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Fine-scale genetic structure of bottlenose dolphins, *Tursiops truncatus*, in Atlantic coastal waters of the Iberian Peninsula

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Abstract In the NE Atlantic, evidence has been found of genetic discontinuities between Iberian bottlenose dolphins and those of Scotland and the Mediterranean. Here, we explored the genetic relationships between resident populations of dolphins from southern Galicia (NW Spain) and the Sado estuary (S Portugal), and their relationship with dolphins inhabiting neighbouring areas. A total of 91 skin and muscle samples were taken from stranded and biopsied animals between 1994 and 2008 in southern Galicia ($N = 29$), the Sado estuary ($N = 5$) and five other geographical locations ($N = 57$) including sites around the Iberian Peninsula, the Canary Islands and the Azores. Individuals were genotyped at 10 microsatellite loci and sequenced at the highly variable mitochondrial control region. From individual-based analyses of microsatellite data, dolphins from southern Galicia and the Sado estuary were assigned to an individual genetic population, though nine dolphins were identified as possible migrants between putative populations as their genetic makeup did not correspond with their geographical stranding location. Pairwise estimates of genetic differentiation (F_{ST}) based on mitochondrial and nuclear DNA also revealed genetic differences between populations. The existence of fine-scale population substructure should be considered in the future designation of Special Areas of Conservation

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(SACs) for the species, as required by the European Habitats Directive.

Keywords Genetic differentiation · Resident populations · Cetaceans · Migrants · Conservation

Introduction

The bottlenose dolphin, *Tursiops truncatus*, has a worldwide distribution from temperate to tropical seas, displaying strong behavioural and ecological plasticity that allows it to inhabit marine and estuarine ecosystems, even ranging into rivers (Wells & Scott, 2002). Satellite tracking has demonstrated that individual dolphins can travel very large distances in short periods of time (2,000–4,000 Km in 43–47 days) (Wells et al., 1999). A high propensity for dispersal, coupled with an apparent lack of barriers to movement, suggests that high levels of gene flow over large geographic areas are possible. Such predictions have been confirmed by low levels of population structure among oceanic bottlenose dolphins (Quérouil et al., 2007). However, many studies have shown significant population genetic structure, frequently without correlation with geographical distance. There is clear genetic structure extending from the Black Sea to Scotland (UK), with discontinuities separating Iberian animals from those around Scotland and the Mediterranean (Natoli et al., 2005). Genetic differentiation is also apparent between offshore and coastal populations in the NW Atlantic (Hoelzel et al., 1998; Natoli et al., 2005), as well as within ecotypes over short geographic distances (Krützen et al., 2004; Sellas et al., 2005; Segura et al., 2006; Parsons et al., 2006; Tezanos-Pinto et al., 2009). Strong site fidelity, with resident groups inhabiting coastal inlets and estuaries, plus resource specialisation, as a consequence of different social and behavioural strategies, could be some of the factors leading to genetic structure (Hoelzel, 1998; Wiszniewski et al., 2009).

Around the Iberian Peninsula, bottlenose dolphins are present continuously along the Atlantic coast (VVAA, 2007; Brito et al., 2009) with recognised resident populations in southern Galicia (NW Spain) and in the Sado estuary (S Portugal) (dos Santos & Lacerda, 1987; López, 2003; Brito et al., 2009). The southern Galician population inhabits a series of large inlets or rías, characterised by their considerable width and a SW–NE orientation (Méndez & Vilas, 2005). The Sado estuary is located in the SW coast of Portugal and it is divided in an outer and inner areas separated by a shallow area with tidal flats (Caeiro et al., 2005). Groups of bottlenose dolphins enter the river mouth to forage, play and socialise on a year-round daily basis (dos Santos et al., 2005).

The West coast of Portugal and Galicia is influenced by the NW Africa upwelling system, and therefore upwelling–downwelling dynamics are encountered in the area enhancing productivity (Santos et al., 2001; Figueiras et al., 2002). This seasonal characteristic pattern can vary considerably: in Galician waters the spring transition (from downwelling to upwelling) can occur in February or in April, while the autumn transition shows less variability and generally takes place around October (Figueiras et al., 2002).

The resident populations of bottlenose dolphins from southern Galicia and the Sado estuary are characteristically small (a minimum figure of 123 animals in Galicia based on simultaneous counts (López, 2003), and 25 dolphins with a negative population trend in the Sado estuary (Silva, 2008)), and are likely to face greater threats than offshore dolphin populations from habitat degradation, pollution, and other anthropogenic impacts. Therefore, understanding the structure of these populations and their relationships with other groups/populations should be a pre-requisite to establishing appropriate management units and define future Special Areas of Conservation (SACs) as required by the EU Habitats Directive (92/43/CEE).

Here, the spatial distribution of microsatellite and mitochondrial DNA variation was used to characterise levels of fine-scale and regional population genetic structure among putatively resident populations from southern Galicia and the Sado estuary. Divergence is assessed in relation to differences among more distant locations such as the Canary Islands and the Azores. Both archipelagos are separated from the mainland by thousands of kilometres, including vast areas of low productive waters of depths greater than 5,000 m. As a consequence, isolation by distance (IBD) was expected to occur between insular and continental bottlenose dolphins.

Materials and methods

Study area and sample collection

Skin and muscle samples were obtained from stranded and by-caught bottlenose dolphins from four geographical locations: Galicia (GAL, $N = 48$), mainland Portugal (MPOR, $N = 21$), the Basque Country (BCO, $N = 4$) and the Canary Islands (CAN, $N = 8$). Dolphins stranded in Galicia were further classified as southern (SGAL, $N = 29$), when stranded in the area delimited by the border with Portugal and "Punta Queixal" in the Mount Louro (geographic limit between the southern inlets and the northern Galician coastline), and northern (NGAL, $N = 19$) when stranded in the area extending from "Punta Queixal" to the border with Asturias (Fig. 1). Dolphins stranded in mainland Portugal were divided in non-resident bottlenose dolphins (POR, $N = 16$) and in Sado estuary residents (SAD, $N = 5$). All stranded dolphins classified as Sado estuary residents had been previously photo-identified as locals through the individually distinct marks and nicks present on their dorsal fin by Gaspar (2003). In addition, skin samples from the Azores (AZO, $N = 10$) were collected between 2002 and 2004 using a biopsy darting system (see Quérouil et al., 2007). Sampling locations are shown in Fig. 1.

Samples were preserved in: 20% dimethyl sulfoxide (DMSO) solution saturated with NaCl with subsequent storage at −20°C; 96% ethanol with storage at ambient temperature; or frozen directly at -20°C. DNA was extracted using the DNeasy blood and tissue extraction kit (Qiagen Ltd) according to the manufacturer's instructions with dilution of DNA in sterile water to ~ 20 ng μl^{-1}. It should be noted that as the majority of the samples came from stranded dolphins, state of preservation at the time of sample collection varied considerably between animals.

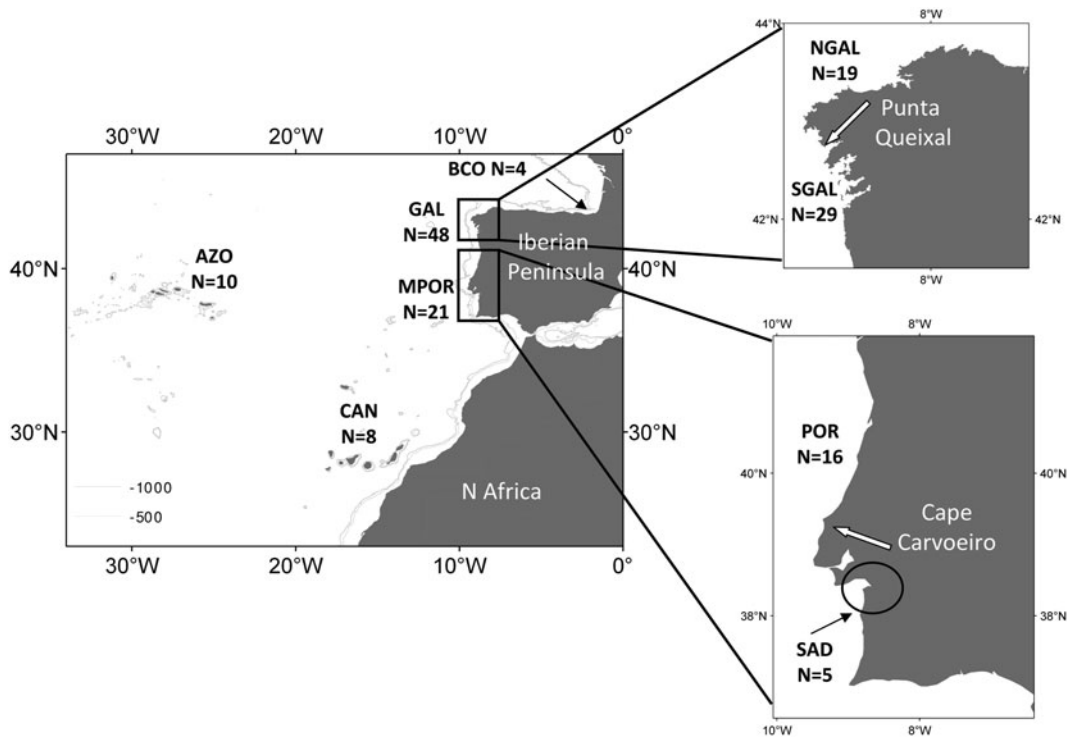

Fig. 1 Locations from which *T. truncatus* samples were obtained: The Basque Country (*BCO*), Galicia (*GAL*), mainland Portugal (*MPOR*), the Azores (*AZO*), and the Canary Islands (*CAN*). Detailed maps of sample origin for GAL, divided into northern and southern (NGAL and SGAL, respectively) and MPOR, divided into Sado Estuary (SAD) resident dolphins and other mainland Portuguese strandings (POR) are shown

Microsatellite genotyping

Individuals were genotyped at ten microsatellite loci: Dde59, Dde65, Dde72 (Coughlan et al., 2006); Ttr04, Ttr19, Ttr34, Ttr48, Ttr58, Ttr63, and TtrRC11 (Rosel et al., 2005). PCRs were carried out in a 10 µl volume containing 20 ng of DNA, 1.5 mM $MgCl_2$, 75 mM Tris–HCL pH 9.0, 20 mM $(NH_4)_2SO_4$, 0.01% Tween-20, 0.2 mM dNTP's, 5 pmol of each primer and 0.5 units of Taq polymerase (Bioline Ltd). The thermal profile included an initial denaturation step at 95°C for 3 min, followed by 30 cycles of: 35 s at 95°C, 30 s at the specified annealing temperature (see Rosel et al., 2005; Coughlan et al., 2006), 30 s at 72°C and 3 min a 72°C. All loci were fluorescently tagged on the 5′ terminus, allowing alleles to be resolved on an ABI3730 automated DNA sequencer and allele sizes were determined using an internal size standard.

Microsatellite analysis

Evidence for the presence of null alleles or large allele dropout was tested using the program MICRO-CHECKER 2.2.3. (Van Oosterhout et al., 2004). Allele frequencies per locus and sample were calculated using the software FSTAT 2.9.3. (Goudet, 2001).

The program *STRUCTURE* 2.2. (Pritchard et al., 2000) was used to determine the most probable number of putative populations (K) that best explained the pattern of genetic variability. Correlated allele frequencies and an admixture model were assumed and performed with a burn-in period and number of simulations that ranged from 100,000 and 10,000 to 500,000 and 100,000 repetitions, respectively. Due to the different geographical origin of the samples, that could represent different genetic units, we used values of K between 1 and 6. Ten replicates for each proposed value of K were performed and consistency of results was assessed. *STRUCTURE* 2.2. was also used to assign individuals among the putative genetic groupings.

There is considerable debate in the population genetics literature about the relative merits of maximising numbers of loci or individuals in analysis of population genetic structure (Kalinowski, 2002; Ryman et al., 2006). This issue is especially pertinent in studies where the quality of DNA obtained amongst individuals varies such that unambiguous genotypes cannot be obtained for all individuals at all loci. This was the case in this study, and as such, parallel *STRUCTURE* analyses were undertaken on two datasets, one based on 10 loci for 51 individuals, followed by a second analysis with samples size increased to 71 after addition of 20 individuals with genotypes obtained from 5 loci. Congruent outcomes derived from both datasets were taken to indicate biologically meaningful patterns. Prior to the *STRUCTURE* analysis, a power analysis was undertaken based on 5 microsatellite loci with the software POWSIM 4.0. (Ryman & Palm, 2006). The method uses multilocus allele frequency data to assess the probability of detecting significant differentiation using Fisher's exact tests and the more conservative χ^2 test. A specified level of genetic divergence is assumed by applying the formula $1 = (1 - 1/N_e)^t$ where t is the time since divergence, and N_e is the effective population size assuming complete isolation between populations. Simulations were run assuming two (sample sizes of $N = 25$ and $N = 45$) and six subpopulations (sample sizes of $N = 25$, $N = 30$, $N = 35$, $N = 40$, $N = 45$ and $N = 50$). Various combinations of N_e and t were used, leading to F_{ST} of 0.025–0.05, which approximates the values obtained from empirical data based on 10 loci (see below). Six simulations were performed for each of the two levels of population substructure; three simulations for an F_{ST} of 0.025 using N_e/t of 500/25, 1000/40 and 2000/101 and three for an F_{ST} of 0.05 using N_e/t of 500/51, 1000/102 and 2000/205. Additional simulations were performed omitting the drift steps ($t = 0$, $N_e = 500$, $F_{ST} = 0$) to approximate the type I error (null hypothesis is incorrectly rejected). A thousand replicates were run for each simulation and power was estimated as the proportion of these 1,000 tests that indicated significant genetic differentiation ($P < 0.005$).

Principal coordinates analysis (PCoA) was undertaken using the program GENALEX 6 (Peakall & Smouse, 2006). Genetic distances were calculated between pairs of individuals and translated into ordination distances in a low-dimensional space. As such, samples with similar genetic genotypes are expected to be closer together in the ordination plot. The presence of Isolation by Distance (IBD) was tested with GENALEX 6. A matrix of geographical distances between pairs of sampled dolphins was created based on the latitudes and longitudes of

stranding positions. This matrix was correlated against a genetic distance matrix between pairs of dolphins and tested using Mantel permutations (10,000 iterations).

Genetic diversity was assessed as the number of alleles, number of private alleles and allelic richness using the software FSTAT 2.9.3 (Goudet, 2001). Observed (Ho) and expected (He) heterozygosities were calculated at each loci and population and deviations from the Hardy–Weinberg equilibrium were tested using the Markov chain method (chain length; 1,000,000, dememorization steps; 100,000) with ARLEQUIN 3.11. For each population, the overall deviation from the Hardy–Weinberg equilibrium and the degree of inbreeding were estimated based on F_{IS} values (10,000 randomisations) using the software FSTAT 2.9.3. A classical estimation of between-population F_{ST} was calculated using ARLEQUIN 3.11. Sex-biased dispersal was examined by determining sex-specific F_{ST} values using FSTAT 2.9.3 and tested using 10,000 randomizations. Populations were defined based upon their geographic stranding location (Fig. 1).

Mitochondrial DNA sequencing

A 549 bp fragment of the mitochondrial control region was PCR amplified using the primers L15926 (5′-ACACCAGTCTTGTAAACC-3′; Eggert et al., 1998) and H16498 (5′-CCTGAAGTAAGAACCAG ATG-3′; Rosel et al., 1995). Amplification reactions were carried out in a 25 µl volume containing 50 ng DNA, 2 mM $MgCl_2$, 75 mM Tris–HCL (pH 9.0), 20 mM $(NH_4)_2SO_4$, 0.01% Tween-20, 0.2 mM dNTP's, 5 pmol of each primer and 0.5 units of Taq polymerase (Bioline Ltd). The thermocycle profile followed a 10° "touchdown" procedure which included 2 min of initial denaturation at 95°C, followed by 20 cycles of: 30 s denaturation at 92°C, 30 s annealing at 60°C (which decreases 0.5°C in each of the 20 cycles) and 45 s extension at 72°C, then 20 cycles of: 30 s denaturation at 92°C, 30 s annealing at 50°C and 45 s extension at 72°C, with a final 2.5 min extension at 72°C. PCR products were purified using a Qiaquick PCR purification kit (Qiagen). Samples were sequenced in both directions on an ABI3730 automated sequencer using the L15926 and H16498 PCR primers as the sequencing primers.

The dataset was augmented with 19 published mtDNA bottlenose dolphin sequences from mainland Portugal and the Azores obtained from Genbank (accession numbers: DQ073641, DQ073644, DQ073646, DQ073647, DQ073669, DQ073688, DQ073699, DQ073700, DQ073706, DQ073710, DQ073718, DQ073720, DQ073722–DQ073725, DQ073727–DQ073729; Quérouil et al., 2007). Given these sequences were shorter than those obtained in this study, all subsequent analyses are based upon a 426 bp truncated alignment.

Mitochondrial DNA analysis

All sequences were aligned using Clustal W within MEGA 4.0 (Tamura et al., 2007). Phylogenetic relationships among the mtDNA haplotypes were inferred from a median-joining network constructed using the program NETWORK 4.5 (Bandelt et al., 1999; http://www.fluxus_engireering.com). Population differentiation was estimated as F_{ST} using the program ARLEQUIN 3.11. Haplotype diversity (Hd) and nucleotide diversity (π) were estimated using DnaSP vs5 (Librado & Rozas, 2009).

Results

Given variability in the quality of DNA obtained from samples, successful PCR amplifications were not achieved for every sample at every locus. Details of the number of animals included in microsatellite and mtDNA data analyses are given in Table 1. For 20 individuals, microsatellite genotypes were only obtained for five of the loci, so separate statistical analyses were performed including and excluding these individuals. Power analysis based on these five loci, two subpopulations and an F_{ST} of 0.025 showed that in >98% of the runs the tests detected a genetic difference between subpopulations. When F_{ST} was set to 0.05, genetic differentiation was found in 100% of the tests. Considering six subpopulations, genetic differentiation was found in 100% of the runs independent of the F_{ST} value. When F_{ST} was set to 0, between 3 and 6% of the runs returned evidence of genetic differentiation which approximates the expected Type I error rate of 5%. Among the microsatellite loci, there was no evidence for null alleles or large allele dropout.

Table 1 Composition of the set of samples

Population	SGAL	SAD	BCO	NGAL	POR	AZO	CAN	TOTAL
N	29	5	4	19	16	10	8	91
mtDNA	25	4 (2[a])	2	18	16 (7[a])	10[a]	6	81
Microstatellites	24	4	4	14	14	6	5	71

SGAL South Galicia, *SAD* Sado estuary, *BCO* Basque Country, *NGAL* North Galicia, *POR* Mainland Portugal, *AZO* The Azores, *CAN* Canary Islands

[a] Number of sequences obtained from Genbank

Microsatellite DNA

STRUCTURE analyses based on either 51 (10 loci) or 71 samples (10 and 5 loci genotypes) indicated that the most probable number of populations was two (Ln $Pr(X/K) = -1539.27 \pm 1.81$ for 51 samples and $P(X/K) = -1887.28 \pm 1.18$ for 71 samples; Fig. 2). Individuals from the different sampling locations were apportioned among these two populations according to Fig. 3a for the analysis involving 51 samples and Fig. 3b for the analysis involving 71 samples.

Genetic differences were found when 51 dolphins (10 loci) were examined. Nineteen of the 22 SGAL dolphins were identified as belonging to a single population together with four animals stranded in NGAL and the single Sado estuary (SAD) resident dolphin. All the other dolphins were classified as belonging to a second population. Thus, seven Galician animals (three from SGAL and four from NGAL) were identified as possible migrants between populations as their genetic signature did not correspond to the majority of individuals from their geographical stranding location (Fig. 3a). When 71 samples were considered, only two individuals remained poorly resolved, showing proportions of coefficients of admixture between populations higher than 0.25 (sample 54 and sample 60; Fig. 3b). In this case, the seven potential migrant individuals previously identified can still be recognised. All the AZO animals are classified as belonging to the second undifferentiated population while dolphins from the Sado estuary (SAD) appear to be genetically similar to the SGAL population. However, among the 20 new individuals included in the analysis (five loci samples), two new potential migrants are found between the SAD population and the POR groups (Fig. 3b).

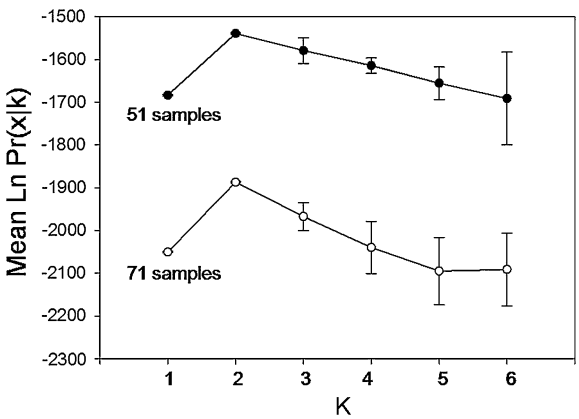

Fig. 2 Results of the *STRUCTURE* analysis, showing mean (±SD) probabilities of the data (Ln $Pr(x|k)$) based on 10 *STRUCTURE* replicated runs plotted as a function of the putative number of clusters (*K*). *Black dots* 51 samples analyzed; *white dots* 71 samples analyzed

Only those individuals from which results over 10 loci were available were used in further statistical analyses, therefore Azorean individuals and all but one SAD dolphin were excluded from additional calculations. The PCoA analysis confirmed a level of population structure among samples (Fig. 4). Analyses show most of the SGAL animals and the single SAD dolphin grouped towards one side of the ordination plot, with the two first principal coordinates explaining 50.5% of the variation. Mantel tests demonstrate the existence of IBD ($P = 0.003$) which was expected due to the inclusion of distant dolphins from the Canary Islands. However, IBD was also patent when these insular dolphins were excluded from the analysis ($P = 0.001$).

Pairwise estimates of genetic differentiation (F_{ST}) were calculated between SGAL, NGAL and POR. SGAL animals were significantly different to the other two groups although no significant difference was found between NGAL and POR dolphins (Table 2). Sex-specific F_{ST} were calculated between

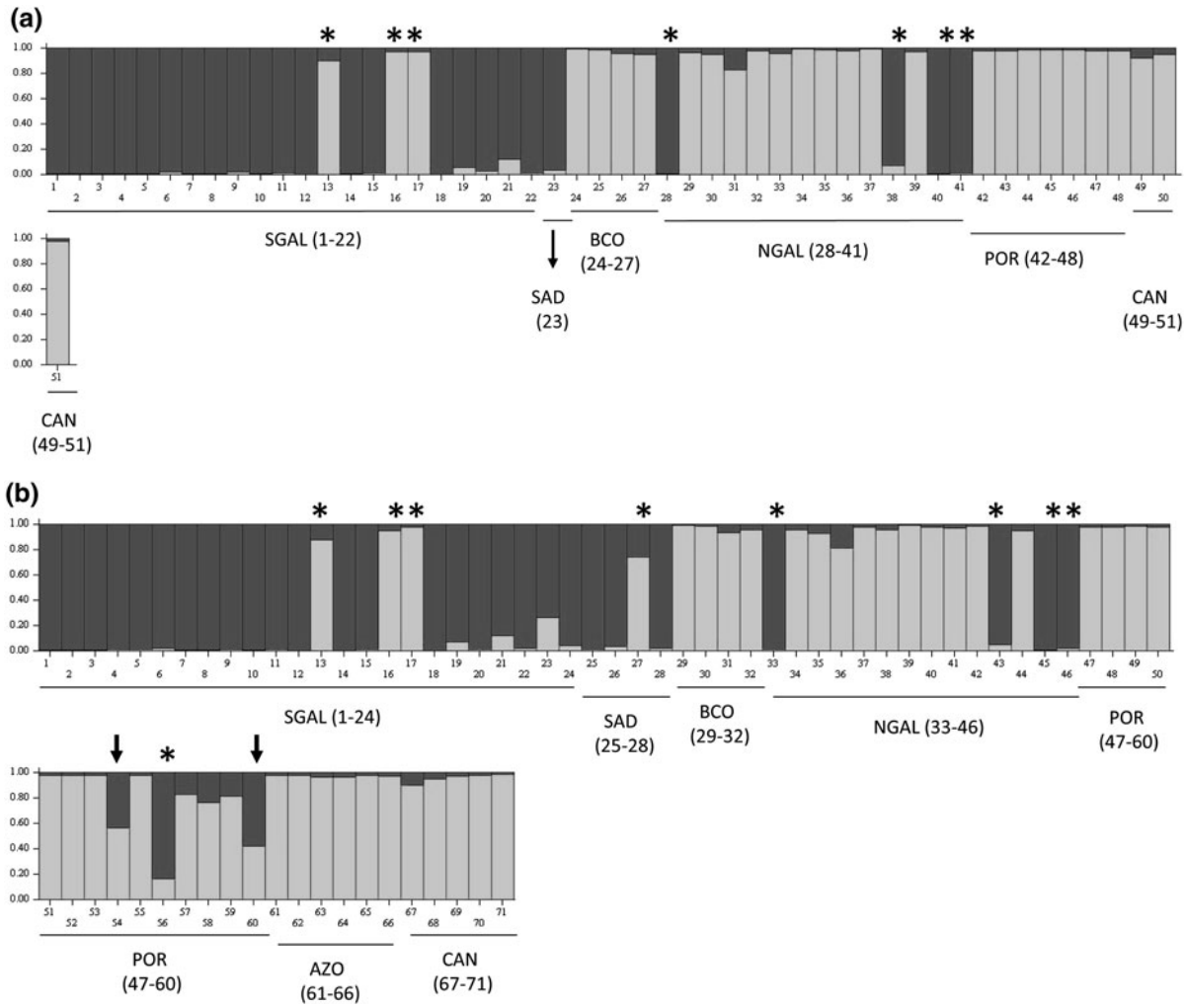

Fig. 3 Estimated proportions of the coefficient of admixture of each individual's genome that originated from population *K*, for $K = 2$. Each individual is represented by a column. *Asterisk* indicates individuals identified as possible migrants. *Down arrow symbol* indicates animals poorly resolved (proportions of coefficients of admixture between populations higher than 0.25). **a** Analysis including 51 samples. **b** Analysis including 71 samples (for 20 individuals only results over 5 loci were available). *SGAL* Southern Galicia, *SAD* Sado Estuary, *BCO* the Basque Country, *NGAL* northern Galicia, *POR* Portugal, *CAN* the Canary Islands, *AZO* the Azores

these same groups and females showed only slightly higher F_{ST} values than males (F_{ST} females $= 0.091$, F_{ST} males $= 0.089$; $P = 0.967$) suggesting no evidence of sex-biased dispersal.

The highest allelic richness was found in NGAL and POR dolphins even when sample sizes were considerably smaller than that from SGAL (Table 3). The number of private alleles found in SGAL dolphins was much lower than that found in NGAL or CAN which had much smaller sample sizes, especially the latter (Table 3).

No deviation from the Hardy–Weinberg equilibrium was found based on F_{IS} values for any of the groups considered (SGAL, NGAL, POR, BCO, and CAN). However, when dolphins stranded outside SGAL and SAD were grouped as a single population, as identified by the *STRUCTURE* analysis, F_{IS} estimates showed a significant deviation from the Hardy–Weinberg equilibrium ($F_{IS} = 0.094$, $P = 0.0004$) reflecting a deficiency of heterozygote genotypes. NGAL and MPOR populations showed deviations from the Hardy–Weinberg equilibrium at a single

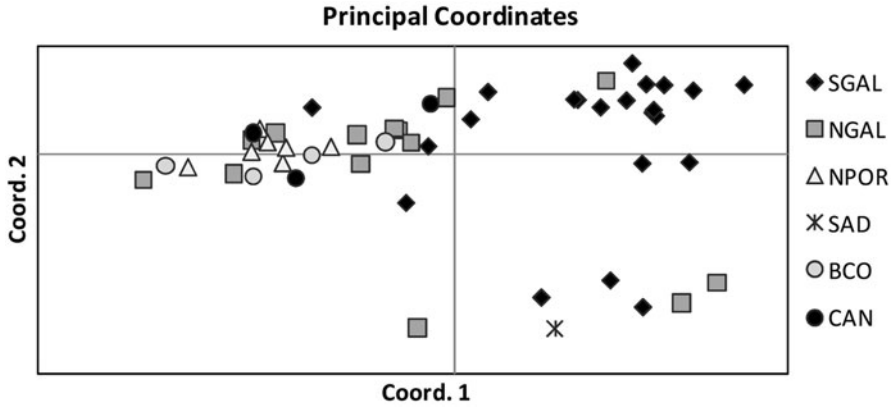

Fig. 4 Principal coordinates analysis, PCoA, based on genetic distances between individuals, showing main patters of data variation over 10 loci: 50.5% of the variability explained by principal coordinates 1 and 2. *SGAL* Southern Galicia, *SAD* Sado Estuary, *BCO* the Basque Country, *NGAL* northern Galicia, *POR* Portugal, *CAN* the Canary Islands

Table 2 Estimates of population differentiation expressed as F_{ST} based on microsatellite length polymorphism (below diagonal) and mtDNA haplotype frequencies (above diagonal) where * $P < 0.05$ (not significant after Bonferroni correction) and *** $P < 0.00001$

Population *N*:	*N*	SGAL 25	NGAL 18	POR 16	CAN 6	AZO 10
SGAL	22	–	0.124***	0.471***	0.434***	0.461***
NGAL	14	0.063***	–	0.137*	0.131*	0.098
POR	7	0.132***	0.015	–	0.144*	0.104***

Sample sizes for the microsatellite and mitochondrial analyses for each group are given in the second column and row, respectively. Only groups with a sample size higher than $N = 5$ were considered for the present analyses

SGAL South Galicia, *NGAL* North Galicia, *POR* Mainland Portugal, *CAN* Canary Islands, *AZO* The Azores

Table 3 Within population measures of nuclear DNA genetic diversity calculated over 10 loci

	SGAL	NGAL	POR	BCO	CAN
N	22	14	7	4	3
Overall F_{IS}	0.063	0.066	0.119	0.107	0.211
Private alleles	2	9	5	2	9
Average ± SD					
N alleles	5.9 ± 1.969	7.6 ± 1.647	6.3 ± 1.829	4.1 ± 1.101	3.7 ± 0.675
Allelic richness	5.648 ± 1.975	7.205 ± 1.753	5.932 ± 1.531	3.775 ± 0.690	3.160 ± 0.386
Ho	0.568 ± 0.226	0.762 ± 0.195	0.804 ± 0.148	0.733 ± 0.232	0.717 ± 0.261
He	0.604 ± 0.229	0.786 ± 0.124	0.832 ± 0.074	0.807 ± 0.077	0.850 ± 0.074

SGAL South Galicia, *NGAL* North Galicia, *POR* Mainland Portugal, *BCO* Basque Country, *CAN* Canary Islands

locus, Ttr19 ($P = 0.036$) and Dde59 ($P = 0.004$), respectively, due to heterozygote deficiency.

Mitochondrial DNA sequences

Thirty-four different mtDNA haplotypes were found among 81 individuals (Table 4); sixteen haplotypes were newly discovered (Accession numbers GU599885-GU59899 and HM236171; Table 4). From thirty-four polymorphic sites, 23 were transitions (one transition being a transversion for two animals), seven were transversions and four were insertions–deletions. Unique haplotypes were found for all geographic regions (Table 4). The most common haplotype was present in 30 dolphins from SGAL, NGAL, SAD and AZO, which matches the

Table 4 Mitochondrial control region haplotype polymorphic nucleotides and haplotype frequencies

Hapl.	Nucleotide position																																	
	1	16	25	26	51	60	64	71	72	74	75	76	80	85	89	90	103	105	109	149	150	160	205	244	256	277	310	313	321	351	361	373	377	393
H_02	G	–	G	T	C	G	T	G	G	A	G	A	G	G	T	A	G	G	G	A	G	A	G	A	G	A	G	G	A	–	G	T	A	G
H_01[a]	A	A	.	.	T	.	A	.	.	.	.	.	.	.	G	.	.	.	A	G	.	.	A	G	A	.	.	.	G	.	.	.	.	.
H_03	.	.	.	.	.	.	.	.	.	.	A	G	.	.	.	.	.	.	A	G	.	G	.	.	A	.	.	.	.	.	.	.	.	.
H_04[a]	A	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	A	C	.
H_05	.	.	.	.	T	.	.	.	A	.	.	G	.	A	G	.	.	.	A	.	.	.	A	G	A	.	.	.	G	.	.	.	.	.
H_06	.	.	.	.	T	.	.	.	.	.	.	G	.	.	A	.	.	.	A	G	.	.	A	G	A	.	.	.	G	.	.	.	.	.
H_07	.	.	.	.	.	A	.	.	.	.	A	G	.	.	.	.	.	.	A	G	.	.	.	.	A	.	.	.	.	.	.	.	.	.
H_08[a]	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	A	.	A	.	.	T	.	.	.	.
H_09	.	.	.	.	.	.	.	.	.	.	A	.	–	.	.	.	.	.	A	G	.	.	.	.	A	.	.	.	.	.	.	.	.	.
H_10[a]	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H_11[a]	.	.	.	.	.	A	.	A	.	.	A	.	.	.	.	.	.	.	.	G	.	.	.	.	A	.	.	.	.	.	.	.	.	.
H_12	.	.	.	.	.	.	.	.	A	.	.	G	.	.	.	.	.	.	.	G	.	.	.	.	A	.	A	.	.	T	.	.	.	.
H_13[a]	A	A	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H_14	.	.	.	.	.	.	.	.	.	.	A	G	.	.	.	.	.	.	A	G	.	.	.	.	A	.	.	.	.	.	.	.	.	.
H_15	.	.	.	.	.	A	.	.	A	.	A	G	.	.	.	.	.	.	A	G	.	.	.	.	A	.	.	.	.	.	.	.	.	.
H_16[a]	.	.	.	.	T	.	.	A	.	.	.	G	.	T	G	G	.	.	A	.	.	.	A	.	A	.	.	.	.	T	.	.	.	A
H_17	.	.	A	.	.	.	.	.	.	.	A	G	.	.	.	.	.	.	A	G	.	.	.	.	A	.	.	.	.	.	.	.	.	.
H_18[a]	.	.	.	.	T	.	.	.	.	.	.	G	.	.	G	.	.	.	.	G	.	.	A	G	A	.	.	.	G	T	.	.	.	.
H_19	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	G	.	.	.	.	A	.	.	.	.	.	.	.	.	.
H_20[a]	.	.	.	.	T	.	.	.	.	.	.	G	.	.	G	.	A	.	.	G	.	.	A	G	A	.	.	.	G	.	.	.	.	.
H_21[a]	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H_22[a]	.	.	.	.	T	.	.	.	A	.	.	.	.	.	G	.	.	.	A	G	.	.	A	G	A	.	.	.	G	.	.	.	.	.
H_23[a]	.	.	.	.	T	.	.	.	A	.	.	.	.	.	G	.	.	.	A	G	.	.	A	G	A	.	.	T	G	.	.	.	.	.
H_24	.	.	.	.	T	.	.	.	.	.	.	.	.	.	G	.	.	.	A	G	.	.	A	G	A	.	.	.	G	.	.	.	.	.
H_25	.	.	.	.	T	.	.	.	.	.	.	.	.	T	G	G	.	.	A	.	.	.	A	A	A	.	.	.	.	T	.	.	.	.
H_26	.	.	.	.	.	.	.	.	.	.	A	G	–	.	.	.	.	.	A	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.
H_27	.	.	.	.	T	.	.	.	A	.	.	G	.	A	G	.	.	A	A	.	.	.	A	G	A	.	.	.	G	.	.	.	.	.
H_28	.	.	.	.	T	.	.	.	.	.	.	G	.	.	A	.	.	.	A	G	.	.	A	G	A	–	.	.	G	.	.	.	.	.
H_29	.	.	.	.	T	.	.	.	A	G	.	G	.	A	G	.	.	.	A	.	.	.	A	G	A	.	.	.	G	.	.	.	.	.
H_30	.	.	.	.	T	.	.	.	.	.	.	G	.	.	G	.	.	.	.	G	.	.	A	G	A	.	.	.	G	.	.	.	.	.
H_31[a]	.	.	A	.	.	A	.	.	A	.	A	G	.	.	.	.	.	.	A	G	.	.	.	.	A	.	.	.	.	.	.	.	.	.
H_32[a]	A	A	.	.	.	A	.	.	.	.	A	G	.	.	.	.	.	.	A	G	A	.	.	.	A	.	.	.	.	.	.	.	.	.
H_33[a]	A	A	.	A	T	.	A	.	A	.	.	G	.	A	G	.	.	.	.	.	.	.	A	G	A	.	.	.	G	.	.	.	.	.
H_34[a]	A	.	.	.	.	A	.	.	.	.	A	G	.	.	.	.	.	.	A	G	.	.	.	.	A	.	.	.	.	.	.	.	.	.

Hapl.	Location						
	SGAL	SAD	BCO	NGAL	POR	AZO	CAN
H_02	20	2		7		1	
H_01[a]	1						

Table 4 continued

Hapl.	Location						
	SGAL	SAD	BCO	NGAL	POR	AZO	CAN
H_03				1	1		
H_04[a]	1						
H_05				2			
H_06				1			
H_07				1	5		
H_08[a]				1			
H_09				1	1	1	
H_10[a]				1			1
H_11[a]				1			
H_12	1			1		1	
H_13[a]	1						
H_14	1				2		
H_15					1		
H_16[a]					1		
H_17					1	1	
H_18[a]			1				
H_19			1				
H_20[a]					1		
H_21[a]							1
H_22[a]				1			
H_23[a]					1		
H_24					1	1	
H_25					1		
H_26		2					
H_27						1	
H_28						2	
H_29						1	
H_30						1	
H_31[a]							1
H_32[a]							1
H_33[a]							1
H_34[a]							1

[a] The correspondent haplotypes have been registered in Genbank with accession numbers GU599885–GU59899 and HM236171

Hapl. Haplotype, *SGAL* South Galicia, *SAD* Sado estuary, *BCO* Basque Country, *NGAL* North Galicia, *POR* Mainland Portugal, *AZO* The Azores, *CAN* Canary Islands

haplotype that Parsons et al. (2002) identified as the most common among UK bottlenose dolphins (GenBank accession no. AF268357).

Pairwise estimates of genetic differentiation (F_{ST}) were calculated between those groups with sufficient sample size ($N > 5$; SGAL, NGAL, POR, CAN and AZO). After the Bonferroni correction was applied, ssignificant mtDNA population differentiation was observed between SGAL dolphins and all the other groups and also between POR and AZO individuals

Table 5 Within population measures of mitochondrial DNA genetic diversity: nucleotide diversity, π (±SD) and haplotype diversity, Hd (±SD)

Population	SGAL	SAD	NGAL	POR	BCO	CAN	AZO
N	25	4	18	16	2	6	10
π (±SD)	0.005 ± 0.002	0.006 ± 0.002	0.013 ± 0.002	0.012 ± 0.002	0.012 ± 0.006	0.018 ± 0.004	0.015 ± 0.002
Hd (±SD)	0.367 ± 0.122	0.667 ± 0.204	0.856 ± 0.079	0.908 ± 0.063	1.000 ± 0.500	1.000 ± 0.096	0.978 ± 0.054

SGAL South Galicia, *SAD* Sado estuary, *BCO* Basque Country, *NGAL* North Galicia, *POR* Mainland Portugal, *AZO* The Azores, *CAN* Canary Islands

(Table 2). Overall haplotypic diversity (Hd) among the 81 bottlenose dolphins was estimated to be 0.855 (±0.037) although clear differences between putative populations do exist (Table 5). Lowest nucleotide (π) and haplotypic (Hd) diversities were found in the SGAL and SAD populations ($\pi = 0.005 \pm 0.002$, Hd $= 0.367 \pm 0.122$ and $\pi = 0.006 \pm 0.002$, Hd $= 0.667 \pm 0.204$, respectively).

The genetic relationships among haplotypes are given as a median-joining network in Fig. 5. Two main clusters, separated by five mutational steps, were identified, with most of the SGAL and SAD dolphins (except haplotype H_01; Genbank accession number GU599885) represented in one of the groups. The SAD population unique haplotype differs by one mutational step from haplotypes present in dolphins from other geographical areas while SGAL exclusive haplotypes were 1 to 6 mutational steps apart from sequences present in other groups (Fig. 5). The SGAL population showed very low levels of variation, with most of the individuals sharing the same haplotype (H_02) (Table 4).

Discussion

Both microsatellite and mtDNA analyses indicate genetic structure within our sample set. Individual-

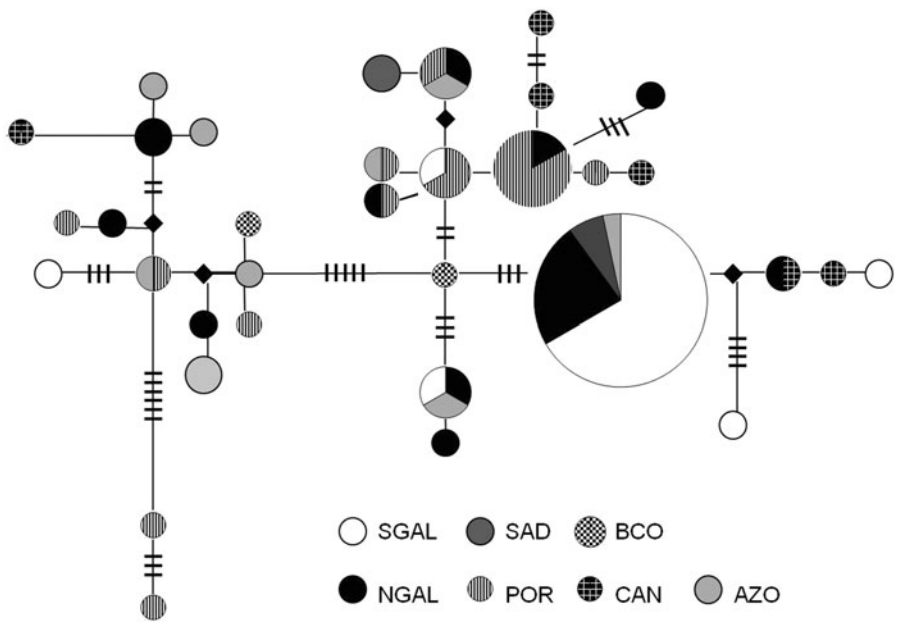

Fig. 5 Median-joining network of bottlenose dolphin mtDNA haplotypes. Higher weights were applied to insertions–deletions. *Circle size* is approximately proportional to the number of individuals exhibiting the corresponding haplotype. Connector length is proportional to the number of mutations between haplotypes. *Black diamonds* indicate potential intermediate haplotypes that were not sampled. *Hatch marks* indicate total number of mutations between haplotypes when more than one mutation is present. *Numbers* correspond to haplotypes from Table 4. *SGAL* Southern Galicia, *SAD* Sado Estuary, *BCO* the Basque Country, *NGAL* northern Galicia, *POR* Portugal, *CAN* the Canary Islands, *AZO* the Azores

based analysis of microsatellite genotypes identified southern Galician and Sado estuary animals as more similar to each other than to dolphins sampled elsewhere. However, the existence of strong gene flow between both sites is unlikely. Around 500 km of coastline with continuous presence of bottlenose dolphins separate the two resident communities and, as shown by this study, gene flow between resident and immediate neighbouring populations in our research area is limited. More likely, low sample size precluded accurate estimates of population structure. Bigger sample sizes (especially for Sado resident dolphins) and a higher number of genetic markers are needed to better understand the relationships between these resident populations. Previous genetic studies carried out with samples from the area did not find evidence of population structure among bottlenose dolphins from mainland Portugal and Galicia (Natoli et al., 2005). Differences between this article and conclusions from Natoli et al. (2005) may be also due to an uneven distribution of animals (with fewer animals coming from southern Galicia or the Sado estuary), the small sample size used in Natoli's study ($N = 35$), some inherent bias caused by the use of stranded individuals for which the true provenance of samples (i.e. where the animals lived as opposed to where the carcases were found) is unclear, or a combination of the three. It should be noted that carcases may be transported considerable distances along the coast before being stranded (Peltier et al., 2009) and some of these biases could also apply to this study.

The high mobility of marine species and the lack of obvious barriers to gene flow in the oceans should translate in low levels of population genetic structure in marine animals (Fontaine et al., 2007). However, cetaceans often show local structure at regional or fine scales that is not necessarily related to physical features or geographic separation. Frequently, these patterns are attributable to complex behaviours such as local resource specialisation, philopatry or social organization into kin groups (Hoelzel, 1998; Fontaine et al., 2007). In this study, we found evidence of IBD. However, the strong genetic differentiation found between southern Galician dolphins and animals from neighbouring locations may reflect more complex reasons such as resource partitioning. Indeed, bottlenose dolphins from northern and southern Galicia were found to display different proportions of the main prey species (e.g. blue whiting, *Micromesistius poutassou*) in their diet and dissimilar stable isotope signatures (Fernández, 2010; Fernández et al., 2011). It is also generally recognised that higher levels of genetic diversity usually occur towards the centre of a species' range while subsequent founder events could originate a stepwise loss of microsatellite diversity (Hoffman et al., 2009). The low levels of genetic diversity found in dolphins from southern Galicia suggest that, despite the lack of physical barriers, this population inhabits peripheral areas and gene flow with adjacent sites is restricted. Philopatry and kin association among individuals within resident populations can also act to reduce genetic diversity.

Bottlenose dolphins live in fission–fusion societies (Connor et al., 2000). Therefore, even within resident groups, some individuals may move frequently between schools, contributing to gene flow and influencing the genetic make-up of the community. In this study and despite the genetic substructure found, nine possible migrants between populations were identified. The existence of migrants in Galician waters is not unlikely given the high mobility of the bottlenose dolphin community in the region. Based on stomach contents analyses Santos et al. (2007) suggested that bottlenose dolphins in the area could perform foraging trips towards offshore waters, while López (2003), based on photo-identification matches, registered dolphin movements between distant inlets (rías). Two animals were also identified as potential migrants between the Sado estuary and other sites in mainland Portugal, indicating medium and/or long distance movements between locations. In this case, the Sado estuary dolphin with distinct genetic makeup was a well-known resident male named Nune. The other migrant dolphin was found stranded in the proximities to Cape Carvoeiro (Fig. 1), although it was genetically similar to southern Galician and Sado estuary dolphins. These results indicate that cases of immigration and emigration between populations currently occur.

The most common haplotype found within southern Galician dolphins matches the most frequent haplotype observed among UK bottlenose dolphins (Parsons et al., 2002; GenBank accession no. AF268357). This haplotype represents one of the only two sequences found among 15 samples from Moray Firth (Scotland, UK) resident dolphins (Parsons et al., 2002). In this study, and despite the shorter mtDNA sequence, six different haplotypes were recorded from 25 dolphins

stranded in southern Galicia. However, we found an uneven distribution of haplotypes, with most of the dolphins sharing the same sequence and, as a result, levels of diversity were similar to the ones reported in the Moray Firth (Parsons et al., 2002). Measures of mtDNA diversity were low for southern Galician and Sado estuary dolphins but comparable to levels found in other studies targeting small local populations of bottlenose dolphins (Krützen et al., 2004; Sellas et al., 2005).

Inherently low sample size makes further portioning of the microsatellite data to assess sex-biased dispersal problematic. Notwithstanding, divergence estimates were comparable between males and females (though F_{ST} values were slightly higher for females than for males) suggesting limited dispersal of both sexes. Moreover, divergence estimates for mtDNA were also significant.

Previous studies found high levels of gene flow between animals from the Azores and dolphins from Madeira and mainland Portugal (Quérouil et al., 2007) which agrees with individual-based analysis from the present *STRUCTURE* analysis, in which Azorean and Canary Island animals are identified as a single genetic group together with non-resident dolphins found throughout the Iberian coastline. In this paper, one Azorean animal included in the *STRUCTURE* analysis had been identified as resident (Silva, personal communication) although no apparent genetic differences between this individual and non-resident dolphins were highlighted in our results. As suggested by Silva et al. (2008), the extensive ranging behaviour exhibited by some Azorean resident bottlenose dolphins and the apparent lack of territoriality may allow genetic interchange to occur, thus preventing genetic divergence. In addition, both Azorean and Canary Island dolphins shared one haplotype with dolphins stranded in the mainland and the median-joining network did not show any clear clustering patterns based on the geographic origin of the samples. Nonetheless, IBD was found and significant differences in F_{ST} based on mtDNA haplotype frequencies were shown between animals from mainland Portugal (excluding Sado estuary dolphins) and dolphins from the Azores. Given the remoteness of the Azorean archipelago, IBD may prevent complete panmixia between insular and continental dolphins. In addition, certain degree of heterozygosity deficit was found when dolphins from locations different than southern Galicia and the Sado estuary were grouped as a single population that could suggest cryptic local structure (Wahlund effect).

The bottlenose dolphin is recorded in the Habitats Directive as a Species of Special Interest (Directive 92/43/CEE) which protection requires the designation of special areas of conservation (SACs) by the Member States. The Sado estuary is already a Marine Protected Area (MPA) and candidate SAC (Hoyt, 2005) based on its resident population of bottlenose dolphins. On the other hand, in southern Galicia only a set of small islands located towards offshore waters is currently protected and proposed as a SAC (Hoyt, 2005). The bottlenose dolphin is also identified as a key species to be preserved in southern Galicia although the legally protected sites represent a very small fraction of the habitat used by dolphins in the region. Coastal cetaceans could face extra threats than offshore ones such as geographically restricted ranges, relatively narrow ecological niches, and dependence on resources that are also used intensively by humans (Reeves et al., 2003). Indeed, a previous extinction of an isolated, coastal population of *T. truncatus* in European waters has been reported (Nichols et al., 2007). Genetic isolation could represent an added risk for the southern Galician and Sado estuary populations and ensuring the viability of both local populations must be guaranteed in future conservation plans including the designation of SACs.

Conclusion

This study adds to the growing body of evidence that bottlenose dolphins display fine-scale genetic structure, and that detailed genetic and demographic studies are needed to accurately determine the complex patterns of gene flow within any particular area. In this case, the resident population in southern Galicia is genetically distinct which needs to be considered when defining the most appropriate scale of management, especially given that such resident individuals may be facing added threats relative to non-resident dolphins.

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