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Running title: High gene flow in oceanic dolphins

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Abstract

Several cetacean species exhibit fine-scale population structure despite their high dispersal capacities and the apparent continuity of the marine environment. In dolphins, most studies have focused on coastal areas and continental margins, and they revealed differentiated populations within relatively small geographic areas, sometimes in conjunction with a specialization for different habitats (ecotypes). We analysed the population genetic structure of short-beaked common dolphins (*Delphinus delphis*) and Atlantic spotted dolphins (*Stenella frontalis*) in the Azores and Madeira, the two most isolated archipelagos of the North Atlantic. The archipelago of the Azores is divided into three groups of islands and stands 900 km away from Madeira. It is not known whether individuals migrate between groups of islands and archipelagos, nor whether distinct ecotypes are present. These questions were investigated by genetic analyses of 343 biopsy samples collected on free-ranging dolphins. The analyses consisted in sequencing part of the mitochondrial hyper-variable region, screening up to 14 microsatellite loci, and molecular sexing. Results did not unravel any population structure at the scale of the study area. Lack of differentiation matches expectations for spotted dolphins, which are transient in both archipelagos, but not for common dolphins, which are present year-round in the Azores and potentially resident. Absence of genetic structure over hundreds and even thousands of kilometres implies the existence of gene flow over much larger distances than usually documented in small delphinids, which could be achieved through individual movements. This finding indicates that population structure in oceanic habitat differs from that observed in coastal habitat.

Key-words: *Delphinus delphis*, *Stenella frontalis*, Azores, Madeira, population genetics

Introduction

In the face of global warming and increasing anthropological pressure, conservation of marine ecosystems has become one of the major concerns of this century. Cetaceans, as long-lived top predators, are particularly vulnerable (cf. Simmonds and Hutchinson 1996). Their conservation is considered a priority at the international level (e.g. the CITES Convention, Washington, 1973; the Bern and Bonn Conventions, 1979; the European Union Habitats Directive, 1992). The definition of conservation policies requires previous knowledge on stock structure. Nowadays, molecular genetic techniques are commonly used to identify conservation units and estimate gene flow between demes (cf. Mills and Allendorf 1996; Frankham et al. 2002). Such techniques have revealed fine-scale population structure in various cetacean species, in spite of the large size and high dispersal capacities of these animals (Hoelzel et al. 2002). Population differentiation can occur over a few dozen or hundreds of kilometres, as a result of isolation by distance (e.g. western Australian bottlenose dolphins, *Tursiops* sp., Krützen et al. 2004), tight social structure (e.g. South Pacific spinner dolphins, *Stenella longirostris*, Oremus et al. 2007) or ecological specialisation in relation with habitat features (e.g. killer-whales, *Orcinus orca*, Hoelzel et al. 1998a; common bottlenose dolphins, *Tursiops truncatus*, Hoelzel et al. 1998b; Natoli et al. 2004, pantropical spotted dolphins, *Stenella attenuata*, Escorza-Treviño et al. 2005). In the latter case, forms specialised for different habitats (called "ecotypes") are often both genetically and morphologically differentiated (e.g. Hoelzel et al. 1998a,b). For instance, in common bottlenose dolphins, coastal forms tend to be resident and form small groups (Connor 2000), and populations are differentiated over small geographic distances (Sellas et al. 2005; Parsons et al. 2006). On the contrary, offshore forms of common bottlenose dolphins tend to be transient, form large groups (Connor 2000), and maintain high gene flow over wide geographic areas (Quérouil et al. 2007). It is likely that other species exhibit similar patterns, but very little information is available on the population structure of dolphins in pelagic environment, especially in distant offshore regions.

The present study focuses on the two most isolated archipelagos of the North Atlantic Ocean: the Azores and Madeira. These archipelagos are situated 1500 km and 580 km away from the mainland, respectively, and separated by 900 kilometres of deep waters. They are characterised by an absence of continental slope and the occurrence of deep waters at short distance from the coast with scattered seamounts (Santos et al. 1995; Caldeira et al. 2002; Morato et al. 2008). The existence of large stretches of deep waters between islands suggests that populations may be differentiated between groups of islands and archipelagos. Distinct ecotypes can be expected to occur in nearshore vs. offshore waters given that ecotype differentiation is widespread amongst delphinids in other parts of the world.

The archipelagos of the Azores and Madeira host more than 20 species of cetaceans (Gonçalves et al. 1996; Santos-Reis and Mathias 1996). The most abundant species are the short-beaked common dolphin, *Delphinus delphis*, Linnaeus 1758, the Atlantic spotted dolphin, *Stenella frontalis* (Cuvier 1829) and the common bottlenose dolphin (Silva et al. 2003; Freitas et al. 2004). Whilst the genetic structure of the latter species was studied recently in the Azores and Madeira (Quérouil et al. 2007); there is virtually no information available on the stock structure of the former two species in that region. Preliminary data indicate that the common dolphin is resident in the Azores, but its abundance fluctuates seasonally (Quérouil et al. 2008). In Madeira, it occurs only in winter and spring (Freitas et al. 2004). The spotted dolphin is a seasonal visitor in both archipelagos, where it is present essentially during summer months (Freitas et al. 2004; Quérouil et al. 2008). The common dolphin seems to prefer coastal waters, while the spotted dolphin tends to prefer deep offshore waters (Silva et al. 2003; Freitas et al. 2004). Little information exists on the seasonal movements and stock structure of these species in the pelagic waters of the North Atlantic,

and it is not known whether the individuals ranging in both archipelagos belong to the same stock. Further information is needed to define management and conservation policies regarding these two important cetacean species in the Macaronesian region (Azores, Madeira and Canary Islands).

In common dolphins, two species are presently recognized: the short-beaked common dolphin, *D. delphis*, and the long-beaked common dolphin, *D. capensis* (Heyning and Perrin 1994; Rosel et al. 1994). In the North Atlantic, a wide spectrum of beak lengths encompassing those of *D. delphis* and *D. capensis* is present along the northern West African coast (Pinela et al. 2008). North of the 25th parallel, all common dolphins are considered to be of the short-beaked type (Pinela et al. 2008), even though in the Azores, individuals resembling long-beaked common dolphins in colour pattern and rostrum length are occasionally sighted (Azorean team, unpublished data). The short-beaked common dolphin has a world-wide distribution and can be found in both coastal and offshore waters, from tropical to temperate latitudes. Molecular and morphological data indicate that there is at least one population of *D. delphis* on each side of the North Atlantic (Westgate 2005, 2007; Murphy et al. 2006; Natoli et al. 2006; Mirimin et al. 2009). Body size and rostrum robustness differ between the eastern and western North Atlantic, and a few populations of the eastern North Atlantic appear to be morphologically differentiated from the others (Murphy et al. 2006). Mitochondrial and nuclear DNA analyses indicate genetic differentiation between the eastern and western North Atlantic, and little or no population structure within each region (Natoli et al. 2006; Mirimin et al. 2009). Apart from a few common dolphin samples that were included in a large-scale mitochondrial DNA study (Natoli et al. 2006), there is no information on population structure in the mid-Atlantic.

Atlantic spotted dolphins, *S. frontalis*, are endemic to warm temperate and tropical waters of the Atlantic. Two morphotypes have been described within this species: a larger, more heavily spotted form occurring in continental shelf waters, and a smaller, less spotted form occurring in pelagic waters and around oceanic islands (Perrin et al. 1987). Both forms can be found in the western North Atlantic, where they appear to be genetically differentiated based on mitochondrial and nuclear DNA markers (Adams and Rosel 2006). In the Azores and Madeira, spotted dolphins are expected to belong to the smaller form, although no formal study has examined the possibility that the larger form is also present. Information is missing on the population structure of *S. frontalis* over most of its distributional range apart from the western North Atlantic (Adams and Rosel 2006).

We used a combination of mitochondrial DNA (mtDNA) sequences and microsatellite markers to test predictions about the population structure of common and spotted dolphins around the oceanic islands of the Azores and Madeira. We searched for genetic differentiation between groups of islands and archipelagos, as well as between potential ecotypes. We expected to find some differentiation between groups of islands and archipelagos in common dolphins, given that this species is present all year round in the Azores and tends to prefer coastal waters. In contrast, we predicted an absence of genetic structure within and between archipelagos in spotted dolphins, given that this species is a seasonal visitor in both archipelagos and tends to prefer offshore waters.

Material and methods

Study sites

The archipelago of the Azores is located in the North Atlantic Ocean, about 1500 km away from the nearest coast (Fig. 1). It lies between the 37th and 41st northern parallels and the 25th

and 31st western meridians, extending more than 480 km along a Northwest-Southeast axis and crossing the Mid-Atlantic Ridge. It is composed of nine volcanic islands divided into three groups (the eastern, central and western groups) separated by deep waters (*ca*. 2000 m) with scattered seamounts (Santos et al. 1995; Morato et al. 2008). Shallow waters (< 200m) occur only at very short distances from the coast. Most field work was conducted in the central group of islands (from the harbour of Horta - 38.53°N and 28.63°W). Cruises to other islands were conducted in order to cover the entire archipelago.

The archipelago of Madeira is located in the North Atlantic Ocean, 580 km West of Morocco, Africa (Fig. 1). It is separated from the Azores by 900 km of deep waters. It is composed of two main volcanic islands, Madeira and Porto Santo, which are separated by a stretch of 35 km with a maximum depth of 3000 m. It also comprises two sub-archipelagos, Desertas Islands and Selvagens Islands, located 11 km Southeast and 300 km South of Madeira, respectively. Fieldwork was conducted on the southern coast of Madeira and West of the Desertas Islands (from the harbour of Machico - 32.73°N and 16.73°W).

Sample collection and DNA extraction

Skin samples were collected either by biopsy darting (a 125-lb Barnett crossbow, with darts and tips specially designed for small cetaceans by F. Larsen, Ceta-Dart, cf. Mathews et al. 1988) or by skin swabbing. The second method was employed in the Azores only and for a small number of samples (n = 31). Although less invasive, it generated stronger reaction by the dolphins (such as boat avoidance, jumps and leaps), especially in *D. delphis*. In addition, 16% of the samples obtained by this method did not contain enough tissue for DNA analyses. Most samples were from adult-size individuals, even though 10 to 30 % were probably from immature individuals. In the Azores, samples were obtained during summer 2004 to 2006 in all three groups of islands and around seamounts located 40 km south of the central group. All *D. delphis* samples were from individuals of the typical short-beaked type. In Madeira, samples were collected year-round between 2004 and 2006. Due to the seasonal occurrence of each species, samples of *D. delphis* were obtained between January and June and samples of *S. frontalis* between June and November. Most fieldwork was done within 10 km from the coast of the main island. Thus, the sampling scheme did not allow examining population structure within the archipelago. Four additional samples were obtained from individuals that stranded on the coast of Madeira between 1997 and 2006.

All samples were stored in 90 % ethanol. They were processed at the INETI, Lisbon, Portugal. DNA extractions were performed following the protocol of Gemmel & Akiyama (1996) or using the DNeasy tissue isolation kit (Qiagen) following the manufacturer's recommendations. About 1-2 mm^3 of skin were minced and rinsed in dd-water prior to extraction. Digestion by recombinant proteinase K was extended overnight at 56°C.

Mitochondrial DNA sequences

Acquisition of sequences

Part of the tRNA-Thr, the tRNA-Pro and the most variable part of the mitochondrial D-loop were amplified using the primers Dloop-16^L (Hoelzel et al. 1991) and H00034 (Rosel et al. 1994). For 24 *S. frontalis* samples, a longer fragment was obtained with the primers Dloop-16^L and Dloop-19^H (Hoelzel et al. 1991). Longer sequences were used to assess the impact of using shorter sequences on haplotype diversity. They were truncated before subsequent analyses.

PCR reactions were carried out in a 25 µl volume using 0.75 units of *Taq* DNA polymerase (MBI Fermentas) and 2mM $MgCl_2$. The number of cycles was set to 35 and the annealing temperature to 52°C. PCR products were purified with the GFX PCR DNA purification kit (Amersham Biosciences). Sequencing was done on an ABI-prism capillary sequencer (Applied Biosystems) by Macrogen, Korea. All samples were sequenced with the 16^L primer, using an annealing temperature of 55°C. Four randomly chosen samples per species were also sequenced with the reverse primer and no ambiguities were found. All sequences were double-checked for errors. Sequences were deposited in GenBank, under accession numbers EF682507 to EF682840. Alignment was performed visually. Three gaps were identified in *D. delphis* and four in *S. frontalis*. Aligned sequences were 611 base pair (bp) long in both species.

Population structure

Population structure was analysed using Arlequin 3.1 (Excoffier et al. 2005). Gene diversity (H) and nucleotide diversity (π) were calculated for each archipelago. Shared haplotypes between archipelagos were identified. Genetic distances between and within archipelagos were estimated using the Tamura-Nei formula (Tamura and Nei 1993). Corrected distances accounting for intra-population variability were also calculated. In order to estimate whether the populations had undergone a size and/or range expansion, we computed the mismatch distributions within each archipelago and the intermatch distribution between archipelagos (following Excoffier 2004). We verified whether the populations from the Azores and Madeira were behaving as a single one by searching for significant correlations between the three distributions by means of Spearman's rank tests. Two demes belonging to the same range expansion are expected to have similar mismatch distributions, which closely overlap with the intermatch (Excoffier 2004). We also performed Fu's test of neutrality (Fu 1997), which is the most powerful test to detect population growth when sample size is large (c.a. 50 individuals or more; Ramos-Onsins and Rozas 2002). Significance was estimated using a coalescence simulation algorithm with 10,000 randomization steps. It was considered significant at $P < 0.02$, as recommended by the author.

Genetic differentiation among potential populations was assessed taking into account nucleotide differences between haplotypes (Φ_{ST}, Weir and Cockerham 1984), after correction by the Tamura-Nei formula (1993). Because male-biased dispersal (as often happens in cetacean species; Hoelzel et al. 2002) could obscure the geographical pattern of female-transmitted mtDNA (Tiedemann et al. 2000), Φ_{ST} was measured for all samples and for females only. Significance was assessed by a permutation procedure (10,000 permutations). A sequential Bonferroni correction was applied to compensate for multiple tests (Rice 1989). Populations with less than five samples were not considered (namely, *D. delphis* from the eastern group of Azorean islands, $n = 2$). Attempts were made to calculate asymmetric estimates of migration rates (Nm) between populations under a maximum likelihood framework using Migrate 2.0 (Beerli 2004). Despite our efforts to achieve convergence through long runs and multiple chains requiring several weeks of computation, acceptance ratios were low, mixing was poor and convergence between runs was limited. Thus, the process was abandoned.

Haplotype networks

We investigated the phyletic relationships between haplotypes using network-building methods. These methods are especially efficient for intra-specific comparisons, when genetic distances between individuals are small and the number of equally parsimonious connections

is high (Templeton et al. 1992; Crandall 1996). We used the Median Joining approach (MJ), implemented by the software Network4 (Bandelt et al. 1999). The homoplasy parameter (ε) was set to zero. Two weighting schemes were compared: 1/ equal weight for all classes of changes, and 2/ weight of 10 for transitions and 30 for transversions and gaps, as recommended by the authors for a tenfold difference in mutation rates between substitution classes.

Microsatellites and molecular sexing

Data acquisition

Fourteen polymorphic dinucleotide microsatellite loci were analysed: d08, d22 (Shinohara et al. 1997), EV14, EV37 (Valsecchi and Amos 1996), FCB1, FCB17 (Buchanan et al. 1996), Kwm2a, Kwm12a, Kwm9b (Hoelzel et al. 1998b), Mk6, Mk8 (Krützen et al. 2001), Sw10, Sw19 (Richard et al. 1996) and TexVet5 (Rooney et al. 1999). Loci TexVet5 and Kwm2a were not analysed in *S. frontalis* because they failed to amplify. PCR reactions were performed in multiplex whenever possible, using 25 cycles and a touch-down decrease in annealing temperatures (0.1°C per cycle): Sw10 and Sw19 (52 → 49.5°C); EV14 (56 → 53.5°C); FCB1, FCB17 and EV37 (56 → 53.5°C); d22, Mk6 and Mk8 (56 → 53.5°C); TexVet5 (49.5 → 47°C); Kwm2a (49.5 → 47°C); d08, Kwm12a and Kwm9b (57 → 54.5°C). Fragments were scanned on an ABI 310 capillary sequencer using the size marker ROX350 (Applied Biosystems). Molecular sexing was performed by co-amplification of a short fragment of the male-specific SRY gene (CSY, 157 bp, Abe et al. 2001) and a tetranucleotide microsatellite used as a PCR control for positive identification of females (GATA028, 99/103 bp, Palsbøll et al. 1997). Unsuccessful PCR reactions were repeated up to three times. DNA extraction and genotyping were repeated whenever a sample was found not to amplify or to be homozygous at more than three loci. Samples that could not be re-analysed successfully were removed from the data set. In order to make sure that samples obtained by skin swabbing were correctly genotyped, we compared the proportion of homozygous loci in both kinds of samples by means of a two-sided non parametric Mann and Whitney U-test for unpaired samples. The skin swabbing samples appeared to be no more homozygous than the biopsy samples (exact P = 0.495 for *D. delphis* and 0.913 for *S. frontalis*).

Polymorphism control

Genotypes were checked for potential errors and replicated individuals using Microsatellite Tools (MsTools, Park 2001). Five potential replicates were identified: two differing by one allele and three with identical genotypes. Otherwise, the highest matching score between distinct individuals was 64.3 % of allelic identity for *D. delphis* and 68.2 % for *S. frontalis*. Extraction and genotyping were repeated for all potential replicates, and perfect matching was found for all pairs of samples. Thus, the initial error rate was 1.5% for these ten samples. One sample per pair was removed from all datasets prior to analyses.

For each archipelago, polymorphism was estimated as the number of alleles per locus, observed heterozygosity (H_O) and unbiased expected heterozygosity (H_E), using Arlequin 3.1. Departure from Hardy-Weinberg frequencies was tested by an exact test using Genepop on the Web (http://genepop.curtin.edu.au/index.html; Genepop 3.3 by Raymond and Rousset 1995) with default settings. A sequential Bonferroni correction was applied to compensate for multiple tests (Rice 1989). Null allele frequencies were estimated under the hypothesis that all the deviation to Hardy-Weinberg Equilibrium (HWE) was due to null alleles, using Cervus 2.0 (Marshall et al. 1998). For loci with a high estimated frequency of null alleles, 20 randomly chosen homozygote samples were re-analysed. The second amplification confirmed

the initial diagnostic in all cases. Finally, we verified that loci were not in linkage disequilibrium using Arlequin 3.1, applying a sequential Bonferroni correction.

Population structure

Inbreeding coefficients (F_{IS}) were calculated for each archipelago, and their significance evaluated by a randomisation procedure (10,000 permutations), using Genetix 4.03 (Belkhir et al. 2001). Global F_{IS} was also calculated over all samples, as a means to evaluate the hypothesis that individuals from the Azores and Madeira belonged to the same population. As recent fluctuations in population size could affect genetic population structure, we tested for the existence of a significant excess or deficit of heterozygosity in each archipelago using the program Bottleneck 1.2 (Cornuet and Luikart 1996). A significant excess of heterozygosity (or "gene diversity") is expected under a situation of recent bottleneck, while a significant deficiency is expected under population expansion. Significance was evaluated by the Wilcoxon sign-rank test. Simulations were based on the Two Phase Model of evolution of microsatellites (TPM, DiRienzo et al. 1994), with default parameters. This choice was justified by the fact that only half of the loci seemed to conform to the uni- or bi-modal distribution of allele sizes expected under the Stepwise Mutation Model (SMM, Ohta and Kimura 1973). Other loci presented multimodal distributions and/or large gaps in allele sizes, more in agreement with the Infinite Allele Model (IAM, Kimura and Crow 1964) or the Two Phase Model (TPM, DiRienzo et al. 1994).

The influence of allele size on population differentiation was tested by the allele size permutation test implemented in SPAGeDi 1.2d (Hardy and Vekemans 2002). It is noteworthy that, although R_{ST} is designed especially for microsatellites and accounts for differences in allele sizes, F_{ST} is more reliable than R_{ST} when sample size is limited (Gaggiotti et al. 1999) and when gene flow is high (Balloux and Goudet 2002). As allele size did not contribute to population differentiation ($P = 0.877$ for *D. delphis* and 0.165 for *S. frontalis*), differentiation among potential populations was estimated based on the IAM model (F_{ST}, Weir and Cockerham 1984) using FSTAT 2.9.3 (Goudet 2001). Its significance was tested by an exact G-test (Goudet et al. 1996) and a 95% confidence interval (95% CI) was obtained by bootstrapping over loci. Because sex biased dispersal could impede detecting population structure in the phylopatric sex, we searched for a difference in F_{ST} between males and females using the randomisation procedure implemented in FSTAT 2.9.3. Finally, as for mitochondrial DNA, attempts were made to calculate asymmetric estimates of migration rates between populations using Migrate 2.0, and the process was abandoned.

We examined the possibility of an undetected population structure, potentially associated with ecotype differentiation, using the software Structure 2.1 (Pritchard et al. 2000). We carried out MCMC simulations with no prior information on the origin of samples, using the admixture model. The maximum number of populations (K) was assumed to vary between 1 and 10. For each potential value of K, five replications were performed, with a number of steps equal to 100,000 for the burnin process and 1,000,000 for the simulations. The probability that a given value of K was the best one was calculated based on mean ln Pr(X/K), following the recommendations of the authors. As simulations have shown that ln Pr(X/K) did not always peak at the actual value of K, we also determined the most likely value of K following the procedure described by Evanno and collaborators (2005). These authors recommend using the mode of the distribution of ΔK, a statistic based on the variability and rate of change of ln Pr(X/K) between successive values of K. A ΔK of at least 25 can be expected at the true K for a hierarchical island model with about 20 individuals per sub-population, under partial sampling of populations and using as little as 5 microsatellite loci

(Fig. 4F in Evanno et al. 2005). It is noteworthy that this statistic cannot be calculated for the lowest or highest value of K (here, K = 1 or 10).

We tested for the effect of geographic distances on population structure by means of a Mantel test. A significant correlation can be expected when dispersal distances are short compared to population range. Given the difficulty of defining geographic boundaries and the high mobility of dolphins, we performed the test at the individual level, using Alleles In Space 1.0 (AIS; Miller 2005). The genetic distance implemented in AIS is an analogue of Nei's distance (Nei et al. 1983) applied to pairs of individuals. Log-transformed geographic distances were used to account for the two-dimensional distribution of the sampling locations. Significance was assessed by 10,000 permutations.

Because haplotypes clustered in distinct groups separated by large genetic distances (cf. result section), we suspected that haplotype groups could represent distinct ecotypes or populations. A Molecular Analysis of Variance (AMOVA) was performed to determine how microsatellite diversity was partitioned between these haplotype groups, using Arlequin 3.1 (Excoffier et al. 2005).

Results

Duplicated samples and sex ratio

In total, 150 *D. delphis* and 193 *S. frontalis* samples were successfully analysed. Search for duplicated samples revealed five cases of perfect matching, all of which originated from the central group of islands of the Azores. In *D. delphis*, one female was sampled twice on successive days, 5 km away from the place where she had been sampled initially; one male was sampled twice at four days interval and 14 km of distance, and another male at one year interval and 18 km of distance. In *S. frontalis*, one male and one female were sampled twice during the same sighting. The final number of different individuals analysed was 147 for *D. delphis* and 191 for *S. frontalis* (Table 1).

Molecular sexing indicated a sampling bias in favour of males in both species (Table 1). Bias was very high in Madeira, where a male to female ratio of 8.3:1 was measured for *D. delphis* and 3.3:1 for *S. frontalis*. In the Azores, the male to female ratio was 1.7:1 for *D. delphis* and 1.3:1 for *S. frontalis*. The factors causing unbalanced sex-ratio were discussed in a recent paper (Quérouil et al. 2010).

Mitochondrial DNA sequences

Variability

Sequencing of short fragments (611 bp) rather than long fragments (1003 bp) of the D-loop resulted in a loss of 8.3 % in the number of distinct haplotypes identified out of 24 *S. frontalis* samples (19 haplotypes instead of 21).

In *D. delphis*, there were 36 distinct haplotypes out of 91 samples from the Azores and 31 haplotypes out of 52 samples from Madeira (four samples from Madeira could not be sequenced). There were 45 variable nucleotide positions in the Azores, 53 in Madeira, and 60 in the whole dataset. Gene diversity was 0.953 in the Azores and 0.975 in Madeira, and nucleotide diversity was 0.013 in both archipelagos.

In *S. frontalis*, there were 76 distinct haplotypes out of 144 samples from the Azores and 35 haplotypes out of 46 samples from Madeira (one sample from Madeira could not be

sequenced). There were 72 variable nucleotide positions in the Azores, 63 in Madeira, and 84 in the whole dataset. Gene diversity was 0.974 in the Azores and 0.990 in Madeira, and nucleotide diversity was 0.018 in both archipelagos.

Population structure within and between archipelagos

In *D. delphis*, 13 haplotypes were shared between the two archipelagos. Mean Tamura-Nei distances were similar within (7.78 % for the Azores and 8.07 % for Madeira) and between archipelagos (8.01 %). The mean distance between archipelagos was 0.08 % after correction for intra-population polymorphism. There was no significant differentiation between the two archipelagos both for the complete sample (Φ_{ST} = 0.010, P = 0.080) and for females only (Φ_{ST} = -0.067, P = 0.948). In *S. frontalis*, 19 haplotypes were shared between the two archipelagos. The mean Tamura-Nei distance was similar within (11.02 % for the Azores and 10.79 % for Madeira) and between archipelagos (10.83 %). The mean distance between archipelagos was -0.07 % after correction for intra-population polymorphism. There was no significant differentiation between the two archipelagos either for the complete sample (Φ_{ST} = -0.007, P = 0.856) or for females only (Φ_{ST} = -0.028, P = 0.918). When the Azores were subdivided into groups of islands, fixation indexes indicated a lack of population differentiation between all sampling sites in both species after application of a Bonferroni correction, both for the complete sample and for females only (Table 2).

In each species and archipelago, the mismatch distribution was not significantly different from that expected following either a population size expansion or a range expansion model (Table 3). The mismatch distributions obtained for the Azores and Madeira were significantly correlated one with the other and with the intermatch between archipelagos (Fig. 2, Table 3). The three distributions were almost perfectly overlapping in *S. frontalis*. Fu's test of neutrality was significant in each species and archipelago (Table 3).

In both species, haplotypes clustered independently of sampling location in the Median Joining networks (Fig. 3A and 3B). The application of differential weights did not significantly alter the phyletic relationships between haplotypes. In *D. delphis*, various "satellite" haplotypes were separated by large genetic distances from the main core of closely related haplotypes. In *S. frontalis*, a single cluster of "satellite" haplotypes was separated from the main core by a very large genetic distance.

Microsatellites

Variability and HWE

The selected loci presented high levels of allelic diversity and heterozygosity (Table 4). For the whole dataset, allelic diversity ranged between 5 and 27 in *D. delphis* (mean = 12.9 +/- s.d. = 6.1) and between 5 and 22 in *S. frontalis* (mean = 14.3 +/-5.4). Variability at each locus differed between species. After correction for sample size, allelic richness was similar in both populations. Private alleles were found in at least one of the two populations at all loci but one. In *D. delphis*, private alleles were scored once, twice, or exceptionally three times. In *S. frontalis*, most private alleles were rare, but one was scored seven times in the largest population. Two loci per species appeared not to be in HWE after application of a sequential Bonferroni correction (Table 4): EV14 and TexVet5 in *D. delphis* (in both archipelagos) and d22 and Sw10 in *S. frontalis* (in the Azores only for d22). Locus TexVet5 had already been shown not to be in HWE in *D. delphis* (Natoli et al. 2006). The unbalanced loci presented a high estimated proportion of null alleles, ranging from 9 to 25.3% depending on species and

location. Therefore, they were removed from the datasets for subsequent analyses. There was no linkage disequilibrium between the remaining loci in either species.

Population structure within and between archipelagos

Within each population, the inbreeding coefficients calculated over all loci were not significant, except for *D. delphis* in the Azores (*D. delphis*: F_{IS} = 0.025, P = 0.032 for the Azores and F_{IS} = 0.015, P = 0.175 for Madeira; *S. frontalis*: F_{IS} = 0.011, P = 0.169 for the Azores and F_{IS} < 0.001, P = 0.471 for Madeira). The global inbreeding coefficient calculated over all samples and loci was significant for *D. delphis* (F_{IS} = 0.023, P = 0.013), but not for *S. frontalis* (F_{IS} = 0.007, P = 0.230). In both species, there was no significant heterozygosity excess or deficit neither in the Azores nor in Madeira (Wilcoxon two-tailed test, *D. delphis*: P = 0.204 for the Azores and P = 0.110 for Madeira; *S. frontalis*: P = 0.492 for the Azores and P = 0.695 for Madeira). The allele frequency distributions were L-shaped, as expected in stable populations.

Fixation indexes revealed a lack of differentiation between the Azores and Madeira in both species (*D. delphis*: F_{ST} = 0.002, 95% CI = [0; 0.004], P = 0.108; *S. frontalis*: F_{ST} = -0.002 [-0.003; 0.002], P = 0.891). When the Azores were subdivided into groups of islands, F_{ST}-values were not significant after application of a sequential Bonferroni correction (Table 5). Furthermore, F_{ST}-values were not significantly different between males and females in either species (*D. delphis*: P = 0.563, *S. frontalis*: P = 0.084).

The AMOVA performed on haplotype groups revealed that most of the microsatellite variance was found within haplotype groups (98.96 % in *D. delphis*, 100 % in *S. frontalis*). F_{ST}-values were low and not significant (*D. delphis*: F_{ST} = 0.010, P = 0.084; *S. frontalis*: F_{ST} = -0.0004, P = 0.528), indicating that microsatellite diversity tended to be higher within than among haplotype groups.

Individual-based Mantel tests were not significant in either species (r = 0.016, P = 0.100 in *D. delphis*; and r = -0.016, P = 0.767 in *S. frontalis*).

In both species, Bayesian analyses failed to uncover any cryptic population structure. The highest values of ln Pr(X/K) were obtained for K = 1 (P almost equal to 1 in each species). The distribution curves of ΔK were bimodal, and the highest ΔK-values were obtained for K = 2 in *D. delphis* (ΔK = 8.28) and K = 3 in *S. frontalis* (ΔK = 3.17). These values were below the threshold of 25 expected for distinct populations. When population assignments were conducted with K = 2 or 3, repsectively, the proportion of samples assigned to each population was roughly symmetric (close to 1/K) and no individual was strongly assigned to any population.

Discussion

Variability

Mitochondrial and nuclear DNA diversities were high and in the same range as those previously reported in short-beaked common dolphins (Natoli et al. 2006; Amaral et al. 2007b; Mirimin et al. 2009) and oceanic populations of Atlantic spotted dolphins (Adams and Rosel 2006). Such high values are typical of large panmictic populations (Frankham et al. 2002). In both species, mtDNA analyses revealed one main cluster of closely related haplotypes and some very distantly related “satellite” haplotypes (Fig. 3A and 3B). These clades were not differentiated at the nuclear-DNA level. Similarly, highly divergent clades of

haplotypes are commonly observed in large migratory fishes (e.g. blue marlins, *Makaira nigricans*: McDowell et al. 2007, and bigeye tunas, *Thunnus obesus*: Gonzalez et al. 2008) and in marine mammals (Hoelzel et al. 2002), including common dolphins of the North Atlantic (Amaral et al. 2007b; Mirimin et al. 2009). They are generally explained by inter-oceanic vicariance during the last Pleistocene maxima, followed by uni- or bi-directional dispersal. In the present case, two alternative hypotheses are also possible: incomplete lineage sorting associated with retention of past polymorphism or introgressive hybridization occurring between *D. delphis* and *S. coeruleoalba* (cf. Amaral et al. 2007a) or between the two species of spotted dolphins.

Absence of population structure within and between archipelagos

Mitochondrial and nuclear DNA analyses pointed towards a lack of genetic population structure at the scale of the study area in both species. In fact, clustering of mtDNA haplotypes was independent of the geographical origin of samples (Fig. 3A and 3B). Fixation indexes based on mitochondrial and nuclear DNA indicated a lack of population structure (Tables 2 and 5) that was not the fate of higher male dispersal. Individual-based Mantel tests performed on microsatellite data did not show any significant effect of isolation by distance. Bayesian analyses performed on unassigned individuals failed to uncover any cryptic population structure. The mismatch and intermatch mt-DNA distributions were significantly correlated in both species and almost perfectly overlapping in *S. frontalis* (Fig. 2), indicating that the populations of the Azores and Madeira behaved as a single population.

The star-shape mt-DNA haplotype networks, large significant negative Fs-values, and mismatch distributions were suggestive of population size and/or range expansions. On the other hand, tests of microsatellite heterozygosity excess or deficit revealed no effect of recent fluctuations in population size. These latter two results are not inconsistent since mitochondrial DNA keeps track of older events than nuclear DNA. At least, it seems that failure to identify any population structure was not caused by a recent change in population size. There was also no evidence of sex-biased dispersal that could have obscured population structure in the most phylopatric sex. Genetic differentiation was in the same order of magnitude for mt-DNA sequences (female transmission) and microsatellites (biparental inheritance) within each species. There were also no significant differences in sex-specific measures of genetic differentiation between archipelagos based on either kind of marker. In the case of *D. delphis*, this could have been caused by the limited number of female samples from Madeira, but results were consistent with previous studies that shown no sex-biased dispersal at the scale of the Atlantic in that species (Natoli et al. 2006; Mirimin et al. 2009).

It cannot be ruled out that the analyses overlooked weak population structure associated with low allelic differentiation. However, similar studies based on the same mitochondrial gene and almost the same set of microsatellite loci were able to detect differentiation in *D. delphis* in other regions (Bilgmann et al. 2008; Natoli et al. 2008) or in other species (e.g. in *Tursiops* sp.: Hoelzel et al. 1998b; Krützen et al. 2004). Even though it was not possible to obtain reliable estimates of the amount of gene flow, our results indicated that gene flow was sufficient to prevent differentiation within and between archipelagos in common and spotted dolphins at the scale of the study area. In fact, poor mixing and lack of convergence between runs should be expected under weak differentiation.

Lack of population structure was unexpected in the short-beaked common dolphin, because this species occurs year-round in the Azores and tends to prefer nearshore waters. Notwithstanding, the observed pattern agrees with previous studies indicating high gene flow over large geographic distances at the scale of the North Atlantic in this species (Natoli et al. 2006; Mirimin et al. 2009). It cannot be ruled out that failure to detect any population

structure in *D. delphis* was caused by low levels of divergence associated with recent population differentiation following the last glacial maximum. Actually, the significant inbreeding coefficients obtained for the whole sample and within the Azores suggest that populations are not at the mutation-drift equilibrium, not panmictic, or undergoing selection. Deviation from panmixia could be caused by a Wahlund effect, i.e. the existence of undetected sub-populations. In the Azores, it is possible that there are resident and non-resident individuals who do not fully interbreed. It is noteworthy that genotyping revealed three cases of re-sampling of common dolphins that occurred within a small geographic range and with a time interval of up to one year. These events indicate some degree of site fidelity, and suggest that there might be resident individuals in the Azores. Residency has been shown to be associated with population differentiation in other species (e.g., bottlenose dolphins of the species *T. aduncus* in Australia, Möller and Beheregaray 2004; spinner dolphins in the South Pacific, Oremus et al. 2007). However, in the Azores, no population differentiation was found in *T. truncatus* despite the existence of known resident individuals (Quérouil et al. 2007; Silva et al. 2008). The extent of differentiation might depend on the proportion of individuals that are resident. A more detailed genetic study would possibly reveal population differentiation and seasonal variations in *D. delphis* population structure within the Azores.

In the Atlantic spotted dolphins, lack of population structure could be expected, given that they are temporary visitors in the Azores and Madeira and tend to prefer offshore waters. Although spotted dolphins occur in both archipelagos during the same period of the year (Freitas et al. 2004; Quérouil et al. 2008), it is likely that the individuals frequenting the Azores and Madeira belong to the same population. Interestingly, the observed pattern contrasts with the population structure existing in the western Atlantic, where oceanic and coastal populations can be distinguished (Adams and Rosel 2006). This discrepancy is likely due to differences in habitat structure, related to the presence of a continental shelf in the western Atlantic.

Conclusions

Mitochondrial and nuclear DNA did not evidence any genetic structure among common and spotted dolphins of the Azores and Madeira, neither between archipelagos nor between groups of islands nor in relation with habitat features. Similar genetic patterns were observed in both species, independently of their patterns of residency and ecological preferences. Spotted dolphins are temporary visitors in the Azores and Madeira, and it can be hypothesised that they undertake large migrations within and outside the study area. Common dolphins are seasonal visitors in Madeira, but present year-round in the Azores, where some individuals may be resident. We recommend that a long-term study is conducted to investigate seasonal and local population differentiation in *D. delphis*, especially within the archipelago of the Azores.

Absence of genetic structure over hundreds and even thousands of kilometres implies the existence of gene flow over much larger distances than usually documented in small delphinids (e.g., Natoli et al. 2004; Escorza-Treviño et al. 2005; Parsons et al. 2006; Oremus et al. 2007). It has long been controversial whether such amounts of gene flow could be achieved through individual long-distance movements. In coastal areas, dolphin movements are usually at the scale of a few dozens kilometres (cf. Gowans et al. 2007). In common dolphins of the North Atlantic, lack of population structure within the eastern and western basins suggested that individuals might undergo long-distance migration movements (Mirimin et al. 2009). In the Azores, high levels of gene flow were found in bottlenose dolphins of the species *T. truncatus* (Quérouil et al. 2007), and a photo-identification study revealed that some individuals travel between groups of islands and probably come from

outside the archipelago (Silva et al. 2008). In the eastern tropical Pacific, capture-recapture data showed that dolphins of the genus *Stenella* can travel more than 1000 km, and seasonal shifts in distributions suggested that movements could be as wide as 2500 km (Reilly 1990). Spotted dolphins probably undergo such long-distance movements in the study area. It is likely that small delphinids have very large ranges in pelagic waters due to low productivity, variations in water surface temperatures, and their consequences on prey distribution and availability (cf. Gowans et al. 2007). Oceanic dolphin population structure seems to parallel that of other top predators, which are capable of trans-oceanic movements across the Atlantic Ocean (e.g. large migratory fishes such as blue marlins and big eyed tunas: McDowell et al. 2007; Gonzalez et al. 2008).

We recommend that common and spotted dolphins frequenting the Azores and Madeira are provisionally considered as members of one single conservation unit within each species. These conservation units, characterised by high levels of genetic diversity and large population sizes, are probably not threatened in the short term. However, it appears that long term conservation policies regarding these and other large migratory species shall be considered on a global scale. Ocean-wide international regulations are necessary in order to avoid depletion of fish stocks and other marine resources, limit pollution, and ensure sustainable conservation in the North Atlantic.

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Figure 1. Map of the archipelagos of the Azores and Madeira within the North Atlantic Ocean, with enlarged map of the Azores showing the three groups of islands and the seamounts (localized by an X).

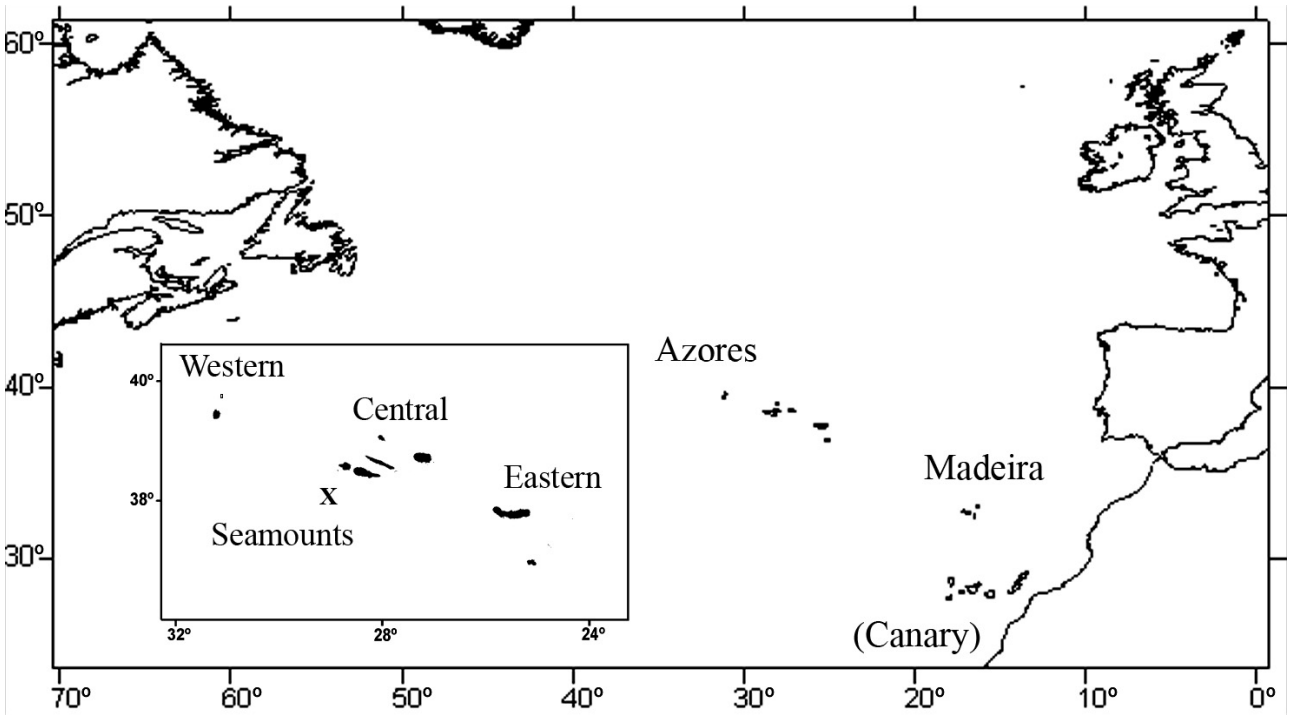
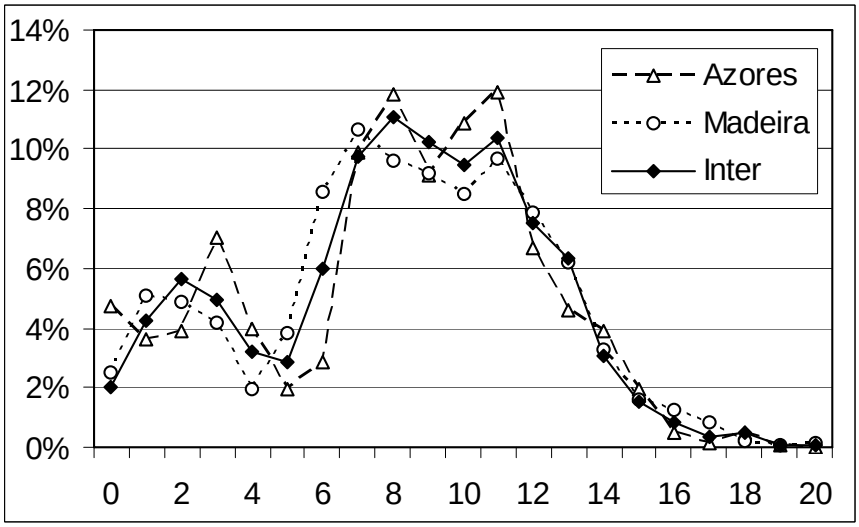


Figure 2. Mismatch distribution of pairwise differences between D-loop sequences of *D. delphis* (A) and *S. frontalis* (B) within the Azores (open triangles) and Madeira (open circles) and intermatch between archipelagos (plain diamonds).

A



B

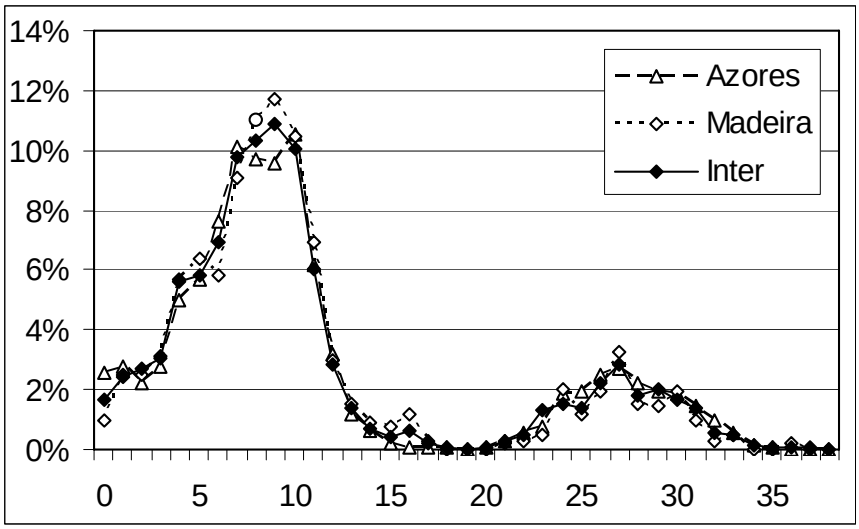


Figure 3A. Median Joining networks obtained with differential weighting of transitions, transversions and indels, for D-loop sequences of *D. delphis* from the Azores (red = eastern, yellow = central, green = western, pink = seamounts) and Madeira (black). Circle size is proportional to the number of samples and connector length is proportional to the number of substitutions. Small open circles represent potential intermediate haplotypes that were not sampled.

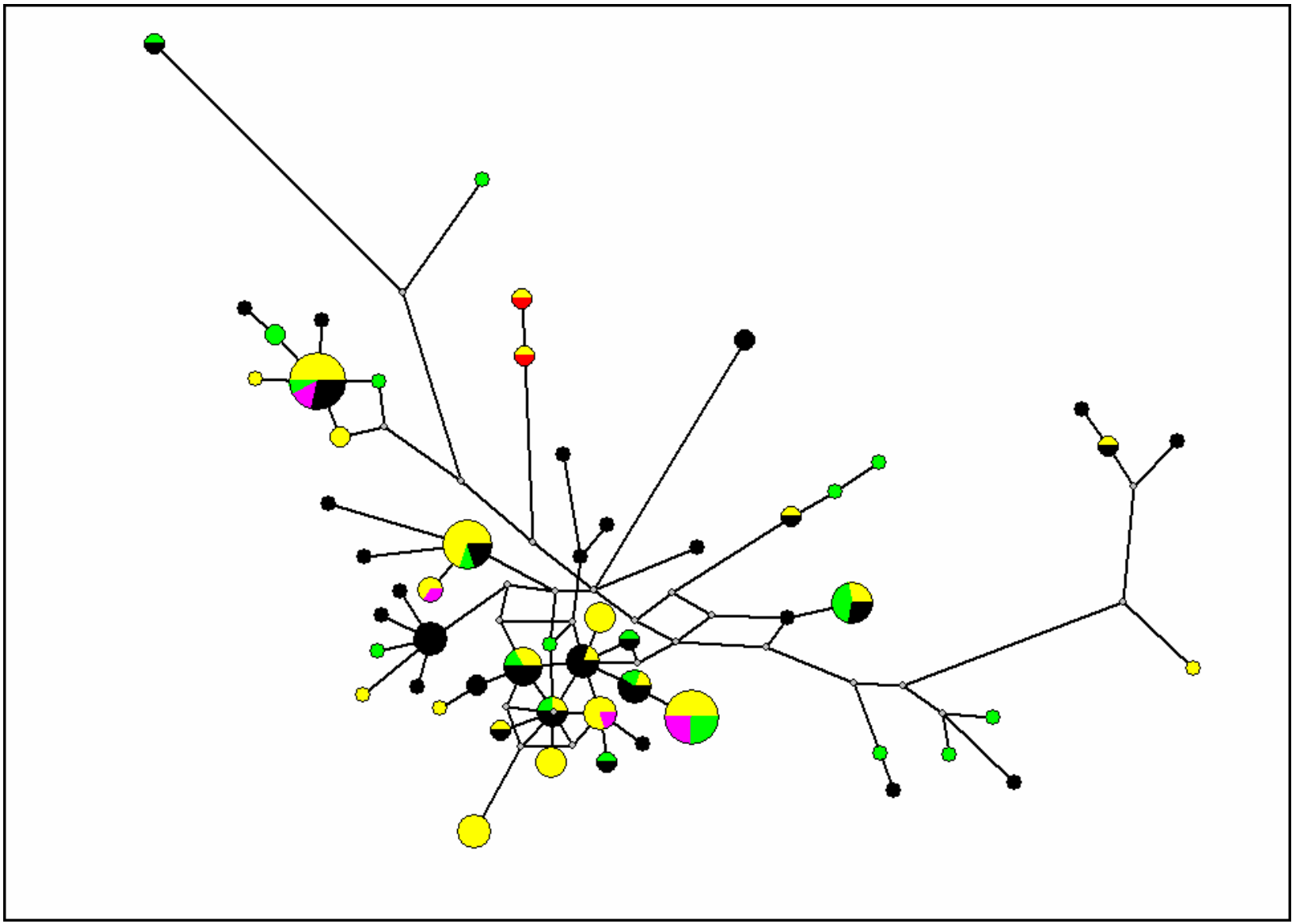


Figure 3B. Median Joining networks obtained with differential weighting of transitions, transversions and indels, for D-loop sequences of *S. frontalis* from the Azores (red = eastern, yellow = central, green = western, pink = seamounts) and Madeira (black).

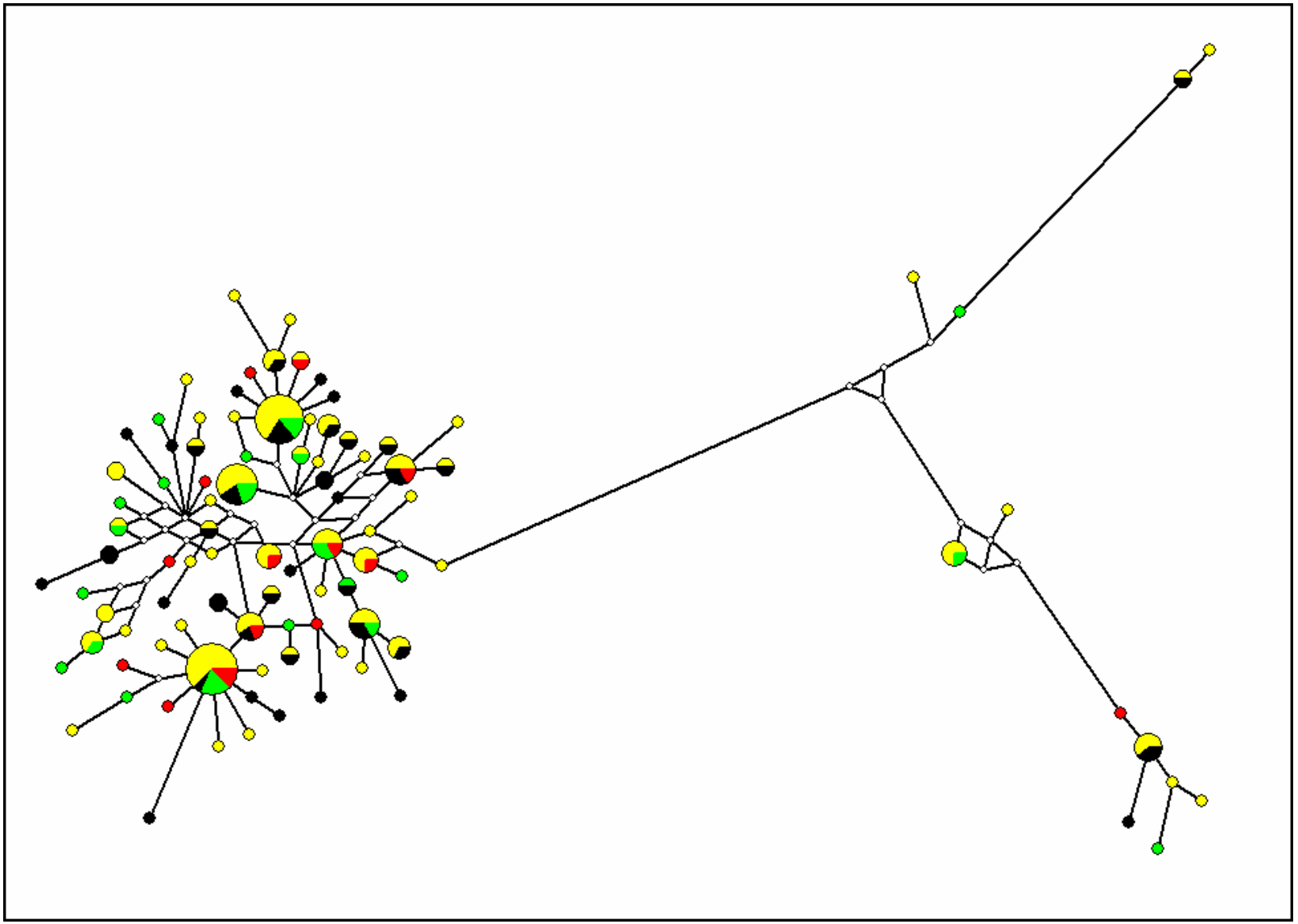


Table 1. Number of distinct individuals analysed per species, sex, group of islands and archipelago.

Archipelago	Azores					Madeira	Total
Island group	Western	Central	Eastern	Seamounts	All	--	--
D. delphis							
males	15	36	1	5	57	50	107
females	10	21	1	2	34	6*	40
total	25	57	2	7	91	56*	147
S. frontalis							
males	16	60	5	0	81	36*	117
females	10	43	10	0	63	11*	74
total	26	103	15	0	144	47*	191

* four samples originated from stranded animals: one female *D. delphis*, two males and one female *S. frontalis*.

Table 2. Population differentiation and gene flow between groups of islands of the archipelago of the Azores and Madeira based on 611 bp-long D-loop sequences: Φ_{ST} (above diagonal: complete dataset; below diagonal: females only) with number of sequenced individuals (*n*) and level of significance (*: $0.01 < P < 0.05$).

D. delphis	*n* total	*n* females	Western	Central	Seamounts	Madeira
Western	25	10	-	0.023	0.014	-0.004
Central	57	21	0.016	-	-0.016	0.019*
Seamounts	7	2	0.003	-0.039	-	0.038
Madeira	52	5	-0.077	-0.049	-0.201	-
S. frontalis	*n* total	*n* females	Western	Central	Eastern	Madeira
Western	26	10	-	-0.014	-0.011	-0.011
Central	103	43	-0.021	-	-0.007	-0.009
Eastern	15	10	0.008	0.011	-	-0.008
Madeira	46	11	-0.029	-0.032	-0.016	-

Table 3. Test of population expansion in each species and archipelago: A. goodness of fit to a model of population size or range expansion; B. correlation between the observed mismatch distributions within the Azores and Madeira and the intermatch (inter) distribution between archipelagos (Spearman's rank test, $n = 21$ for *D. delphis* and 39 for *S. frontalis*); C. Fu's test of neutrality.

	D. delphis		*S. frontalis*	
A. Model fit	P size	P range	P size	P range
Azores	0.320	0.225	0.193	0.205
Madeira	0.809	0.363	0.335	0.156
B. Correlation	R Spearman	P	R Spearman	P
Azores / Madeira	0.856	<0.0001	0.946	<0.0001
Azores / inter	0.915	<0.0001	0.983	<0.0001
Madeira / inter	0.968	<0.0001	0.976	<0.0001
C. Fu's test	Fs	P	Fs	P
Azores	-9.9	0.016	-14.6	0.0005
Madeira	-24.2	0.0004	-16.2	0.0001

Table 4. Locus-specific information: allelic diversity (K, with number of private alleles between parentheses), allelic richness for the largest population based on sample size of the smallest population (R), observed (H_O) and expected (H_E) heterozygosity, and probability of departure from Hardy-Weinberg Equilibrium within each population (HWE, P-value).

	Azores					Madeira			
Locus	K	R	H_O	H_E	HWE	K	H_O	H_E	HWE
D. delphis									
D08	12 (1)	11.5	0.758	0.849	0.160	13 (2)	0.839	0.863	0.709
D22	5 (0)	5.0	0.626	0.630	0.763	6 (1)	0.625	0.584	0.146
EV14	19 (0)	17.9	0.637	0.925	<0.0001	20 (1)	0.661	0.933	<0.0001
EV37	25 (5)	21.5	0.923	0.911	0.896	22 (2)	0.946	0.930	0.965
FCB1	14 (0)	12.2	0.879	0.839	0.904	15 (1)	0.156	0.839	0.311
FCB17	5 (0)	5.0	0.505	0.568	0.128	5 (0)	0.554	0.569	0.039
Kwm2a	16 (1)	14.9	0.901	0.900	0.681	15 (0)	0.893	0.904	0.097
Kwm9a	15 (3)	13.5	0.824	0.852	0.011	14 (2)	0.875	0.893	0.425
Kwm12b	11 (1)	10.1	0.733	0.781	0.233	11 (1)	0.786	0.810	0.672
Mk6	14 (2)	12.7	0.879	0.868	0.736	12 (0)	0.804	0.869	0.302
Mk8	11 (2)	9.9	0.813	0.826	0.806	9 (0)	0.804	0.807	0.660
Sw10	5 (0)	5.0	0.703	0.764	0.375	6 (1)	0.714	0.786	0.304
Sw19	6 (1)	5.8	0.648	0.641	0.996	5 (0)	0.643	0.629	0.415
TexVet5	12 (1)	11.4	0.580	0.840	<0.0001	11 (0)	0.518	0.874	<0.0001
Mean +/- s.d.	12.1 +/-5.8	11.2 +/-5.0	0.743 +/- 0.131	0.800 +/- 0.111		11.7 +/-5.3	0.751 +/- 0.133	0.806 +/- 0.124	
S. frontalis									
D08	19 (7)	15.3	0.854	0.868	0.249	13 (1)	0.894	0.854	0.060
D22	5 (2)	4.5	0.472	0.623	<0.0001	3 (0)	0.489	0.597	0.295
EV14	17 (5)	13.9	0.868	0.866	0.045	12 (0)	0.851	0.874	0.203
EV37	14 (2)	11.8	0.833	0.852	0.465	13 (1)	0.830	0.865	0.725
FCB1	22 (9)	14.1	0.882	0.852	0.741	13 (0)	0.851	0.865	0.257
FCB17	5 (0)	4.8	0.511	0.565	0.008	5 (0)	0.575	0.574	0.394
Kwm9a	19 (5)	15.7	0.874	0.898	0.514	15 (1)	0.893	0.884	0.603
Kwm12b	14 (3)	12.4	0.910	0.880	0.121	11 (0)	0.915	0.857	0.602
Mk6	18 (6)	10.9	0.757	0.761	0.102	15 (3)	0.766	0.795	0.080
Mk8	12 (2)	9.8	0.799	0.783	0.626	11 (1)	0.808	0.822	0.438
Sw10	14 (4)	10.6	0.660	0.815	0.0003	10 (0)	0.510	0.796	<0.0001
Sw19	12 (6)	8.4	0.472	0.518	0.530	7 (1)	0.447	0.418	0.889
Mean +/- s.d.	14.2 +/-5.3	11.0 +/-3.7	0.741 +/- 0.168	0.773 +/- 0.131		10.7 +/-3.8	0.736 +/- 0.177	0.767 +/- 0.151	

Table 5. Population differentiation and gene flow between groups of islands of the archipelago of the Azores and Madeira based on microsatellites: F_{ST} with level of significance based on an exact G-test of population differentiation (*: $0.01 < P < 0.05$, not significant after Bonferroni correction).

D. delphis	*n*	Central	Seamounts	Madeira
Western	25	0.003*	0.013	0.001
		[-0.002; 0.008]	[-0.004; 0.035]	[-0.003; 0.007]
Central	57	-	0.000	0.002
			[-0.014; 0.016]	[-0.001; 0.004]
Seamounts	7		-	0.014
				[-0.002; 0.031]
Madeira	56			-
S. frontalis	*n*	Central	Eastern	Madeira
Western	26	0.002	-0.003	-0.001
		[-0.003; 0.007]	[-0.012; 0.008]	[-0.006; 0.004]
Central	103	-	0.000	-0.001
			[-0.007; 0.008]	[-0.003; 0.001]
Eastern	15		-	-0.005
				[-0.010; 0.001]
Madeira	47			-