijk et al., 2018; Wisniewska et al., 2016).  
79 This species is thus expected to be particularly sensitive to climate changes (Heide-Jørgensen  
80 et al., 2011). In addition, due to their coastal distribution, thousands of harbor porpoises are  
81 also killed accidentally each year by commercial fisheries. This by-catch mortality is  
82 threatening local populations to a level that is still hard to quantify, but it is likely unsustainable,  
83 especially with a highly fluctuating environment (Carlén et al., 2021; IMR-NAMMCO, 2019;  
84 Vingada & Eira, 2018).

85 Harbor porpoises are officially subdivided into three distinct subspecies: *P. p. vomerina*  
86 in the Pacific, *P. p. phocoena* in the North Atlantic, and *P. p. relicta* in the Black Sea (Fontaine,  
87 2016; Read, 1999; Rosel et al., 1995). A fourth lineage or Evolutionary Significant Unit (ESU)  
88 has been suggested for the Afro-Iberian harbor porpoises (IBMA; currently unnamed sub-  
89 species, but possibly *P. p. meridionalis* following Fontaine et al. 2014) owing to their  
90 distinctive ecology, morphology (Donovan & Bjørge, 1995; Smeenk et al., 1992), and genetic  
91 divergence (Fontaine et al., 2014) (Figure 1). However, no formal description or subspecies  
92 taxonomic assignment has been made for this distinct lineage so far (Pierce et al., 2020). IBMA  
93 porpoises are found around the relatively cold and productive upwelling waters off the coasts  
94 of Iberia and on the NW Atlantic coasts of Africa from southern Morocco, to Mauritania, and  
95 Senegal (Fontaine, 2016; Gaskin, 1984; IMR-NAMMCO, 2019; Read, 1999). They are  
96 significantly larger in term of skull morphology, growth curve, and adult body size (typically  
97  $\geq 2$ m long) compared to the two other ecotypes (or subspecies) ( $\sim 1.5$ m long) (Galatius &  
98 Gol'din, 2011; Read, 1999; Sequeira, 1996; Smeenk et al., 1992). Beside their distinctive  
99 morphological features, the IBMA porpoises belong to the Eastern Canary Coastal province  
100 with equatorward surface circulation in the form of the Canary current, rich in eddies and large  
101 upwellings that are permanent along the NW African coast and seasonal along the Iberian coast  
102 (Álvarez-Salgado et al., 2003; Fraga, 1981; Moustahfid et al., 2021; Pierce et al., in revision).  
103 While *P. p. phocoena* in the northern Bay of Biscay and further north in the North Atlantic are  
104 generally considered opportunistic feeder relying primarily on bottom dwelling on the shallow  
105 continental shelf (i.e. in waters of  $< 200$  m depth), the IBMA porpoise feeding ecology is still  
106 poorly known. It is hypothesized that they exhibit similar foraging behaviors as their  
107 counterparts on the continental shelf to the north, but with a greater emphasis on pelagic fish  
108 (Pierce et al., in revision; Pinela et al., 2010; Santos & Pierce, 2003). From a genetic  
109 perspective, the Iberian and Mauritanian porpoises are closely related but they were found

110 genetically differentiated from each other and form two distinct populations separated by at  
111 least 2500 km (Ben Chehida et al., 2021; Fontaine, 2016; Fontaine et al., 2014).

112 The upwelling habitat of the Iberian porpoise (IB) population is at the border of a  
113 biogeographical transition zone between temperate and subtropical waters where species with  
114 different affinities co-occur and reach their respective ecological limits (Bowen, 1997;  
115 MacLeod, 2009). Because species' habitat suitability at the margins between biogeographic  
116 zones are often suboptimal, populations like the Iberian harbor porpoises are expected to be  
117 among the first to show the impacts of environmental changes. This is particularly true with  
118 the small-scale Iberian upwelling system, which is known to fluctuate in response to ongoing  
119 climate change (Casabella et al., 2014; Pires et al., 2013). Such environmental fluctuation may  
120 impact the Iberian harbor porpoises in various ways, possibly leading to dramatic impacts on  
121 the population demography and genetic diversity. Porpoises may adapt locally, for example,  
122 by adapting to various prey species (Heide-Jørgensen et al., 2011), by migrating to more  
123 suitable environments, or the population may also become extirpated if environmental changes  
124 outpace their ability to adapt. Previous population genetic studies (Ben Chehida et al., 2021;  
125 Fontaine et al., 2014; Fontaine et al., 2017; Fontaine et al., 2010), based on samples collected  
126 between 1990 and 2002, showed that the Iberian population was a source population with  
127 highly asymmetric gene flow, sending more migrants to the North into the *P. p. phocoena*  
128 populations on the northern side of the Bay of Biscay and to the South into the Mauritanian  
129 population than in the other direction (Figure 1). In fact, a tension zone was detected in the  
130 northern side of the Bay of Biscay with Iberian genetic ancestry decreasing sharply with the  
131 increasing latitude (Fontaine et al., 2007; Fontaine et al., 2014; Fontaine et al., 2017). If  
132 recruitment does not keep up with the mortality and migration rate, the census size of the  
133 Iberian population may decrease, leading to a gradual reduction in number of reproducing  
134 individuals, in genetic diversity, and thus in effective population size over time.

135 Unlike previous studies, which inferred population structure between the Iberian  
136 population and other populations (Ben Chehida et al., 2021; Fontaine et al., 2014; Fontaine et  
137 al., 2017; Fontaine et al., 2010), the goal of the present study is to assess how mtDNA variation  
138 evolved over time in the Iberian population, comparing sampling cohorts spanning about 3  
139 decades and how this information can guide conservation of the Iberian population. More  
140 precisely, using genetic diversity as a proxy for effective population size variation, we tested  
141 if mitochondrial genetic diversity decreased through time by comparing the genetic  
142 composition of two cohorts of porpoises, one sampled in the 1990's (1990-2002), and the other  
143 one from the 2010's (2012-2015).

144

## 145 **2. Material and methods**

146 *Sampling and molecular analyses.* Tissue samples from 52 by-caught or stranded  
147 individuals collected along the Portuguese coast between 2012 and 2015 were obtained from  
148 the Portuguese Marine Animal Tissue Bank (MATB). These samples form the 2010's cohort  
149 of the Iberian population (also called the 'new' cohort or IB\_N in this study) (**Figure 1, Table**  
150 **1 and S1**). Tissues samples were stored in 70% ethanol and kept at -20°C until molecular  
151 analyses. Genomic DNA was extracted with the DNeasy Blood and Tissue kit (QIAGEN Inc.)  
152 following the manufacturer's protocol. Following Fontaine et al. (2014), we screened five  
153 mitochondrial coding regions (*ATP-6*, *ATP-8*, *Cyt-b*, *ND5*, and *COI*) for genetic  
154 polymorphisms using Sanger sequencing. Target regions were PCR amplified, quality  
155 controlled, and purified following the protocol described in Fontaine et al. (2014). Sanger  
156 sequencing was outsourced to GATC Biotech Ltd. The resulting forward and reverse sequences  
157 were visually inspected, cleaned, and assembled with *Geneious* 10.0.9 (Kearse et al., 2012).

158 We augmented the dataset with the 82 previously published sequences from Fontaine et al.  
159 (2014) encompassing the same five mitochondrial genes. These sequences were obtained for

160 82 harbor porpoises collected between 1990 and 2002 (**Figure 1, Table 1 and S1**). These  
161 included: 14 individuals collected along the French coast of the Bay of Biscay (BB), 23 further  
162 north in the North Atlantic (NAT) from the Channel to the Nordic waters of Norway and  
163 Iceland; 19 from the Iberian coast (*a.k.a.* the 'old' Iberian cohort or IB\_O), 14 from Mauritania  
164 (MA); and 12 from the Black Sea (BS). The 'old' Iberian cohort from Fontaine et al. (2014)  
165 obtained between 1990 and 2002 included 19 stranded and bycaught porpoises from the  
166 Galician coasts of Spain and Portugal (Figure 1 and Table S1). Previous studies based on  
167 mtDNA data and nuclear microsatellite data demonstrated that the Spanish and Portuguese  
168 samples were part of a same panmictic genetic group (Ben Chehida et al., 2021; Fontaine et  
169 al., 2007; Fontaine et al., 2014; Fontaine et al., 2017). The main focus of the present study thus  
170 consists in comparing the temporal evolution of the mtDNA variation between the two Iberian  
171 cohorts of porpoises: *i.e.* the 'new' [2010's: 2012-2015, n=52] *versus* the 'old' [1990's:1990-  
172 2002, n=19]). The temporal coverage of this serial sampling of the Iberian population is thus  
173 at most 25 years, which corresponds to 2.5 generations, assuming a generation time of 10 years  
174 (Read, 1999). The mtDNA sequences from the other populations of Fontaine et al. (2014)  
175 neighboring the Iberian population were included here to provide a broader perspective to  
176 interpret the phylogenetic analyses.

177 One additional sequence from the closest outgroup species, the Dall's porpoise  
178 (*Phocoenoides dalli*) also generated in Fontaine et al. (2014), was included in the present  
179 dataset to root the mitochondrial phylogeny. All mtDNA sequences (n=135) were aligned with  
180 *MUSCLE* (Edgar, 2004).

181

182 *Phylogenetic and population genetic analyses.* In order to place the Iberian porpoises into  
183 a global phylogeographic context, we first reconstructed a maximum-likelihood (ML)  
184 phylogeny with all the 135 mtDNA sequences (including the outgroup). Unique mtDNA



185 haplotypes were identified using *DnaSP* v.5.10.1 (Librado & Rozas, 2009). Then, we used  
186 *PhyML* (Guindon et al., 2010) implemented in *Geneious* to estimate the ML tree based on all  
187 available unique haplotypes identified in the dataset using a nearest neighbor interchange  
188 topology search. We used the Tamura and Nei (1993)'s (or TN93) model of nucleotide  
189 evolution as identified as the best-fitting model for our sequence alignment using the Akaike  
190 Information Criterion (AIC) in jModelTest 2.1.10 (Darriba et al., 2012). Each node's support  
191 was evaluated by conducting 5,000 iterations of bootstrap resampling. The phylogenetic tree  
192 was rooted with the sequence from a Dall's porpoise. In addition to the phylogenetic trees, we  
193 also reconstructed a median-joining haplotype network (Bandelt et al. 1999) using *PopART*  
194 (<http://popart.otago.ac.nz>).

195 Various statistics informative on the mtDNA genetic variation of the two cohorts of  
196 Iberian porpoises ("old" vs. "new") were estimated using the C library *libdiversity*  
197 (<https://bioinfo.mnhn.fr/abi/people/achaz/cgi-bin/neutralitst.c>) and the R package *pegas*  
198 v.1.2 (Paradis, 2010) (**Table 1**). Genetic diversity was estimated using the number of  
199 haplotypes ( $H$ ), haplotype diversity ( $H_d$ ), nucleotide diversity ( $\pi$ ), and Watterson's theta ( $\theta_W$ ).  
200 Differences in sample size can significantly impact the values of some genetic diversity  
201 estimators (Goodall-Copestake et al., 2012). Therefore, a rarefaction approach was applied to  
202 estimate measures of genetic diversity while correcting for difference in sample size (Sanders,  
203 1968) using a custom python script. More precisely, for the two cohorts of Iberian porpoises,  
204 for each statistic and each sample size increment  $k$ , we randomly subsampled 5,000 times with  
205 replacement a number of  $k$  sequences available in each group, with  $k$  ranging between 2 and  
206 the  $k_{max}$  available sequences (up to a maximum of 50). At each sample size increment  $k$ , we  
207 estimated the mean and standard error values per statistic and per group. Subsequently, genetic  
208 differentiation between the two cohorts was assessed using the Hudson's estimator of  $F_{ST}$   
209 (Hudson et al., 1992) and  $\phi_{ST}$  (Excoffier et al., 1992) using respectively *the libdiversity C-*

210 *library* and the R-package *haplotypes v.1.1.3.1*. The significance levels of  $F_{ST}$  and  $\phi_{ST}$  were  
211 assessed by randomization tests (1,000 permutations). Finally, we investigated evidence of  
212 changes in mtDNA effective population size ( $N_e$ ) between the two cohorts (old vs. new) by  
213 calculating values of Tajima's  $D$  (Tajima, 1989) and Achaz's  $Y$  (Achaz, 2008) accounting for  
214 differences in sample sizes by implementing the same rarefaction approach as described for  
215 the diversity statistics. The Achaz's  $Y$  statistic is analogous to the *Tajima's D*, but accounting  
216 for potential sequencing errors (see details in Achaz, 2008).

217

218 *Approximate Bayesian Computation (ABC)*. In order to evaluate the support for different  
219 demographic scenarios and assess whether there is a signal of a recent population decline in  
220 the Iberian population based on the mtDNA data, we applied an ABC approach relying on  
221 coalescent simulations (Lopes & Beaumont, 2010). More precisely, we compared six  
222 demographic scenarios: (1) a population with a large constant effective population size, (2) a  
223 small constant effective population size, (3) an old expansion ( $\geq 150$  generations, gen.), (4) an  
224 old decline ( $\geq 150$  gen.), (5) a recent decline ( $\leq 3$  gen.), and (6) an old expansion ( $> 150$  gen.)  
225 followed by a recent decline scenario ( $\leq 3$  gen.) (see **Figure 2 and Table S2** for details). In the  
226 coalescent simulations, we leverage the time series data information available and specified  
227 explicitly at which time the samplings of the two cohorts were performed.

228 For each of the six scenarios, we ran 10,000 coalescent simulations using *fastsimcoal2*  
229 *v2.6* (Excoffier & Foll, 2011). Details on the coalescent simulations and the mutation model  
230 used are provided in the supplementary **Table S2**. We used the program *Arlsumstat v3.5.2*  
231 (Excoffier & Lischer, 2010) to compute a set of 55 summary statistics listed in **Table S3**,  
232 augmented with the principal axes obtained from a linear discriminant analysis (LDA) on the  
233 summary statistics (Pudlo et al., 2016), to describe and summarize the genetic variation of each  
234 simulation.

235 We employed a random forest classification (RF) procedure for the model selection (Pudlo  
236 et al., 2016). RF is a machine-learning algorithm that uses bootstrapped decision trees to  
237 classify and select the best scenario using the summary statistics as a set of predictor variables  
238 (Breiman, 2001). RF was therefore applied to compare the likelihood of the six competing  
239 scenarios (Figure 2). This procedure provides classification votes that accounts for the number  
240 of times a scenario is selected as the best one among the trees used to constructed the random  
241 forest. Based on a forest of a 1,000 decision trees, the scenario that gathered the highest number  
242 of votes was selected as the most likely.

243 The *prior* error rate and the type-II error rate were estimated using the out-of-bag  
244 simulations (i.e. simulations not used to build the decision trees). In addition, we computed the  
245 confusion matrix, which provides a more global assessment of the performance of our RF  
246 procedure (Schridder & Kern, 2018). The convergence of the results was evaluated over 10  
247 independent RF runs, as recommended by Fraimout et al. (2017).

248 Finally, to check whether the formulated scenarios and associated priors were compatible  
249 with the observed dataset, we plotted both the simulated datasets from the training set and the  
250 observed dataset on the two first principal axes of a Linear Discriminant Analysis (LDA) and  
251 of a Principal Component Analysis (PCA), following the recommendation of Pudlo et al.  
252 (2016). We visually checked whether the summary statistics from the observed data was  
253 located within the clouds formed by those from the simulated data for at least one demographic  
254 scenario. In addition, we assessed the goodness-of-fit between the simulated and the observed  
255 data following Gelman et al. (2004): the summary statistics for which the cumulative simulated  
256 distribution was larger or smaller than 95% CI of the distribution compared to the observed  
257 value were considered as significantly different from the observed data. All ABC-RF analyses  
258 were performed using the R package *abcrf* v.1.8.1 (Pudlo et al., 2016) in R 4.1.2 (R Core Team,  
259 2016).

260

### 261 **3. Results and Discussion**

262 In total, we successfully sequenced 52 Iberian harbor porpoises from the new cohort  
263 (2012-2015) for fragments of five mitochondrial genes including Cytochrome b (Cyt-b: 1,111  
264 base-pairs, bp), and ATP-6 and -8 (842bp), NADH dehydrogenase 5 (ND5: 707bp), and the  
265 Cytochrome c oxidase subunit I (COI: 1,515 bp), When aligned with the 82 previous sequences  
266 of harbor porpoises from Fontaine et al. (2014) and an additional one of the Dall's porpoise  
267 used here as outgroup, the final alignment of 4,175 base-pairs (pb) and included 135 sequences  
268 and contained 384 segregating sites with 267 singletons and 117 parsimony informative sites  
269 defining 61 distinct haplotypes. Without the outgroup, the 134 mtDNA sequences of harbor  
270 porpoise contained 170 segregating sites with 65 singletons and 105 parsimony informative  
271 sites defining 60 distinct haplotypes ([Table 1](#)).

272 Consistent with previous studies, the phylogenetic relationships among mtDNA  
273 haplotypes depicted by the ML tree ([Figure 3 and S1](#)) and by the haplotype network ([Figure](#)  
274 [S2](#)) revealed the three main monophyletic lineages corresponding to the three previously  
275 identified subspecies or ESU's (Fontaine et al., 2014): (i) *P. p. relicta* in the Black Sea; (ii) *P.*  
276 *p. phocoena* composed of the porpoises from the North Sea to the waters of Norway; and (iii)  
277 the Afro-Iberian (IBMA) porpoises (unnamed *ssp.* possibly *P. p. meridionalis*), which  
278 comprises two distinct monophyletic sub-lineages in the upwelling waters of Iberia and  
279 Mauritania, respectively.

280 Evidence of migration from the Iberian to the Mauritanian populations has been previously  
281 documented (Ben Chehida et al., 2021; Fontaine et al., 2014) and is also observed here with  
282 two haplotypes (Hap\_55 and Hap\_59) found in the Mauritanian population, but clustering with  
283 the Iberian lineage ([Figure 3, S1 and S2](#)). However, we also identified one haplotype (Hap\_10  
284 or Haplotype 10, see [Figure 3, S1 and S2](#)) present in two porpoises from the new Iberian

285 cohort, but clustering with the Mauritanian lineage. This haplotype was carried by two  
286 individuals in the southernmost part of the Iberian sampling (red arrow in **Figure 1**). This result  
287 provides the first evidence of possible dispersal (or migration) from the Mauritanian population  
288 into Iberian waters, suggesting that these two populations may not be as isolated as previously  
289 thought. Haplotype 10 could also belong to a distinct unknown population as it is relatively  
290 divergent from the other Mauritanian haplotypes (**Figure 3, S1 and S2**). Indeed, the ML tree  
291 (**Figure 3 and S1**) shows that this haplotype forms a strongly supported (bootstrap support  
292 >70%) distinct lineage from the other Mauritanian porpoises. The haplotype network also  
293 illustrates this divergence (**Figure S2**): while the Mauritanian haplotypes are separated from  
294 each other with at most two mutational steps, at least ten mutational steps separate this  
295 haplotype 10 from the other Mauritanian haplotypes (**Figure S2**). Notably, both porpoises  
296 carrying this haplotype 10 were found on the southern extremity of the Portuguese coasts  
297 (8.78W-37.07N; Praia do Burgau, Portugal), at least 400km south from all other Iberian  
298 sampling locations (**Figure 1**). Stranding records of harbor porpoises were previously reported  
299 in Morocco, on the east coasts of Gibraltar, and in Cadiz (Spain; Rojo–Nieto et al., 2011). If  
300 unsampled populations occur between the Iberian Peninsula and the Mauritanian waters, they  
301 are most likely confined to a narrow and seasonal upwelling area. This relatively divergent  
302 haplotype 10 closely related to the Mauritanian rather than the Iberian porpoises could be  
303 evidence of such unsampled populations. This hypothesis of such unsampled population could  
304 be plausible since harbor porpoise sightings, stranding, and bycatches have been reported from  
305 the North of Morocco to the Gambia (Boisseau et al., 2010; IMR-NAMMCO, 2019).  
306 Haplotype 10 was excluded from subsequent analyses of genetic diversity and coalescent  
307 simulations because it could be part of a distinct population and therefore would artificially  
308 inflate measures of genetic diversity (**Table 1 and S1**). Future studies with additional samples  
309 from these relatively unsampled regions (southern Iberian coast and Morocco) and data from

310 the nuclear genome are needed to confirm whether these two porpoises are genetically distinct  
311 from the other Iberian porpoises, possibly forming a distinct population, and whether or not  
312 they are admixing with the other Iberian porpoises.

313       Aside from the two porpoises carrying the haplotype 10, our results indicate that both  
314 cohorts sampled at different times along the Iberian coasts belong to the same mtDNA gene  
315 pool. We detected no genetic differentiation between the two Iberian cohorts suggesting no  
316 significant differences in haplotype frequencies between them ( $F_{ST}$  and  $\phi_{ST}$  values = 0;  $p$ -value  
317 > 0.54, **Table S4**). This result reflects the high prevalence of haplotype 4 which dominates in  
318 both cohorts (**Figure 3, S1 and S2**). However, once we accounted for differences in sample  
319 size between the two cohorts using a rarefaction method, we observed a significant reduction  
320 in genetic diversity in the new cohort compared to the old one as shown by the lower number  
321 of haplotypes ( $H$ ; **Figure 4a**), and in the two measures of nucleotide diversity ( $\pi$  and  $\theta_w$ ; **Figure**  
322 **4b and 4c**, respectively). Rarefaction curves provided striking evidence of this reduction in  
323 genetic diversity (**Figure 4**). The non-overlapping standard errors from a standardized sample  
324 size beyond eight sequences suggest that the decline of genetic variation is likely highly  
325 significant. For example, the average number of haplotypes ( $H$ ) observed from 19 sampled  
326 sequences in each cohort decreases from 10 to 6 when comparing the old vs. new cohorts. This  
327 suggests that the proportion of rare haplotypes has dropped substantially over the 25-years  
328 sampling period of this study. Consistent with this observation is the rapid loss in nucleotide  
329 genetic diversity as measured by  $\pi$  and  $\theta_w$  (**Figure 4b and 4c**). Such a substantial loss of genetic  
330 variation over 2.5 generations is often attributed to an enhanced effect of genetic drift rather  
331 than other evolutionary forces like selection (Hartl, 2020). This could suggest a dramatic  
332 decline of the Iberian population in term of effective population size, possibly reflecting a  
333 substantial decline in the number of reproducing individuals.

334 Tajima's  $D$  (Tajima, 1989) and Achaz's  $Y$  (Achaz, 2008) statistics estimated for the two  
335 cohorts sampled at different times along the Iberian coast also displayed negative values  
336 (Figure 5). At first glance, such negative values would be indicative of a population that  
337 underwent a "recent" expansion at evolutionary time-scales (Epps & Keyghobadi, 2015; Smith  
338 et al., 2011), most likely due to the foundation of the Iberian population during the period that  
339 followed the Last Glacial Maximum (19kyr ago; Fontaine et al. 2014; Fontaine 2016).  
340 However, the values estimated for the new cohort for both statistics were significantly less  
341 negative (based on non-overlapped standard error estimates) than those from the old cohort.  
342 For example, the Tajima's  $D$  values obtained from 19 randomly sampled sequences increased  
343 from -1.5 for the old cohort to -1.25 for the new (Figure 5a). The change in Achaz's  $Y$  values,  
344 which account for possible sequencing errors (Achaz, 2008), show an even stronger increase  
345 from -1.75 to -0.95 between the old and new cohorts (Figure 5b). It is remarkable to see how  
346 both values have become dramatically less negative in the time lapse of only 2.5 generations  
347 between the two cohorts. This increase in  $D$  and  $Y$  values also provides a strong indication of  
348 a population losing rare haplotypes as it would be expected for a small and isolated population  
349 strongly subject to genetic drift that could be declining.

350 Such a loss of genetic diversity may reflect different demographic scenarios, for example  
351 a small population that did not experience any population size changes, but still experiencing  
352 strong genetic drift. Alternatively, a scenario of ancient expansion and a very recent decline  
353 could be also possible. We used a simulation-based Approximate Bayesian computation  
354 analysis relying on the Random-Forest classifier (ABC-RF) to explored statistically six  
355 plausible demographic scenarios (Figure 2) describing the evolutionary history of the Iberian  
356 population since it diverged from the Mauritanian population within the last 150 generations  
357 (Fontaine et al., 2014). Out of the six scenarios compared, the analysis strongly supported the  
358 SC6 scenario assuming an "old" expansion (>150 generations) followed by recent decline with

359 the past 3 generations (SC6; **Figure 2, S3 to S5**). Ten replicates of the ABC-RF analysis  
360 supported strongly and unanimously this result (**Figure S3**). Scenario SC6 gathered in average  
361 ( $\pm$  SD) over the ten runs  $56.6 \pm 1.3\%$  of the RF votes (**Figure 2 and S3**), with a posterior  
362 probability of  $66.3 \pm 2.5\%$ , a *prior* error rate of  $9.5 \pm 0.0\%$ , and type II errors  $\leq 3.1\% \pm 0.0\%$ .  
363 Furthermore, the confusion matrix (**Figure S5**) depicting the overall performance of the RF  
364 classification analysis indicated high power to distinguish between the competing scenarios  
365 ( $>90\%$ ). Such a high-resolution power to discriminate among the different demographic  
366 scenarios likely comes from the multiple sampling time points included in the ABC analysis.  
367 The goodness-of-fit analysis provided additional support to the demographic model identified  
368 by showing that scenario SC6 was the only scenario that produced simulated values that closely  
369 matched the observed values for each summary statistics used in this analysis (**Table S5,**  
370 **Figure S4 and S6**). This scenario SC6 (Figure 2) suggests that the effective size ( $N_e$ ) of the  
371 Iberian population expanded at least  $\sim 150$  generations ago, thus  $\sim 1500$  years ago, assuming a  
372 rough generation time of 10 years (Fontaine et al., 2014). Then, under this scenario,  $N_e$  values  
373 of the Iberian population would have declined by a factor  $\geq 10$  within the last three generations  
374 (thus 30 years). Thus, this ABC analysis, together with the rarefaction results, adds an  
375 additional line of evidence suggesting a rapid loss of genetic variation over the last three  
376 generations due to a very recent decline in effective population size. The reader should note  
377 however that the effective population size ( $N_e$ ) is a population genetic concept that differs from  
378 and is usually smaller than the *census* population size. It does not represent the number of  
379 individuals actually alive at any given time point. Following Frankham (2019),  $N_e$  is the  
380 number of individuals that would result in the same loss of genetic diversity, inbreeding,  
381 genetic drift, etc., if they behaved in the manner of an idealized population. Such idealized  
382 population would assume non-overlapping generations, random mating, and no migration,  
383 mutation or selection.  $N_e$  would thus roughly represent the number of breeding individuals in



384 this idealized population, or more precisely the number of breeding females since we analyzed  
385 here maternally inherited fragments of the mitogenome that behave as a single non-  
386 recombining haploid unit. Therefore, our results are not intended to estimate exactly how many  
387 Iberian porpoises there were at different points in history, but rather they suggest that the  
388 number of reproducing females may have been substantially declining in the Iberian population  
389 over the last 2.5 generations. However, such interpretation must be taken with caution  
390 considering all the assumptions related to the  $N_e$  concept.

391 In this study, we also confirmed previous evidence of gene flow from the Iberian  
392 population to more northern populations with Iberian haplotypes present in the Bay of Biscay  
393 and in the English Channel where admixture between the two lineages, IBMA (or *P. p.*  
394 *meridionalis*) and *P. p. phocoena*, has been reported (see Figure 3, S1 and S2; Table S4, and  
395 also Ben Chehida et al., 2021; Fontaine et al., 2014; Fontaine et al., 2017). Additionally, we  
396 did not find any evidence of gene flow from the populations located further North into the  
397 Iberian population, as previously reported (Ben Chehida et al., 2021; Fontaine et al., 2007;  
398 Fontaine et al., 2014; Fontaine et al., 2017; Fontaine et al., 2010). Indeed, Fontaine et al. (2014)  
399 estimated very asymmetric values of gene flow using the coalescent approach of MIGRATE  
400 (Beerli, 2006; Beerli, 2009) based on microsatellite data. While no evidence of immigration  
401 was detected toward the Iberian porpoises from the neighboring populations, *ca.* 3 individuals  
402 per generation were estimated migrating from the Iberian to the Mauritanian populations, and  
403 70 per generation from the Iberian to the northern Bay of Biscay populations (see table 2 in  
404 Fontaine et al., 2014). In the northern Bay of Biscay and adjacent waters, a geographically  
405 restricted admixture zone was reported forming a well-delimited tension zone (Fontaine et al.,  
406 2017). Because the population size of *P. p. phocoena* in the North Sea and adjacent waters is  
407 much larger than that of the Iberian population (census  $N$  estimates from the SCANS-III survey  
408 (Hammond et al., 2017):  $\sim 400,000$  vs.  $\leq 3,000$  individuals respectively), the observed

409 northward migration may possibly lead to the assimilation of the Iberian gene pool by *P. p.*  
410 *phocoena*. In the long term, this process could be an additional cause of concern as it could  
411 result in the disappearance of the unique Iberian genetic pool. This scenario is advanced by  
412 Macleod et al. (2009), which used habitat modeling to show that species with a similar range  
413 as the Iberian harbor porpoises (Figure 1C in MacLeod, 2009) may shift their range northward  
414 in response to a 5°C increase in water temperature due to global warming (Figure 1D in  
415 MacLeod, 2009). This model suggests that the future range of IBMA could completely overlap  
416 with the current range of *P. p. phocoena* (Figure 1D in MacLeod, 2009). It is worth mentioning  
417 that Ben Chehida et al. (2021) recently modeled the future distribution of the harbor porpoises  
418 in the North Atlantic under the most aggressive scenario of the Intergovernmental Panel on  
419 Climate Change (IPCC) in 2050 (RCP8.5; Schwalm et al., 2020) and did not capture this strong  
420 northward range shift. The discrepancy between the two models may be related to differences  
421 in assumed temperature increase, 2°C and 5°C, in Ben Chehida et al. (2021) and MacLeod  
422 (2009), respectively. In addition, the environmental niche modeling in Ben Chehida et al.  
423 (2021) did not account for any biotic factors in the simulations. However, environmental niche  
424 modelling such as those conducted by MacLeod (2009) and Ben Chehida et al. (2021) needs  
425 to be taken with some caution since they do not account for local population adaptation. In the  
426 case of IBMA porpoises, their morphological and genetic distinctiveness together with the  
427 peculiar upwelling habitat they inhabit suggest that they are adapted to the local conditions.  
428 Future genomic studies could assess whether selective processes have impacted the genome of  
429 IBMA porpoises and whether there is genetic evidence of such local adaptation process  
430 compared to other populations.

431 The results of this study support the hypothesis that the Iberian population is both small  
432 and isolated. Furthermore, both the sharp decline in mtDNA genetic diversity over the past 25  
433 years and northward migration may represent reasons for concern for the long-term persistence

434 of the Iberian porpoises. One plausible explanation is that the decay of mtDNA genetic  
435 variation we reported here could simply reflect the fact that Iberian porpoises constitute a stable  
436 population with a very low effective population size ( $N_e$ ) (SC2 in Figure 2). This scenario SC2  
437 was not the best model according to our simulation-based ABC-RF analysis, but the third best  
438 one, receiving only 16% of the total RF classification votes, but it remains plausible (**Figure**  
439 **2, S3 to S6, Table S5**). In such a condition, genetic drift would have a disproportionate  
440 influence on the genetic make-up of this population and could lead to the observed loss of  
441 genetic variation over a few generations. Several previous studies showed that the Iberian  
442 population has indeed a very low  $N_e$  at nuclear microsatellite loci ( $N_e < 100$  individuals; Ben  
443 Chehida et al., 2021; Fontaine et al., 2014; Fontaine et al., 2010). The mtDNA employed in  
444 this study has an effective size four times smaller compared with nuclear autosomal loci. This  
445 means that the stochastic loss of genetic variation due to genetic drift would be drastically  
446 increased for mtDNA compared to nuclear loci. Thus, the Iberian population might be at a  
447 stable census size losing rare haplotypes through the enhanced effect of genetic drift at the  
448 mtDNA. Another more plausible explanation receiving the highest support in our ABC-RF  
449 analysis (scenario SC6, **Figure 2, S2 to S6, Table S5**) is that the decay of mtDNA genetic  
450 variation might be associated with a rapid and recent decline of the number of reproductive  
451 females in addition to the overall low  $N_e$  (Lande, 1988), as depicted by the scenarios SC6 in  
452 **Figure 2**. This scenario SC6 support a severe population decline in the last three generations,  
453 leaving at most 10% of the population pre-decline  $N_e$ . This hypothesis is even more plausible  
454 since there is accumulating evidence that the Iberian population is sending more migrants to  
455 adjacent populations than the reverse (Ben Chehida et al., 2021; Fontaine et al., 2014; Fontaine  
456 et al., 2010). Thus, in addition to being small and isolated, the recent and strong population  
457 decline envisioned under SC6 of the ABC-RF analysis would imply that the Iberian porpoises  
458 may become increasingly subject to demographic stochasticity (Allendorf et al., 2012).

459 Because this population is also known to be subject to strong genetic drift and environmental  
460 stochasticity (Casabella et al., 2014; Pires et al., 2013), all these effects can accumulate and  
461 reinforce each other, potentially precipitating the decline of the Iberian porpoises. This so-  
462 called extinction vortex (Allendorf, et al., 2012) can be particularly severe for a long-lived  $K$   
463 strategist species, inhabiting a marginal habitat highly subject to environmental variation like  
464 the Iberian harbor porpoises (Casabella et al., 2014; Pires et al., 2013) and could put them at a  
465 high risk of extinction.

466 The present study relies on a single molecular marker (mtDNA). Therefore, future studies  
467 with additional samples and data from the nuclear genome are highly needed to confirm the  
468 trends highlighted here. Indeed, genome-wide data can provide further insights into the  
469 temporal dynamics of the Iberian population. For example, the reconstruction of historical  
470 demography based on genome-wide data (Liu & Fu, 2020; Schiffels & Wang, 2020; Terhorst  
471 et al., 2017) can be used to test whether these harbor porpoises maintained a long-term low  $N_e$   
472 (Morin et al., 2020; Robinson et al., 2022) since their post-glacial emergence (Fontaine, 2016;  
473 Fontaine et al., 2014; Fontaine et al., 2010), or if on the contrary, the population declined  
474 recently (Hu et al., 2020). A simulation framework, similar to the one used by Robinson et al.  
475 (2022), could be applied to assess whether these porpoises are already facing deleterious effects  
476 of the mutation load, despite their current low  $N_e$ , or if purging processes have been ongoing  
477 similar to those recently reported for the vaquita (*Phocoena sinus*) (Morin et al., 2020;  
478 Robinson et al., 2022).

479 Additional data sources also point to large fluctuations in population size in the Iberian  
480 porpoises and require careful considerations: the SCANS-III survey in summer 2016 estimated  
481 a census size as low as 2,898 animals (CV=0.32) in Iberian waters and Bay of Biscay  
482 (Hammond et al., 2017). Annual aerial surveys in Portuguese continental waters between 2011  
483 and 2015 estimated an overall average abundance of 2,254 porpoises and corresponding

484 density of 0.09 ind/km<sup>2</sup> (CV=21.99%) with large interannual fluctuations. The highest annual  
485 abundance estimated for that period was 3,207 individuals (CV=38.14%) in 2013, followed by  
486 a sharp decrease in 2014, when only 1,653 individuals (CV=43.27%) were estimated (Torres-  
487 Pereira et al., 2022). López et al. (2013) estimated an abundance of 683 (CV=0.63, 95%CI:  
488 345-951) animals for the Spanish part of the IBMA range (Galicia and Bay of Biscay).  
489 Globally, these low census sizes were associated with particularly high bycatches rate (always  
490  $\geq 8\%$ ) with the best estimates varying from 90-197 deaths per year (Pierce et al., 2020) to 130-  
491 330 deaths per year (Read et al., 2020). However, it is important to mention that these estimates  
492 are mainly based on “on-board observer” and aerial surveys data that deliver wide confidence  
493 limits. This is due to low observer coverage of fishing activities in the region, low density of  
494 porpoises, and estimates from various sources (observer data, strandings, aerial survey, and  
495 interviews). Furthermore, Iberian harbor porpoises are regularly found stranded, often with  
496 signs of bycatch mortality, potentially indicating unsustainable mortality rates (IMR-  
497 NAMMCO, 2019; López et al., 2002; Pierce et al., 2020; Read et al., 2020; Torres-Pereira et  
498 al., 2022; Torres-Pereira et al., 2023a; Vingada & Eira, 2018). In fact, based on Portuguese  
499 stranding data, an average of 9.19% (CI=5.25-16.10%) of the porpoise population was  
500 estimated to be removed annually by fisheries between 2011 and 2015, corresponding to an  
501 average of 207 porpoises removed per year, which largely surpasses the Potential Biological  
502 Removal rate conservatively estimated for the same period (22 porpoises, CI: 12-43) (Torres-  
503 Pereira et al., 2023a). An additional cause for concern is related to potential overexploitation  
504 of the feeding resources (Méndez-Fernandez et al., 2013; Santos & Pierce, 2003). A scarcity  
505 in prey availability can be harmful because it enhances interspecific competition, as already  
506 reported between harbor porpoises and other marine mammals, for example with the bottlenose  
507 dolphin *Tursiops truncatus* whose ecological niche largely overlaps with the harbor porpoise  
508 in some portions of Iberian waters (Méndez-Fernandez et al., 2013; Spitz et al., 2006). Hostile

509 interactions between the two species have been documented in Iberian waters and hypothesized  
510 to cause harbor porpoises to disperse to less productive narrow shelf areas (Alonso et al., 2000;  
511 Spitz et al., 2006).

512

### 513 **Conclusions**

514 The Afro-Iberian (IBMA) lineage of harbour porpoises was previously recognized as a  
515 unique Evolutionarily Significant Unit (Crandall et al., 2000; Moritz, 2002) distinct from the  
516 other sub-species devribed in the N. Atlantic and in the Black Sea. IBMA display unique  
517 features in terms of genetics, morphology, ecology and evolutionary history following Fontaine  
518 et al. (2014) (see also the review of Fontaine, 2016). Nevertheless, they are currently not  
519 recognized as a distinct subspecies (Pierce et al., 2022; Pierce et al., 2020). There is also  
520 currently no mention of these porpoises in the IUCN Red List of Threatened Species despite  
521 the multiple threats faced by this vulnerable lineage. Given the results of this study, the  
522 previous findings on their genetic distinctiveness from other populations at both mtDNA and  
523 nuclear markers (Ben Chehida et al., 2021; Fontaine, 2016; Fontaine et al., 2007; Fontaine et  
524 al., 2014; Fontaine et al., 2017; Fontaine et al., 2010), and the previous concerns raised about  
525 their viability (IMR-NAMMCO, 2019; Pierce et al., 2022; Pierce et al., 2020), we recommend  
526 immediate actions and further research from the community in the following areas: (1) a formal  
527 taxonomic description is needed for these IBMA porpoises to be officially recognized as a  
528 distinct subspecies, following the guidelines of the *International Commission on Zoological*  
529 *Nomenclature* as recommended by the Taxonomic Committee of the Society for Marine  
530 Mammalogy; and (2) a better assessment for the Iberian porpoises in term of their life history  
531 traits, ecology, behavior, genetics and genomics, and the threats to the viability for this unique  
532 group. This knowledge will help designing better conservation measures and  
533 recommendations. It is indeed paramount to ensure that all measures are taken to gain

534 additional knowledge and to apply effective conservation efforts to protect the Iberian  
535 population, as recently underline by the OSPAR Marine Mammal Expert Group (OMMEG)  
536 (ICES-WGMM, 2021; Pierce et al., 2022). Recently, both countries (Portugal and Spain)  
537 updated the conservation status of the harbor porpoises inhabiting Iberian waters: from  
538 “Vulnerable” to “Endangered” in Spain (Boletín oficial del estado, 2020) and to “Critically  
539 Endangered” in Portugal (Torres-Pereira et al., 2023b). In Portugal, a new Marine Protected  
540 Area (PTCON0063) in the northwestern coast was recently designated whereas another one  
541 was enlarged in the southwestern coast (PTCON00012) in order to incur higher protection for  
542 harbor porpoise hotspots in the country (Vingada & Eira, 2018). Nonetheless, none of the  
543 conservation and mitigation measures within the areas’ management plan (Portaria 201/2019)  
544 is currently in place and bycatch mortality remains effectively unaddressed. In the future,  
545 improving our understanding of the demographic trends and the threats these porpoises are  
546 facing is required to devise suitable and tailored larger scale conservation plans for the whole  
547 Iberian population. Such measures are timely needed to mitigate the decline of this unique  
548 lineage potentially on the brink of extinction.

549

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## 570 **Conflict of interest**

571 The authors declare that they have no conflict of interest.

572



573

## 574 **Data Availability**

575 The data sets and scripts supporting this study are available in the IRD Porpoises Genetics and  
576 Genomics Dataverse Repository ([https://dataverse.ird.fr/dataverse/porpoise\\_genomics](https://dataverse.ird.fr/dataverse/porpoise_genomics)) at  
577 <https://doi.org/10.23708/GOPRGV>. Mitochondrial haplotypes were also deposited on the  
578 NCBI–GenBank under the Accession Numbers (Accession numbers are listed in Table S1).

579

## 580 **Author Contributions**

581 Conceptualization: MCF. Data curation: MF, ATP, LN, CE, MCF. Formal analysis: YBC,  
582 TS, MCF. Funding acquisition: MCF. Investigation: JT, TS, JPAH, MCF. Methodology: JT,  
583 TS, JPAH, MCF. Project administration: MCF. Resources: JT, TS, JPAH, MCF. Software:  
584 YBC. Supervision: MCF. Validation: MCF, YBC, JT. Visualization: TS, YBC, MCF.  
585 Writing – original draft: YBC, TS, MCF. Writing – review & editing: YBC, MCF with inputs  
586 from all the co-authors.

587

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## Tables and Figures

**Table 1:** Mitochondrial genetic diversity. The statistics display per groups and overall include the mtDNA sample sizes ( $N$ ), the number of segregating sites ( $S$ ), singleton mutations (*Singl.*); sites informative in parsimony (*Parsim.*), number of haplotypes ( $\# Hap$ ), haplotype diversity ( $Hd$ ), the nucleotide diversity ( $\pi$ ), Watterson's theta ( $\theta_w$ ), Tajima's  $D$ ,  $Y$ , Achaz's  $Y$ .

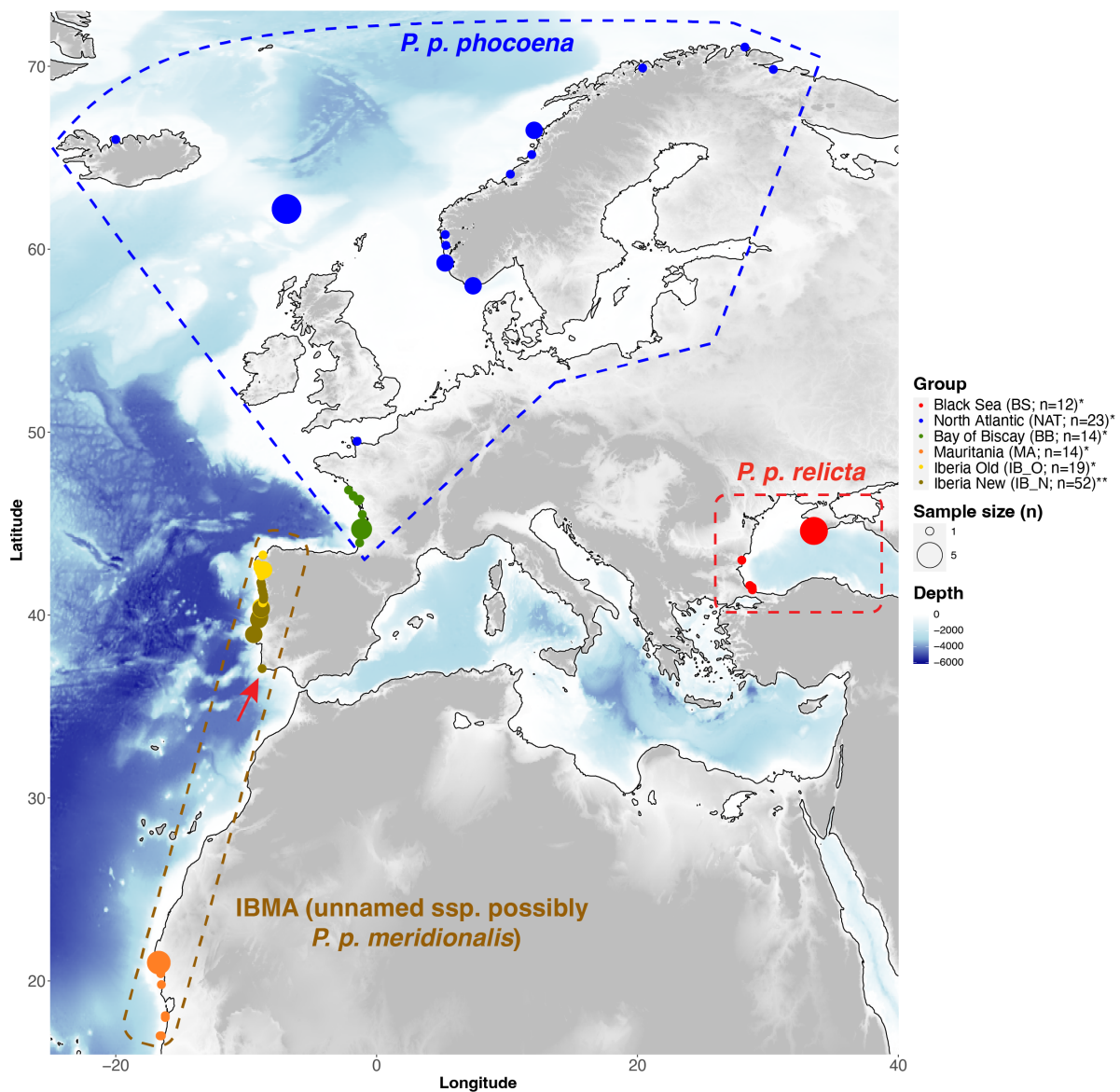
Population codes are as follow: BS=Black Sea; NAT=North Atlantic; BB=Bay of Biscay; MA=Mauritania; IB\_O=Iberian Old 1990's cohort; IB\_N= Iberian new 2010's cohort.

Superscript next to each population code indicate the origin of the mtDNA sequence :  
<sup>1</sup>Fontaine et al. (2014); <sup>2</sup> Fontaine et al. (2014) + this study; 3. This study. See Table S1 for more details on the samples.

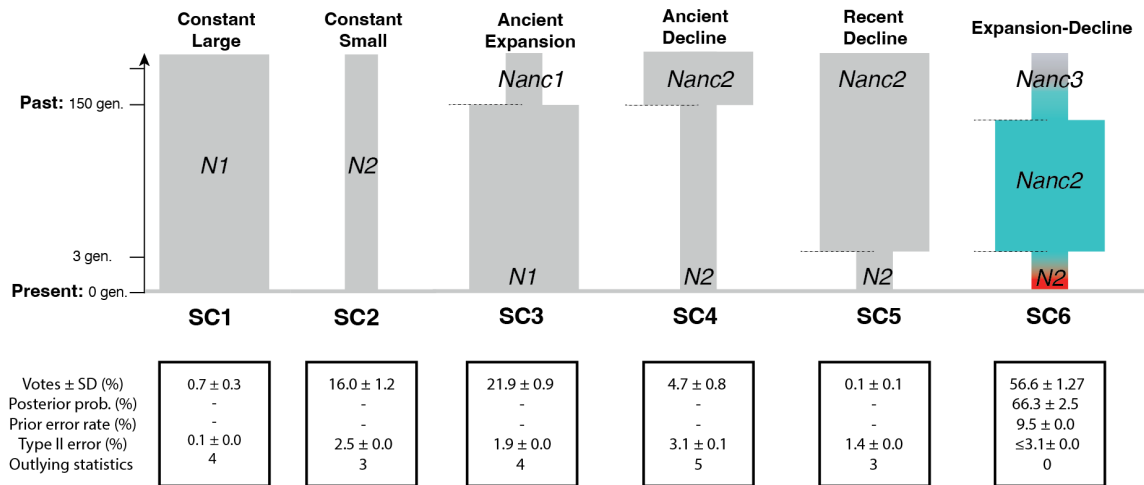
	$N$	$S$	<i>Singl.</i>	<i>Parsim.</i>	$\# Hap$	$Hd$	$\pi$ (%)	$\theta_w$ (%)	$D$	$Y$
<b>BS<sup>1</sup></b>	12	23	21	2	10	0.96	0.10	0.18	-2.04	-1.56
<b>NAT<sup>1</sup></b>	23	99	57	42	18	0.97	0.41	0.64	-1.46	-0.83
<b>BB<sup>1</sup></b>	14	66	19	47	10	0.95	0.52	0.50	0.22	0.05
<b>MA<sup>1</sup></b>	14	11	1	10	7	0.90	0.07	0.08	-0.66	-1.93
<b>All IB<sup>2</sup></b>	71	27	7	20	17	0.72	0.05	0.13	-1.85	-2.12
<b>IB_O<sup>1</sup></b>	19	12	6	6	10	0.74	0.05	0.08	-1.53	-1.77
<b>IB_N<sup>3</sup></b>	52	24	5	19	11	0.70	0.06	0.13	-1.77	-2.19
<b>IB_N*<sup>3</sup></b>	50	14	7	7	10	0.73	0.04	0.08	-1.58	-1.26
<b>All</b>	134	170	65	105	60	0.91	0.40	0.74	-	-

\*Excluding haplotype 10

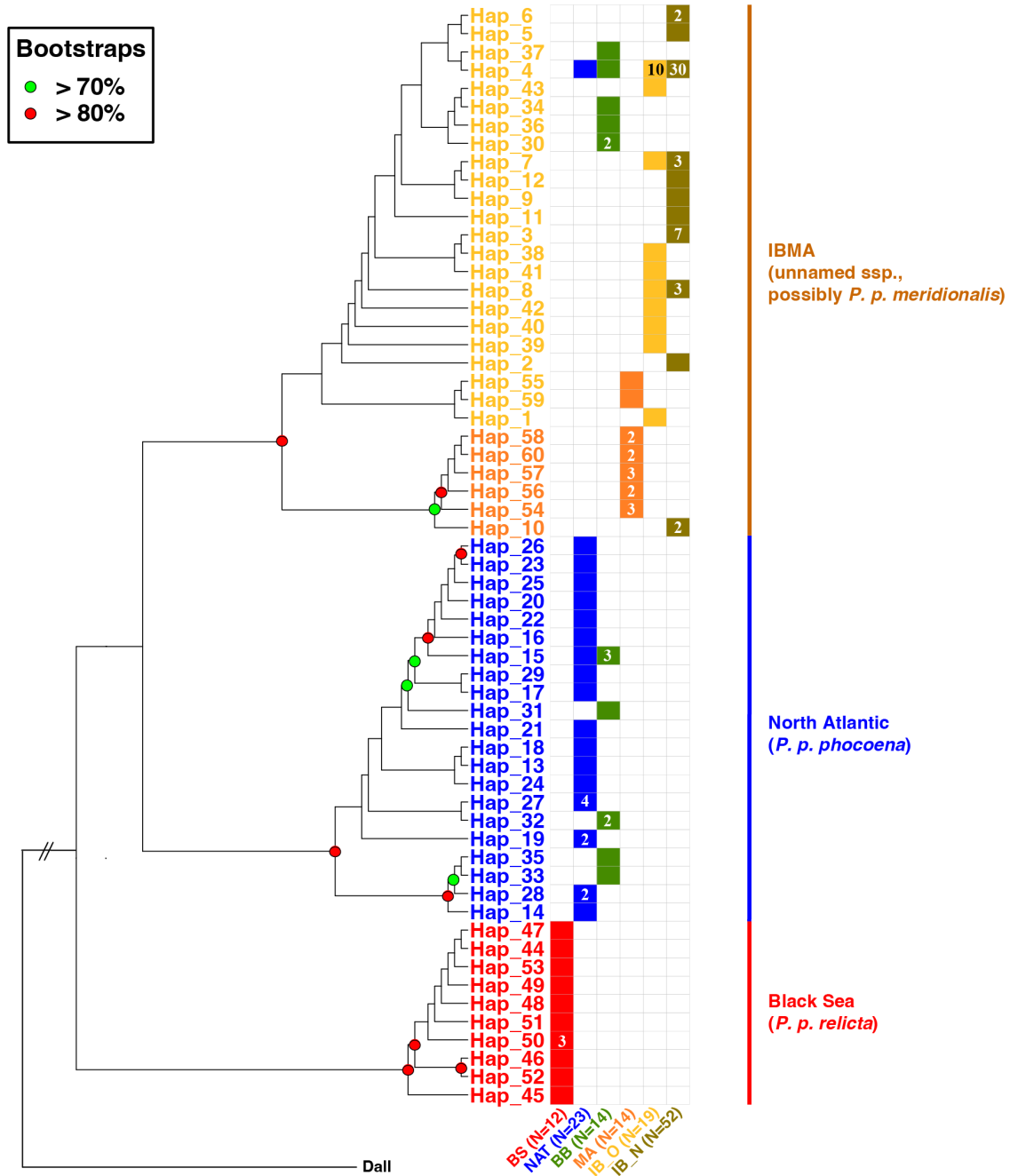




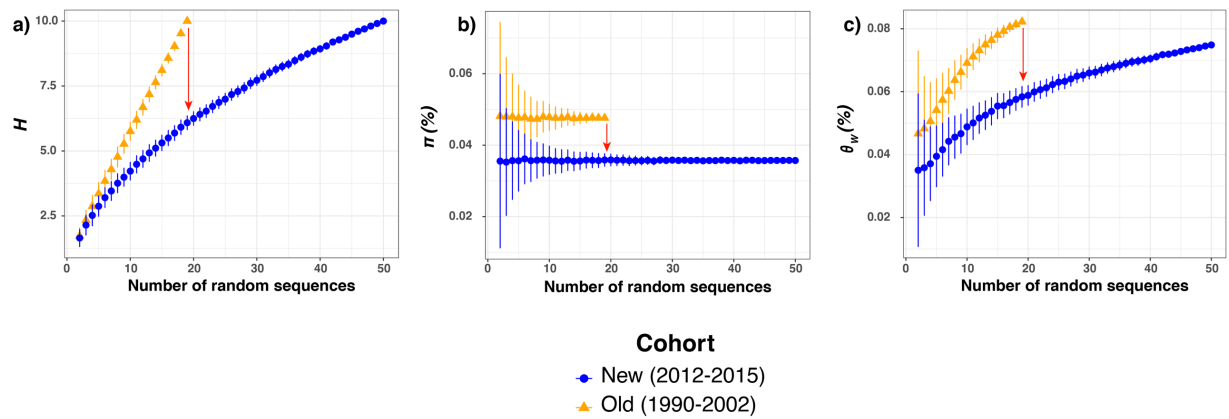
**Figure 1.** Map showing the sampling locations and sample sizes. Geographic locations are based on approximate GPS coordinates or reported discovery locations. The red arrow indicates the geographical location of the two porpoises carrying Haplotype 10. (\* Fontaine et al. (2014); \*\* This study).



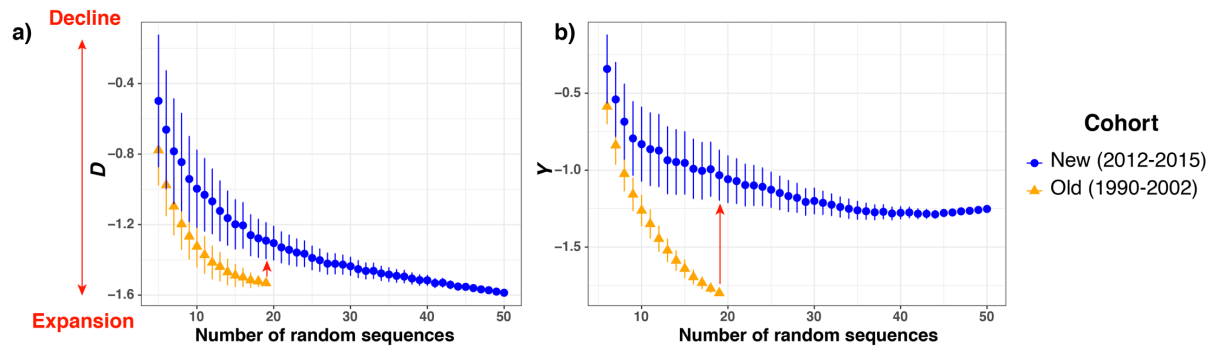
**Figure 2.** Alternative scenarios tested in the ABC-RF procedure. See [Table S2](#) for further details on the model parameters. Below each scenario are provided the main results including the number of Random Forest (RF) votes (in percent), the posterior probability of the best scenario, the prior-error rate, the type-II error rate, and the number of outlying statistics in the goodness-of-fit testing (see [Table S5](#)). The colored scenario, SC6, was the scenario gathering the highest number of Random Forest votes. The time scale is indicated by the arrow on the left, in generations before present (gen.).



**Figure 3.** Cladogram derived from the maximum-likelihood mitochondrial phylogeny among unique mitochondrial haplotypes. A branch length transformation proportional to the number of tips under the node (as implemented in *FigTree*) was applied to facilitate the visualization of the branching patterns (see Figure S1 for the actual phylogenetic tree with branch lengths proportional to sequence divergence). The color-coded labels show the geographic origin of the haplotype. The numbers within the boxes refer to the number of individuals carrying the haplotype. No number in a box means that the haplotype was observed only once. Population code are as follow: BS=Black Sea (N=12)\*; NAT=North Atlantic (N=23)\*; BB=Bay of Biscay (N=14)\*; MA=Mauritania (N=14)\*; IB\_O=Iberian Old (N=19)\*; IB\_N= Iberian new (N=52)\*\*. [\* Fontaine et al. 2014; \*\* This study].



**Figure 4.** Rarefaction curves showing three statistics describing mitochondrial genetic diversity including (a) number of haplotypes ( $H$ ), (b) the nucleotide diversity ( $\pi$ ), and (c) Watterson's theta ( $\theta_w$ ). The red arrow in each panel shows the direction of change between the old and new cohorts. The X-axis shows the number of sequences randomly resampled in the rarefaction procedure to estimate the statistical values. The mean and standard error are provided at each sample size increment ( $k$ ) for each statistic and each group, calculated from 5000 sampling with replacement of the  $k$  sequences available in each group, with  $k$  ranging from 3 to  $k_{max}$  up to 50.



**Figure 5.** Rarefaction curves showing two neutrality indexes indicative of demographic changes including (a) Tajima's  $D$  and (b) Achaz's  $Y$ . The red arrow inside each panel shows the direction of the change between the old and new cohorts. The bidirectional arrow shows the interpretation of  $D$  and  $Y$  in terms of demography. The X-axis shows the number of sequences randomly resampled in the rarefaction procedure to estimate the statistical values.