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Chapter 4

Harbour porpoise losing its edges: genetic time series suggests a rapid population decline in Iberian waters over the last 30 years

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The impact of climate changes on species is expected to be especially visible at the extremities of species distributions, where they meet sub-optimal conditions. Harbor porpoise (*Phocoena phocoena*) is a good model to test this prediction. In the South-Eastern end of the North Atlantic distribution, two genetically isolated populations form a distinct ecotype and are locally adapted to the upwelling waters of Mauritania and Iberia. By analyzing the evolution of mitochondrial genetic variation in the Iberian population between two temporal cohorts sampled between 1990 and 2002 (n=19) and between 2012 and 2015 (n=52), we document here a dramatic decrease in diversity over the past 30 years implying severe population contraction. Phylogenetic analyses including neighboring populations revealed that Iberian porpoises are not as isolated as previously thought and received immigrations from the Mauritanian upwelling population, but not from the northern ecotype. The effective size is declining quickly, likely driven by environmental stochasticity, prey stock depletion, and acute bycatches in commercial fisheries. These results illustrate the value of genetic time series to inform demographic trends and call for additional knowledge to maintain the viability of the upwelling porpoises.

1. Introduction

The impact of climate changes on species is expected to be more visible at their distribution edges, where species are expected to meet their sub-optimal conditions. Marginal populations at the rear (warm) distribution edge are often considered more susceptible to warming than central populations because of the warmer ambient temperatures they experience, although this overlooks the potential for local adaptation.

Cetaceans may be particularly vulnerable to current climate change (MacLeod, 2009) due to their crucial function as apex predators in marine ecosystems (Bowen, 1997). Climate change influences the availability of their prey and modifies the habitat, thereby impacting several (if not all) cetacean species and may even put some of them at a high risk of extinction (Learmonth et al., 2006). As a response, several cetacean species are shifting their distribution ranges to track suitable habitats or adapt locally by switching to different food resources (Lambert et al., 2011; Learmonth et al., 2006; MacLeod, 2009). For example, temperate and subpolar small cetaceans like harbor porpoises (*Phocoena phocoena*) are likely to show a poleward shift (Heide-Jørgensen et al., 2011; MacLeod, 2009). Due to an elevated metabolic rate and fast reproductive turnover, this small cetacean is heavily dependent on a continuous food supply (Hoekendijk et al., 2017; Wisniewska et al., 2016). This species is thus expected to be particularly sensitive to climate changes (Heide-Jørgensen et al., 2011). Due to their coastal distribution, thousands of harbor porpoises are also killed accidentally in commercial fisheries, threatening local populations to a level that is still hard to quantify, but which is assumed to be unsustainable, especially within highly fluctuating environments (Carlén et al., 2021; IMR-NAMMCO, 2019).

Harbor porpoises are officially subdivided into three distinct subspecies: *P. p. vomerina* in the Pacific, *P. p. phocoena* in the Atlantic, and *P. p. relicta* in the Black Sea (Fontaine, 2016). A fourth lineage, IBMA or *P. p. meridionalis*, has been suggested owing to their distinctive ecology, morphology, and genetic divergence (Fontaine, et al., 2014), but no formal description has been made for this distinct subspecies (Pierce, et al., 2020). *P. p. meridionalis* is found around the cold and productive upwelling waters off the coasts of Iberia and Mauritania. Iberian and Mauritanian porpoises are genetically distinct from each other and belong to two separate populations separated by at least 2500 km (Fontaine, et al., 2014; Fontaine, 2016).

The upwelling habitat of the Iberian porpoise is at the border of a biogeographical transition zone between temperate and subtropical waters, where species with different

affinities co-occur and reach respective ecological limits (Bowen, 1997; MacLeod, 2009). Because conditions at the margins between biogeographic zones are often suboptimal, populations like the Iberian harbor porpoise are expected to be among the first to show the impacts of such environmental changes. This is particularly true with the Iberian upwelling which is known to fluctuate due to the ongoing climate change (Pires, et al., 2013; Casabella, et al., 2014). Such environmental fluctuation may impact the Iberian harbor porpoises in various ways, possibly leading to dramatic impacts on its demography and genetic diversity. Porpoises may adapt locally for example by targeting to various preys (Heide-Jørgensen, et al., 2011), by migrating to track suitable environments, or the population may also collapse if environmental changes outpace their ability to adapt. Previous population genetic studies (Fontaine, et al., 2010; Fontaine, et al., 2014; Ben Chehida, et al., 2021) showed that the Iberian population was a source population sending migrants Northward and Southward, but received none. This observation was based on a limited sample size focusing on a cohort obtained about thirty years ago, between 1990 and 2002. If the recruitment does not keep up with mortality and migration rate, the census size of the Iberian population is expected to decrease, leading to a gradual reduction in genetic diversity over time.

In this study, we investigated how the genetic diversity has changed in the Iberian harbor porpoises over the past three decades. More precisely, using genetic diversity as a proxy of population size variation, we tested if their genetic diversity decreased over time by comparing the genetic composition of two cohorts of porpoises, one sampled in the 90's (1990-2002), and the other one from the 2010s (2012-2015).

2. Material and methods

Sampling and molecular analyses. Tissue samples from 52 by-caught or stranded individuals between 2012 and 2015 were obtained from the Portuguese Marine Animal Tissue Bank (MATB) and were stored in 70% ethanol at -20°C until analysis. DNA was extracted with the DNeasy Blood and Tissue kit (QIAGEN Inc.) following the manufacturer's protocol. Following Fontaine et al. (2014), we screened for mitochondrial genetic polymorphism within five coding regions *ATP-6*, *ATP-8*, *Cyt-b*, *ND5*, and *COI* using Sanger sequencing. Target regions were PCR amplified, quality controlled, and purified following the protocol described in Fontaine et al. (2014). Sanger sequencing was outsourced to GATC Biotech Ltd. The resulting forward and reverse sequences were visually inspected, cleaned, and assembled with *Geneious* 10.0.9 (Kearse, et al., 2012). We added 83 previously published sequences from

Fontaine et al. (2014) encompassing the five same mitochondrial genes obtained for 82 harbor porpoises collected between 1990 and 2002, and one sequence from the closest outgroup species, the Dall's porpoise (*Phocoenoides dalli*). All mtDNA sequences were aligned with *MUSCLE* (Edgar, 2004). We grouped the harbor porpoise sequences in five geographical regions (Figure S4.1): North Atlantic (n=23), Bay of Biscay (n=14), Iberia (n=71), Mauritania (n=14) and Black Sea (n=12). We further subdivided the Iberian porpoises into two cohorts: new (n=52, 2012-2015) versus old (n=19, 1990-2002). The temporal coverage of the sampling is thus at most 25 years, which corresponds to 2.5 generations, assuming a generation time of 10 years (Read, 1999).

Phylogenetic and population genetic analyses. In order to place the Iberian porpoises into a global phylogeographic context, we first reconstructed a maximum-likelihood (ML) phylogeny with all the 135 sequences. Unique mtDNA haplotypes were identified using *DnaSP* v.5.10.1 (Librado and Rozas, 2009). Then, we used *PhyML* (Guindon, et al., 2010) implemented in *Geneious* to estimate the ML tree based on all available unique haplotypes identified in the dataset using a Nearest neighbor interchange typology search. The support of each node was assessed using 5000 bootstrap resampling. The phylogenetic tree was rooted with the sequence from a Dall's porpoise. In addition to the phylogenetic trees, we also reconstructed a Median-Joining haplotype network (Bandelt et al. 1999) using *PopART* (<http://popart.otago.ac.nz>).

Then various statistics informative on the genetic variation of the two cohorts of Iberian porpoises (old *versus* new) were estimated using the C library *libdiversity* developed by Dr. Achaz (<http://wwwabi.snv.jussieu.fr/~achaz/cgi-bin/neutraltytst.c>). Genetic diversity was estimated using the number of haplotypes (H), nucleotide diversity (π), and Watterson's theta (θ_w). A rarefaction approach was applied to estimate measures of genetic diversity while correcting for the difference of sample size (Sanders, 1968) using a custom python script. Subsequently, the genetic differentiation between the two cohorts was assessed using Hudson's estimator of F_{ST} (Hudson, et al., 1992) and ϕ_{ST} (Excoffier, et al., 1992) using *DnaSP* v.5.10.1 (Librado and Rozas, 2009) and *Arlequin* v.3.5.2.2 (Excoffier and Lischer, 2010), respectively. The significance levels of F_{ST} and ϕ_{ST} were assessed by randomization tests (1,000 permutations). Finally, we investigated evidence of changes in mtDNA effective population size (N_e) between the two cohorts (old *versus* new) by calculating values of Tajima's D (Tajima, 1989) and Achaz's Y (Achaz, 2008) implementing also a rarefaction approach account for differences in sample sizes.

3. Results and Discussion

In total, we successfully sequenced 52 Iberian harbor porpoises from the new cohort (2012-2015). When aligned with the 83 sequences from Fontaine et al. (2014), the final alignment of 4,175 base-pairs (pb) included 135 individuals and contained 384 segregating sites with 267 singletons and 117 parsimony informative sites defining 61 distinct haplotypes (Table S4.1).

Consistent with previous studies, the phylogenetic relationships among mtDNA haplotypes depicted by the ML tree (Figure 4.1) and the haplotype network (Figure S4.2) revealed the three main monophyletic lineages, corresponding to previously identified distinct subspecies (Fontaine, et al., 2014) : (i) *P. p. relicta* in the Black Sea; (ii) *P. p. phocoena* composed of the porpoises from the North Sea to the waters of Norway; (iii) IBMA or *P. p. meriodonalis* which comprise two distinct monophyletic sub-lineages in the upwelling waters of Iberia and Mauritania.

Evidence of migration from Iberia to Mauritania has been previously documented (Fontaine, et al., 2014) and is also observed here (Figure 4.1 and S4.2). However, we also identified a haplotype (Hap_10 in Figure 4.1 and S4.2) geographically present in the new Iberian cohort, but clustering with the Mauritanian lineage. This haplotype was carried by two individuals. This result provides the first evidence of possible migration from the Mauritanian population into Iberian population, suggesting that these two populations may not be as isolated as previously thought. Haplotype 10 could also belong to a distinct unknown population as it is divergent from the other Mauritanian haplotypes (Figure 4.1 and S4.2). Indeed, the ML tree (Figure 4.1) shows that this haplotype forms a distinct lineage from the other Mauritanian porpoises. The haplotype network also illustrates this haplotype divergence (Figure S4.2): while the Mauritanian haplotypes are separated from each other with at most two mutational steps, at least ten mutational steps separate this haplotype 10 from the remaining haplotypes observed in the Mauritanian porpoises (Figure S4.2). It is noteworthy that both Iberian porpoises carrying this haplotype 10 were found on the southern extremity of the Portuguese coasts (8.78W-37.07N; Praia do Burga, Portugal), at least 400km south from all other Iberian sampling locations (Figure S4.1). Stranding records of harbor porpoises were previously reported in Morocco, on the east coasts of Gibraltar, and in Cadiz (Spain; Rojo–Nieto, et al., 2011). If some unsampled populations occur between the Iberian Peninsula and the Mauritanian waters, they are most likely confined to a narrow and seasonal upwelling area.

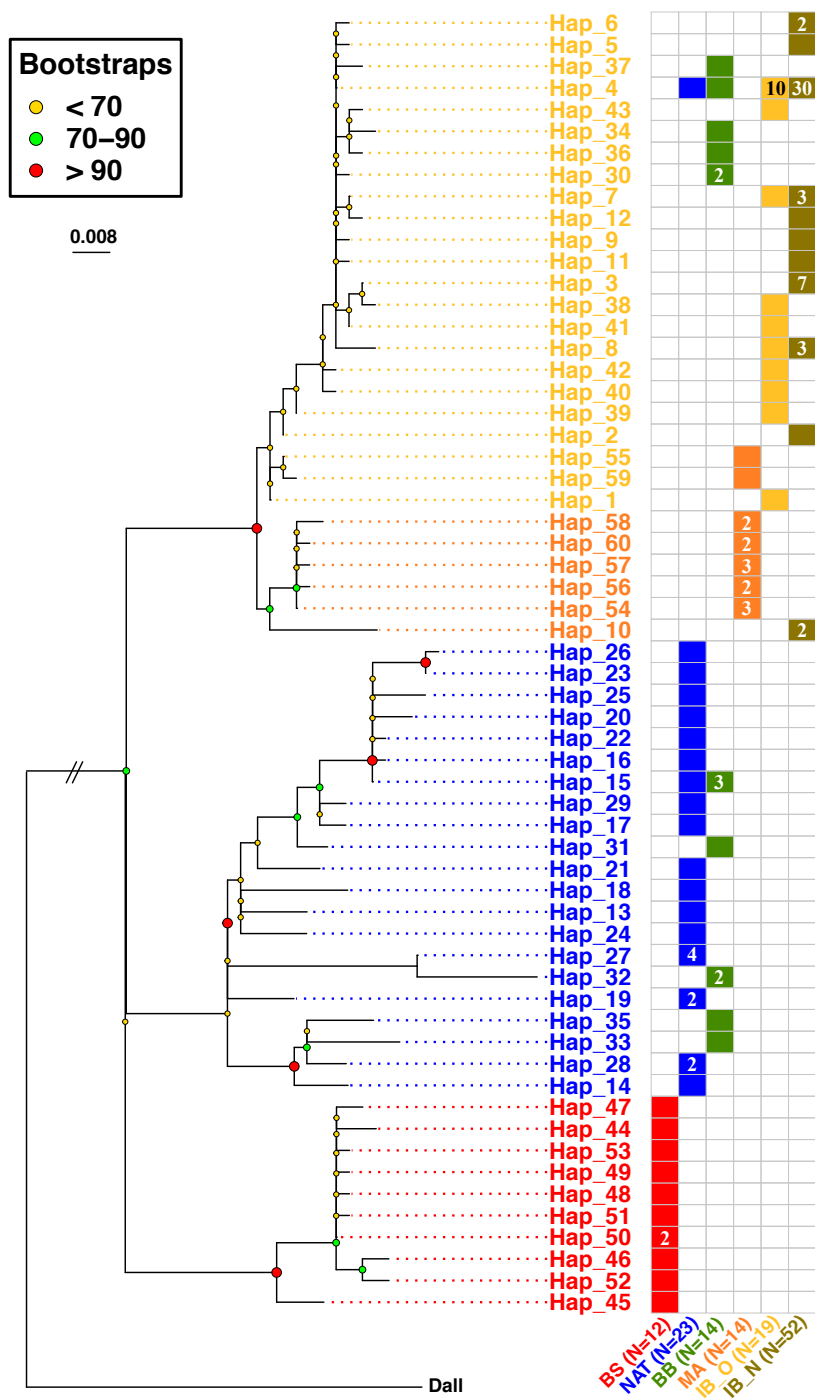


Figure 4.1: Maximum-likelihood mitochondrial phylogeny among unique mitochondrial haplotypes. Poorly supported nodes with less than 60% bootstrap support were collapsed. The color-coded labels show the geographic origin of the haplotype. The numbers within the boxes refer to the number of individuals carrying this haplotype. No number within a box means that the haplotype was observed only once. BS=Black Sea; NAT=North Atlantic; BB=Bay of Biscay; MA=Mauritania; IB_O=Iberian Old; IB_N= Iberian new.

This relatively divergent haplotype 10 closely related to the Mauritanian rather than the Iberian porpoises could be an evidence of these unsampled populations. This haplotype was thus excluded from subsequent analyses of genetic diversity because it could be part of a distinct population and therefore would artificially inflate measures of genetic diversity (Table S4.1). Future studies with additional samples and data from the nuclear genome are needed to confirm whether these two porpoises in the southernmost part of Iberia are genetically distinct from the other Iberian populations, possibly forming a distinct population, and whether or not they are mixing the other Iberian porpoises.

Aside from the two porpoises carrying haplotype 10, our results indicate that both cohorts sampled at different times along the Iberian coasts belong to the same mtDNA genetic pool. We detected no genetic differentiation suggesting no significant differences in haplotype frequencies between them (F_{ST} and ϕ_{ST} values < 0.0025 ; p -value > 0.08). This result reflects the high prevalence of haplotype 4 in both cohorts (Figure 4.1 and S4.2). However, we observed a dramatic reduction in genetic diversity in the new compared to the old cohort as shown by the lower number of haplotypes (H), and in the two measures of nucleotide diversity (π and θ_w ; Figure 4.2a). The rarefaction procedure applied to account for differences in sample size between the two cohorts provided additional evidence of this reduction (Figure 4.2a). The non-overlapping standard errors from a standardized sample size equal to (or larger than) eight sequences in the rarefaction curves suggest that the decline of genetic variation is highly significant. For example, the average number of observed haplotypes (H) observed with 20 randomly sampled sequences in each cohort decreases from 10 to 6 when comparing the old *versus* new cohorts. This suggests that the proportion of rare haplotypes has dropped dramatically over the past 25 years. Consistent with this observation is the rapid loss in genetic diversity as measured by π and θ_w (Figure 4.2a). A stochastic loss of genetic variation of this magnitude is often attributed to an enhanced effect of genetic drift (Hartl, 2020).

Tajima's D (Tajima, 1989) and Achaz's Y (Achaz, 2008) statistics estimated for the two cohorts sampled at different times along the Iberian coast present negative values (Figure 4.2b). At first glance, such negative values would be indicative of a population that underwent a "recent" expansion at evolutionary time-scales (Smith, et al., 2011; Epps and Keyghobadi, 2015), most likely due to the foundation of the Iberian population during the period that followed the Last Glacial Maximum (19kyr ago; Fontaine et al. 2014; Fontaine 2016). However, the values estimated for the new cohort for both statistics were significantly less negative than those from the old cohort. For example, the Tajima's D values obtained with 19 randomly sampled sequences increased from -1.5 for the old cohort to -1.25 for the new (Figure

4.2b). Likewise, values of Achaz's Y statistics increased from -1.75 to -0.95 between the old and new cohorts, respectively (Figure 4.2b). It is remarkable to see how both values have become dramatically less negative in such a short time span between the two cohorts. This increase in D and Y values also provides a strong indication of a population losing rapidly rare haplotypes as expected for a small and isolated population strongly subject to genetic drift that could be declining.

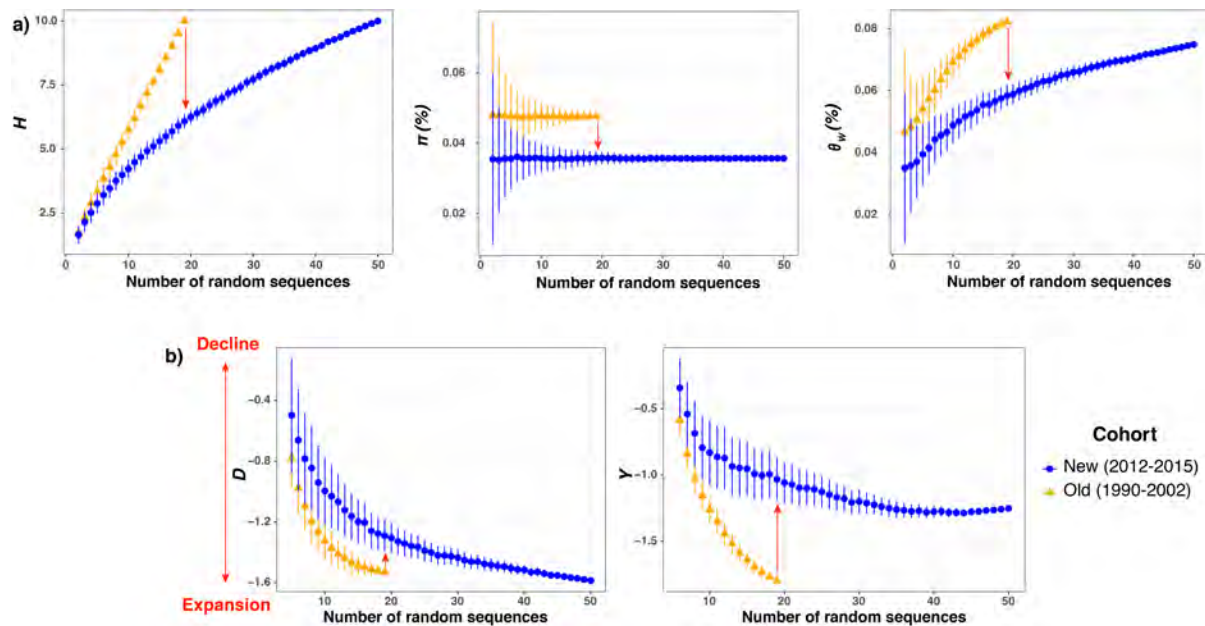


Figure 4.2: Rarefaction curves in the top panels (a) show three statistics describing mitochondrial genetic diversity including the number of haplotypes (H) and two measures of nucleotide diversity (π and θ_w). The bottom panels (b) show neutrality indexes indicative of demographic changes including the Tajima's D and the Achaz's Y . The red arrows inside the different insets are indicative of the direction of the change between the old and new cohorts. In panel b), 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.

We confirmed previous evidence of migration from Iberia to more northern regions (Fontaine, et al., 2014; Fontaine, et al., 2017) with Iberian haplotypes present in the hybrid zone between *P. p. meridionalis* and *P. p. phocoena* as well as in the North Sea (Figure 4.1 and S4.1). Because the population size of *P. p. phocoena* populations in the North Sea is much larger than of the Iberian population, such a northward migration may lead to the assimilation of the Iberian gene pool by *P. p. phocoena*. This process can lead in the long term to the disappearance of the unique Iberian genetic pool. This scenario is plausible as Macleod *et al.* (2009) showed using habitat modeling that species with a similar range as the Iberian harbor

porpoises (figure 1C in MacLeod, 2009) will shift their range northward as a response to an increase in water temperature of 5°C due to global warming (figure 1D in MacLeod, 2009). This model suggests that the future range of *P. p. meridionalis* will be totally confounded with the current range of *P. p. phocoena* (figure 1D in MacLeod, 2009). It is worth mentioning that Ben Chehida et al. (2021) recently modeled the future distribution of the harbor porpoises in the North Atlantic under the most aggressive (RCP8.5; Schwalm, et al., 2020) scenario of the IPCC in 2050 and did not capture this strong northward range shift. A plausible explanation of the discrepancy between the two studies is explained by the temperature increase assumed by the two models, 2°C and 5°C, in Ben Chehida et al. (2021) and MacLeod (2009), respectively. On the other side, the environmental niche modeling in Ben Chehida et al. (2021) did not account for any biotic factors in the simulations.

Hence, all the results of this study support the hypothesis that the Iberian population is small and isolated. Additionally, both the sharp decline of genetic diversity over three generations (30 years) and northward migration can represent sources of concern for the long-term survival of the Iberian porpoises.

On the one hand (hypothesis 1), the decay of genetic variation we reported can simply reflect the fact that the Iberian porpoises constitute a stable population with a very low effective population size (N_e). In such a condition, genetic drift has a disproportionate influence on this population leading to the highlighted loss of genetic variation over a few generations. Several previous studies showed that the Iberian population has indeed a very low N_e at nuclear microsatellites loci ($N_e < 100$ individuals; Fontaine, et al., 2010; Fontaine, et al., 2014; Ben Chehida, et al., 2021). Furthermore, the mtDNA locus employed in this study has an effective size four times smaller compared with nuclear microsatellite loci. This means that the stochastic loss of genetic variation due to genetic drift is expected to be drastically increased here for the mtDNA compared with the nuclear microsatellites (used in previous studies). Thus, the Iberian population might be at a stable census size losing rare haplotypes through the enhanced effect of genetic drift at the mtDNA locus.

On the other hand (hypothesis 2), the decay of genetic variation might be associated with a rapid decline of the number of reproductive females in addition to the overall low N_e (Lande, 1988). This is all more plausible since there is accumulating evidence that the Iberian population is sending more migrants to adjacent populations than the reverse. Therefore, this second hypothesis is more concerning because, in addition to being small and isolated, the decline of the census population would imply that the Iberian porpoises are becoming increasingly subject to demographic stochasticity (Allendorf, et al., 2012). Because this

population is also known to be subject to strong genetic stochasticity (i.e. genetic drift; this study) and environmental stochasticity (Pires, et al., 2013; Casabella, et al., 2014), all these effects can accumulate and reinforce each other, potentially precipitating the decline of the Iberian porpoise. This phenomenon (i.e. the so-called extinction vortex; Allendorf, et al., 2012) can be particularly severe for K strategist species, living in changing sub-optimal conditions like the Iberian harbor porpoises (Pires, et al., 2013; Casabella, et al., 2014) and could put them at a high risk of extinction.

However, because this study relies on a single molecular marker (mtDNA), future studies with additional samples and data from the nuclear markers are needed to tease apart these two hypotheses. Furthermore, genome-wide data can provide more insights into the temporal dynamics of the Iberian population. For example, the reconstruction of the historical demography based on genome-wide data (Terhorst, et al., 2017; Liu and Fu, 2020; Schiffels and Wang, 2020) can be used to test whether these harbor porpoises, since their post-glacial emergence (Fontaine, et al., 2010; Fontaine, et al., 2014; Fontaine, 2016), maintained a long-term low N_e (Robinson, et al., 2019; Morin, et al., 2020) or if, on the contrary, they recently declined (Hu, et al., 2020). The simulation framework developed by Robinson et al. (2019) could as well be applied to evaluate whether these porpoises managed to escape the deleterious effects of the mutation load despite their current low N_e or not (Robinson, et al., 2019; Morin, et al., 2020).

Additional sources of concern possibly related to a severe decline in the Iberian population require careful considerations: the SCANS II project estimated a census size as low as 2,357 animals ($CV=0.92$) in Iberian waters (Hammond et al., 2013). This low census size was associated with both particularly high bycatch rates (with estimates varying from 90-197 deaths per year (Pierce et al., 2020)) and with overexploited feeding resources (Méndez-Fernandez et al., 2013; Santos & Pierce, 2003). A scarcity in prey availability for example can be harmful because it enhances interspecific competition, as already reported between harbor porpoises and other marine mammals, for example with the bottlenose dolphin *Tursiops truncatus*, which occupies an ecological niche that largely overlaps with that of harbor porpoise in Iberian waters (Méndez-Fernandez et al., 2013; Spitz et al., 2006). Hostile interactions between the two species have been documented in Iberian waters and hypothesized to cause harbor porpoises to disperse to less productive narrow shelf areas (Alonso et al., 2000; Spitz et al., 2006). Furthermore, Iberian harbor porpoises are regularly found stranded or in fisheries bycatch, leading to unsustainable mortality rates (IMR-NAMMCO, 2019; López et al., 2002; Pierce et al., 2020).

Despite its uniqueness in terms of genetics, morphology, and ecology, Iberian harbor porpoises are currently still not recognized as a distinct subspecies (Pierce et al., 2020). For example, there is no mention of these porpoises in the IUCN Red List of Threatened Species or the List of Marine Mammal Species and Subspecies of the Society of the Marine Mammalogy. Given the results of this study and the serious concerns raised about their viability already raised in the NAMMCO reports and Pierce et al (2020), we recommend immediate actions from the community and the authorities to officially recognize these upwelling porpoises as a distinct subspecies, and ensure that all the measures are taken to gain additional knowledge on the Iberian population. Improving our understanding of the demographic trends and the threats these porpoises are facing is paramount to devise suitable and tailored conservation plans. Such measures are timely needed to mitigate the decline of this unique cetacean potentially on the brink of extinction.

Supplementary Material Chapter 4

Table S4.1: Mitochondrial genetic diversity

	All (with outgroup)	All (no outgroup)	BS	NAT	BB	MA	All IB	IB_O	IB_N	IB_N (excl. hap 10)
<i>N</i>	135	134	12	23	14	14	71	19	52	50
<i>S</i>	384	170	23	99	66	11	27	12	24	14
<i>Singl.</i>	267	65	21	57	19	1	7	6	5	7
<i>Parsim.</i>	117	105	2	42	47	10	20	6	19	7
<i># Hap</i>	61	60	10	18	10	7	17	10	11	10
π (%)	0.50	0.40	0.10	0.41	0.52	0.07	0.05	0.05	0.06	0.04
θ_w (%)	1.68	0.74	0.18	0.64	0.50	0.08	0.13	0.08	0.13	0.08
<i>D</i>	-	-	-2.04	-1.46	0.22	-0.66	-1.85	-1.53	-1.77	-1.58
<i>Y</i>	-	-	-1.56	-0.83	0.05	-1.93	-2.12	-1.77	-2.19	-1.26

N, mtDNA sample sizes; *S*, segregating sites; *Singl.*, singleton; *Parsim.*, sites informative in parsimony; *# Hap*, number of haplotypes; π , nucleotide diversity; θ_w , Watterson's theta; *D*, Tajima's *D*; *Y*, Achaz's *Y*. BS=Black Sea; NAT=North Atlantic; BB=Bay of Biscay; MA=Mauritania; IB_O=Iberian Old; IB_N= Iberian new.

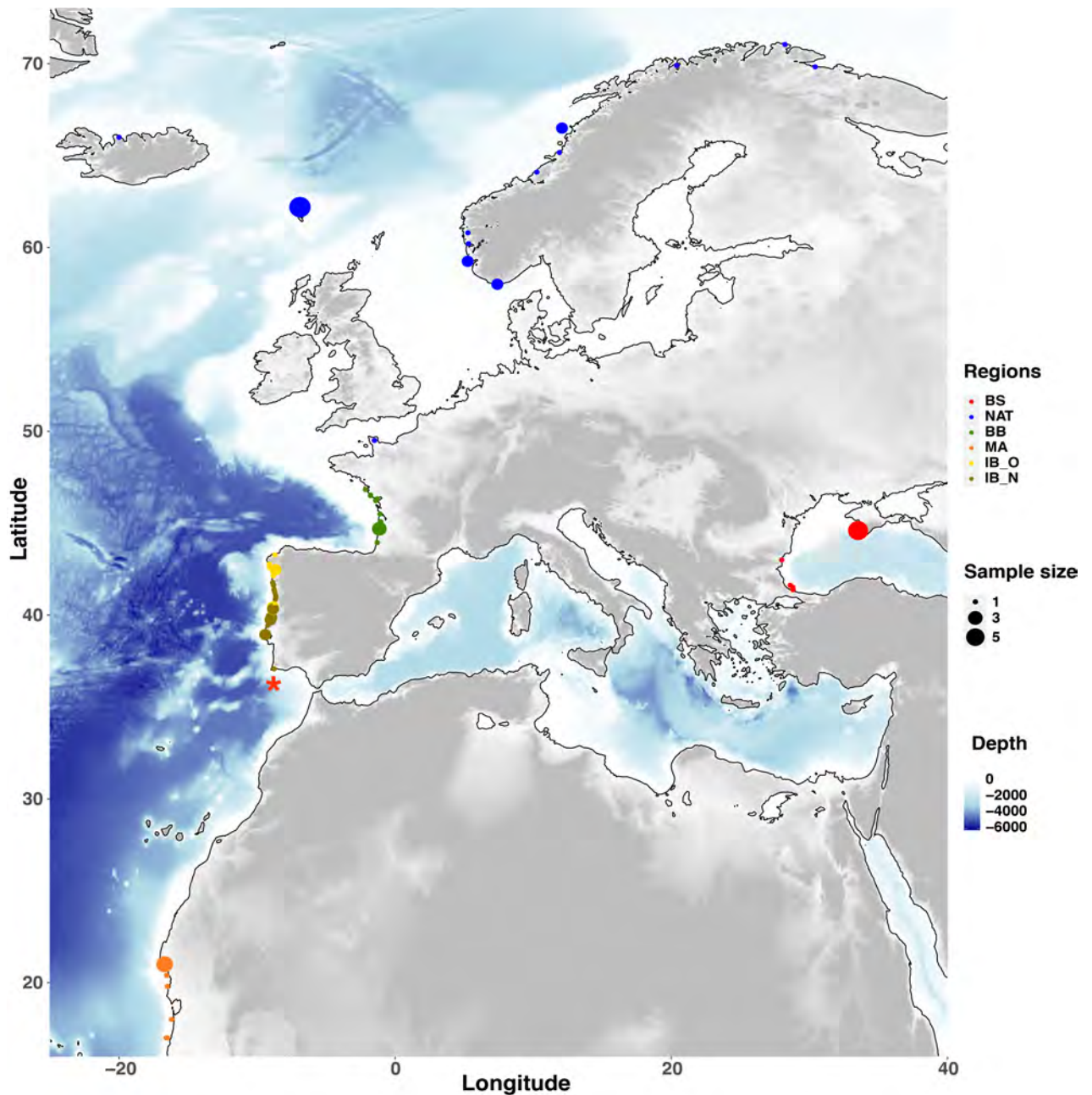


Figure S4.1: Map showing the sampling locations and sample sizes. Geographic locations are based on approximate GPS coordinates or reported discovery locations. BS=Black Sea; NAT=North Atlantic; BB=Bay of Biscay; MA=Mauritania; IB_O=Iberian Old; IB_N= Iberian new. The red star indicates the geographical location of the two porpoises carrying Hap_10.

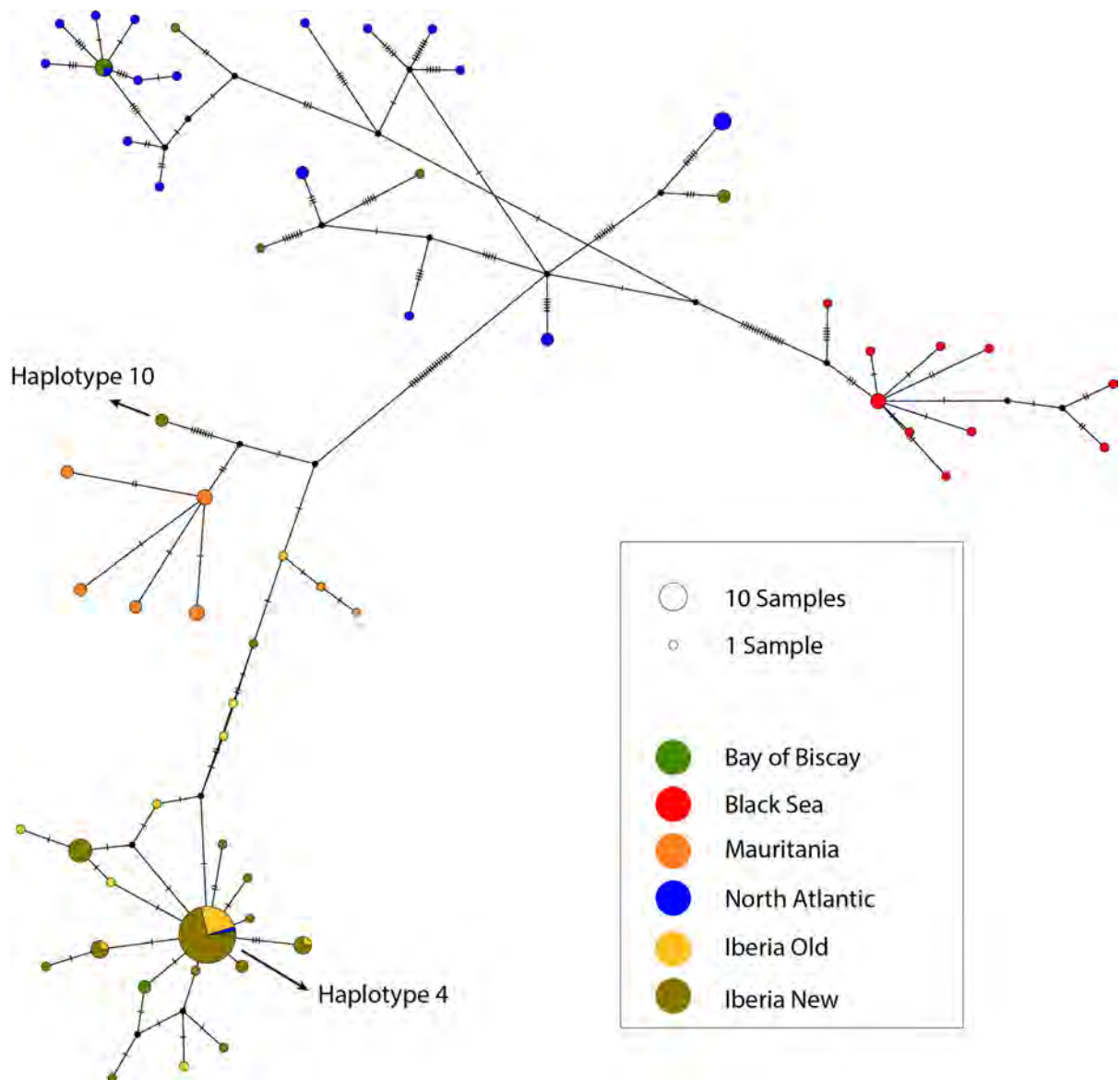


Figure S4.2: Mitochondrial median-joining haplotype network. Each circle represents a haplotype. The size of each circle is proportional to the haplotype frequency observed in the total sampling, and each pie slice is proportional to the number of each haplotype observed per geographic location. Each dash represents a unique mutational step between haplotypes. A black dot represents a hypothetically unsampled or extinct node. The arrows highlight haplotypes 4 and 10 because they are discussed in the main text.

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