Worldwide phylogeography of rough-toothed dolphins *(Steno bredanensis)* provides evidence for subspecies delimitation

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Abstract

Rough-toothed dolphins have a global tropical and subtropical distribution with oceanic, neritic, and island-associated populations. To inform conservation and management for this species, we used sequences from the mtDNA control region (*n=360*), mitogenomes (*n=19*), and six nuclear introns (*n=35*) to provide multiple lines of evidence to critically evaluate the potential taxonomic status of rough-toothed dolphins. Using samples from the Pacific, Indian, and Atlantic oceans, we examined the null hypothesis that rough-toothed dolphins are one panmictic species and the alternate hypothesis of oceanic subspecies. Phylogenetic analyses of mitogenomes revealed a private Atlantic clade sister to a larger cosmopolitan clade including individuals from all tropical and subtropical oceans. We dated the split between the Atlantic clade and the cosmopolitan clade to 890,000 years ago. We determined that Atlantic rough-toothed dolphins could be correctly diagnosed with 98% accuracy with the mtDNA control region and calculated the net nucleotide divergence as 0.02. Population level analyses revealed significant genetic differentiation using mtDNA among most regions, while significant differentiation using nuclear markers occurred only between the Atlantic and the Indian/Pacific regions. Therefore, the oceanic divergence and diagnosability of rough-toothed dolphins in the Atlantic and the Indian/Pacific oceans meet proposed criteria for recognition as two subspecies.

KEY WORDS: biogeography, diagnosability, intron, mitogenome, mtDNA, phylogeography, rough-toothed dolphin, subspecies

1 INTRODUCTION

Divergence of species is often associated with biogeographic events that generate barriers between or among populations (Briggs and Bowen, 2012). Even in the marine environment where barriers are not as obvious, they are still responsible for differentiation and limited gene flow leading to speciation (Daly-Engel et al., 2012; Rocha et al., 2007). Some of these barriers include the closing of seaways, most notably the Isthmus of Panama, and the shallow restriction through southeast Asia in the Indo-Pacific (Steeman et al., 2009). Less visible barriers include ocean temperatures and currents. The southern margin of South America represents a thermal barrier for tropical species restricted to ocean temperatures greater than 21^{o}C, limiting their latitude range to about 37^{o}S (Daly-Engel et al., 2012; Rocha et al., 2007; West et al., 2011) and thus their ability to disperse around the continent. Even the vast open ocean distances between the eastern tropical Pacific (ETP) and the islands of the central Pacific form the eastern Pacific barrier, representing a barrier for many fish species due to lack of habitat for recruitment and the low probability of migrants encountering mates after crossing (Lessios and Robertson, 2006).

Biogeographic provinces specific to cetaceans were first discussed by Davies (1963) who hypothesized that the Isthmus of Panama, the eastern portion of the Pacific Ocean basin, and the continent of Africa were significant barriers to marine species and could thus drive speciation. For tropical cetacean dispersal, Davies (1963) suggested an Indo-western Pacific Core with offshoots that continue to the west into the Atlantic and to the east into the ETP. These biogeographic patterns have been found to be concordant with phylogeographic structure of circumglobally distributed species of cetaceans (Leslie and Morin, 2018), reef fish (Rocha et al., 2007), pelagic fish (Bowen et al., 2016), and sharks (Cardeñosa et al., 2020; Daly-Engel et al., 2012).

Among cetaceans with worldwide distributions, the combination of factors limiting gene flow are complex, driven by processes as varied as behavioral specializations, historical environmental changes, and biogeographic barriers (Hoelzel, 1998; Steeman et al., 2009).

Significant genetic differentiation between geographic regions and/or coinciding with biogeographic boundaries has been previously found for several dolphin species complexes with worldwide distributions, leading to the description of multiple subspecies and species designations (Leslie and Morin, 2018; Morin and et al., 2010; Natoli et al., 2006; Natoli et al., 2003; Tezanos-Pinto et al., 2009).

The Agulhas Current and the fluctuating temperature around Cape Agulhas at the southernmost tip of Africa present a biogeographic barrier for circumtropical species and is aptly named the South African Species Gate (Perrin, 2007). Due to the strong Agulhas Current running southwest from the Indian Ocean into the South Atlantic Ocean, and the prevailing Benguela Current flowing north along the west coast of Africa, a dolphin from the South Atlantic Ocean would need to travel several thousand kilometers against currents to cross from the Atlantic into the Indian Ocean. This barrier is thought to have isolated several lineages of cetaceans (Perrin 2007): the humpback dolphin on the Atlantic coast of South Africa (*Sousa teuszii*) and its sister taxa on the Indian coast of South Africa (*Sousa plumbea*); and the Atlantic spotted dolphin (*Stenella frontalis)* found only in the North and South Atlantic Oceans.

Although there has been a concerted focus on how and when to recognize new species of cetaceans (Reeves et al., 2004), less attention has been given to criteria for delimiting subspecies. Part of the challenge is due to the issue of subspecies experiencing ongoing gene flow, making it imperative to establish operational thresholds (Taylor et al., 2017a). A workshop on cetacean taxonomy in 2003 provided new definitions and criteria for species and subspecies and emphasized concordance across sequence characters within a locus, multiple genetic markers (nuclear and mitochondrial DNA), biogeographic regions, and morphology (Reeves et al., 2004). The criteria that came out of this workshop have been used for diagnosis of several new cetacean species (Caballero et al., 2007; Dalebout et al., 2002) and subspecies (Archer et al., 2013; Jackson et al., 2014; Morin and et al., 2010).

Since the workshop the criteria for subspecies have been further refined to include evolutionary divergence and diagnosability using mitochondrial DNA (mtDNA) markers. Although this maternal marker cannot measure male-mediated gene flow, some gene flow is assumed to occur between subspecies, therefore the marker is considered appropriate (Taylor et al., 2017a). Moreover, corroboration from additional independent markers or other lines of evidence can provide additional justification for the use of mtDNA (Martien et al., 2017a). Evolutionary divergence can be measured using net nucleotide divergence, d_A (Nei, 1987; Tamura and Nei, 1993), of mitochondrial markers between two populations correcting for within-population genetic diversity (Rosel et al., 2017b). An empirical analysis of recognized subspecies by Rosel et al. (2017a) established a subspecies threshold for d_A of 0.002 – 0.04 for the mtDNA control region. Populations bracket the lower taxonomic level with values of d_A below 0.002, while species have values greater than 0.04. Diagnosability is defined by Archer et al. (2017) as "a measure of the ability to correctly determine the taxon of a specimen of unknown origin based on a set of distinguishing characteristics." The subspecies threshold recommended by Archer et al. (2017) and further supported in Rosel et al. (2017b) for diagnosability is 80%-90%. However, Taylor et al. (2017a) argue that a one in five chance of misidentifying an individual to subspecies is too high and instead suggest using 95% diagnosability for consistency with what would be acceptable in morphological studies.

Rough-toothed dolphins (*Steno bredanensis*) have a worldwide tropical and subtropical distribution in the North and South Atlantic, North and South Pacific, and Indian Oceans, as well as in the Mediterranean, Caribbean, and Red seas, and the gulfs of Mexico and Oman (Jefferson, 2008; Notarbartolo di Sciara et al., 2017; Watkins et al., 1987). Rough-toothed dolphins are one of the few delphinids with a worldwide distribution that has not been subject to an extensive taxonomic review by either genetic or morphological analyses. Although they are considered an oceanic species (e.g., observed from large ship surveys in the Indian Ocean, Gulf of Mexico, ETP, and

offshore Hawaiian waters) (Ballance and Pitman, 1998; Bradford et al., 2017), they are also observed around oceanic islands in the North Atlantic, North and South Pacific, Caribbean, and Mediterranean, and in depths of less than 20 m off the coasts of Japan, Brazil, Mauritania, and the Canary Islands (Baird et al., 2008; Carvalho et al., 2021; da Silva et al., 2015; Jefferson, 2008; Kerem et al., 2016; Mignucci-Giannoni, 1998; Poole, 1993; Ritter, 2005). The genus *Steno* is monotypic and there are no subspecies currently recognized by the Society of Marine Mammalogy's Committee on Taxonomy (Committee on Taxonomy 2021), nor by the International Union for Conservation of Nature (IUCN). As a worldwide species, rough-toothed dolphins are listed by the IUCN as Least Concern (Kiszka et al., 2019). Despite this listing, mass stranding events off the United States Eastern Seaboard and coasts of Hawai'i (Ewing et al., 2020; Mazzuca et al., 1999; Nitta and Henderson, 1993), Senegal (Cadenat, 1949), and elsewhere, as well as fishery interactions around the Hawaiian, Society, and Samoan archipelagos and off the coast of Brazil (Baird, 2016; Di Benediito et al., 2001; Monteiro-Neto et al., 2000; Nitta and Henderson, 1993) and elsewhere continue to be documented, potentially resulting in higher impacts to these populations than is currently known. Furthermore, off the coast of Brazil where rough-toothed dolphins inhabit neritic waters, additional anthropogenic impacts including plastic ingestion and organochlorine compound accumulation are a concern (da Silva et al., 2015; Lailson-Brito et al., 2012; Lemos et al., 2013). Previous studies on rough-toothed dolphins identified significant genetic differentiation among island groups in the North and South Pacific (Albertson et al., 2017; Oremus et al., 2012) and subpopulations in the western North and South Atlantic (Carvalho et al., 2021; da Silva et al., 2015; Donato et al., 2019) as well as social organization and site fidelity differences within various island groups in the Pacific and North Atlantic Oceans (Albertson, 2014; Baird et al., 2008; Oremus et al., 2012; Ritter, 2005). A total from compiled abundance estimates is 221,186, but as noted by Kiszka et al. (2019), this is underestimated as several large parts of their range have not been surveyed. One survey conducted from 1986 to 1990 (Wade and Gerrodette, 1993) estimated 145,900

(CV=0.32) in the ETP based on shipboard line-transect surveys. A more recent survey completed of the Hawaiian Islands estimated the abundance of rough-toothed dolphins to be 76,375 (CV=0.41) (Bradford et al., 2021).

Here we describe the worldwide phylogeography of rough-toothed dolphins. Specifically, we quantify the genetic diversity and differentiation of rough-toothed dolphins at multiple hierarchical levels, including explicitly testing for evidence of subspecies based on delimitation criteria proposed by Taylor et al. (2017a). Our study evaluates the concordance between genetic isolation and oceanographic regions and includes both mitochondrial and nuclear markers across a comprehensive geographic area spanning three ocean basins. As with other studies of widely distributed species (e.g., Dalebout et al. 2005), access to samples for genetic analyses was a limiting factor. To help compensate for this limitation, we assessed phylogeography and delimitation at two levels: 1) broad but shallow: using many samples (both oceanic and neritic) across the globe analyzed using one marker (319 bp of the mtDNA control region); and 2) deep but narrow: using a subset of these samples further analyzed using concatenated protein-coding genes of the mitogenome and six nuclear introns.

2 METHODS

2.1 Sample collection

Tissue samples from rough-toothed dolphins (*n*= 336, Figure 1, Table S1) were collected by several collaborators from different sources. Samples from the Hawaiian Islands, Society Islands of French Polynesia and Samoa (near the island of Savai'i) were obtained using a modified veterinary capture rifle and biopsy dart (Krutzen et al., 2002) or a crossbow and arrow biopsy system (Lambertsen, 1987). Skin samples were obtained from fishery bycatch around American Samoa (island of Tutuila) and from mass stranding events around western Florida and the western North Atlantic Ocean, as well as the coast of Oman. The Caribbean samples were collected from individually

stranded dolphins around Puerto Rico. Samples were preserved either frozen at –80°C, or preserved in a 70% ethanol or a 20% salt-saturated DMSO (dimethyl sulfoxide) solution. Samples in ethanol or DMSO were also stored at –80°C.

Teeth samples (*n*=43, Figure 1, Table S1) were obtained in collaboration with the Smithsonian Institution in the U. S., the Port Elizabeth Museum and Oceanarium at Bayworld in South Africa, and the Museum of New Zealand Te Papa Tongarewa. The Smithsonian samples were collected from mass strandings in the western North Atlantic Ocean near Florida and North Carolina and in the North Pacific Ocean from Maui, Hawai'i, as well as from fishery bycatch in the ETP and near Isla Gorgona, Colombia. Bayworld and Te Papa Tongarewa samples were collected from individually stranded dolphins on the southeast coast of South Africa and the east side of the North Island of New Zealand respectively.

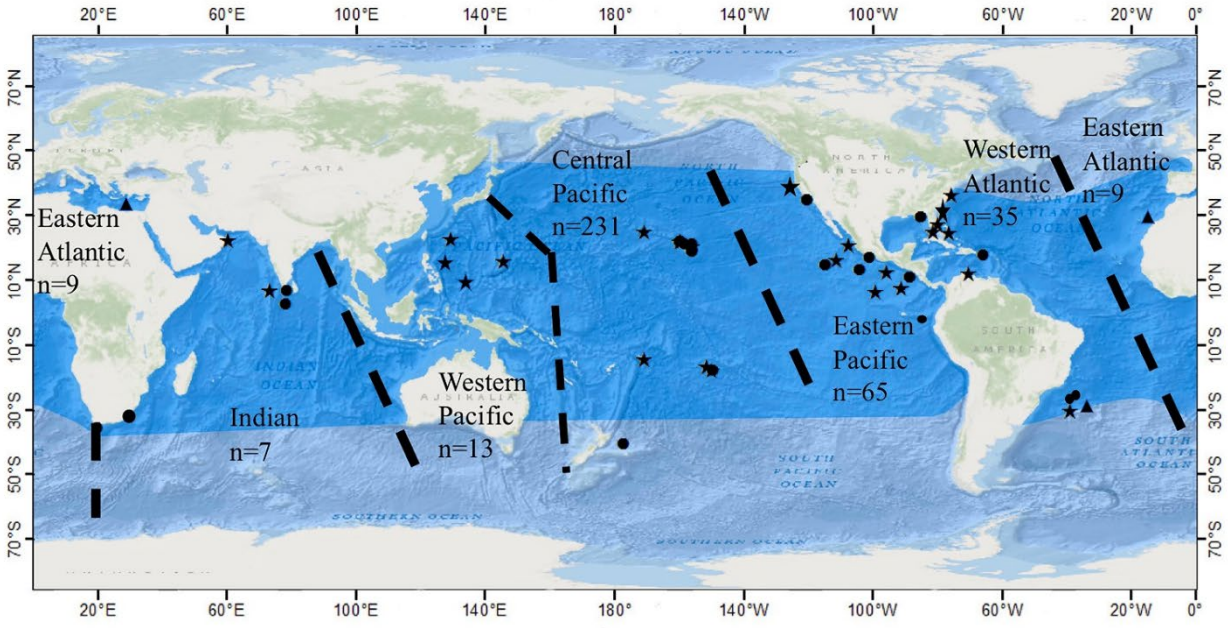

Figure 1. Sampling regions for the worldwide mtDNA data set of the rough-toothed dolphin (*Steno bredanensis*). The boundaries were designated relative to the biogeographic barriers described by Rocha et al. (2007). Relevant boundaries from that study are shown in dashed lines. The mtDNA control region data set (319 bp) was evaluated using the six regions shown here (Indian, Western Pacific, Central Pacific, Eastern Pacific, Western Atlantic, and Eastern Atlantic) as well as the combined biogeographic regions: Atlantic, Indian/Western Pacific, Central/Eastern Pacific. The intron data set was evaluated using the three broader regions only and the mitogenome data set was evaluated using the Atlantic and Indian/Pacific regions only due to limited sample size.

198 Locations of sample collection are shown as circles (teeth and tissue) and triangles (sequences) for
199 the control region only (319 bp) and stars for the mitogenome/intron data sets. Brighter blue
200 shading between approximately 40 degrees S and 50 degrees N represents habitat range of the
201 species. See Methods section for details on sample and sequence collection. Ocean Basemap
202 (http://esriurl.com/obm).

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205 **2.2 Sample location delineations**

206 We divided our sample locations into three biogeographic regions; Indian and western
207 Pacific (Indian/West Pacific), central and eastern Pacific (Central/East Pacific), and North and
208 South Atlantic (Atlantic), and further into six subregions following Bowen et al. (2016) (Figure 1).
209 The delineation of these regions aligns with biogeographic barriers defined in previous studies
210 (Briggs and Bowen, 2012; Cardeñosa et al., 2020; Rocha et al., 2007), as well as constraints from
211 the number of samples and sample locations. The Indian/West Pacific region is represented by
212 individuals sampled in the western tropical Indian Ocean, Oman, Maldives, and Sri Lanka, in
213 addition to Japan, Taiwan and the Mariana Archipelago in the western North Pacific Ocean. The
214 Central/East Pacific region is represented by individuals sampled in the northwestern and main
215 Hawaiian Islands (including up to 370 km offshore), Society and Samoan Islands, and New Zealand.
216 These central Pacific samples were combined with individuals from the eastern Pacific including
217 the ETP and nearshore along North, Central, and South America. The Atlantic region is represented
218 by individuals sampled in the western South Atlantic near Brazil and in the western North Atlantic
219 near the eastern coast of the U.S. (Florida to Virginia), Caribbean islands (Grand Bahama, Aruba,
220 Puerto Rico), and the Gulf of Mexico (Table S1). Samples also included sequences from the eastern
221 North Atlantic (Canary Islands, n=6), and the Mediterranean Sea (n=3). For additional
222 phylogeographic comparisons and to define haplotypes shared among populations, we also
223 included available mtDNA sequences originating from French Polynesia (Oremus et al. 2012), the
224 Hawaiian Islands (Albertson et al. 2017), and the western South Atlantic (Cunha et al. 2011). We

were unable to use four GenBank sequences (accession numbers KM260653 – KM260657, from Da Silva et al. 2015) from the South Atlantic due to incomplete overlap with the mtDNA sequences used here.

2.3 DNA Extraction and mtDNA amplification

Total DNA was extracted from skin and tissue samples using either a Qiagen DNeasy Blood and Tissue Kit or a standard phenol:chloroform extraction protocol (Sambrook et al., 1989), modified for small samples (Baker et al., 1994). A negative control, or blank, was included in each batch of extractions and amplifications to ensure the extraction was free from detectable contamination. DNA was quantified with pico-green fluorescence and normalized to 15 ng/µl. An 800 bp fragment of the 5' end of the mtDNA control region (CR) was amplified using the primers Dlp1.5 and Dlp8 (Baker et al., 1998; Dalebout et al., 2004) and Polymerase Chain Reaction (PCR) conditions as described in Oremus et al. (2007).

The DNA extracted from teeth followed standard protocols for ancient DNA extraction (Pimper et al., 2009). Total DNA was extracted from teeth samples in a lab separate from modern cetacean DNA. A laminar flow chamber and UV radiation were used to provide sterile surface conditions and minimize the risk of contamination. Reagents were made up in a "DNA-free" positive pressure room separate from other laboratories. Teeth were submerged in liquid nitrogen for 20 s and then crushed with a sterilized hammer. The resulting powder was subsampled and stored in a -20°C freezer. DNA was extracted from 0.1 g of tooth powder beginning with a protein digestion with 200 µl of 10% SDS, 100 µl DTT (10mg/ml) and 100 µl Proteinase K (20 mg/ml) and incubated at 37°C overnight, followed by one hour at 50°C. Samples were then centrifuged, and the rest of the extraction procedure followed Pimper et al. (2009), including silica suspension (Boom et al., 1990). A negative control was run every fifth sample, and a maximum of eight samples and two blanks

were extracted at one time (batch). A 450 bp region of the mitochondrial DNA control region was amplified via PCR in a 25 µl reaction using primers M13Dlp1.5 and Dlp5 (Dalebout et al., 1998), 1 U (1mg/ml) Bovine Serum Albumin (BSA) and 5 µl of DNA template as described in Pimper et al. (2009). This was followed by a semi-nested amplification using 3 µl of a 1:10 dilution of the first reaction using the primers Dlp1.5 and Dlp4 (Dalebout et al., 2004) under the same conditions, except no BSA was added.

2.4 Nuclear intron amplification

Nuclear introns are noncoding regions from nuclear DNA shown to be useful in taxonomic studies of dolphins (Caballero et al. 2007) and whales (Gaines et al., 2005). Six nuclear short-range (<1,500 bp) introns (Actin-1, CAT, CHRNA, GBA, IFN and sex marker DBY7; references provided in Table S2) were amplified for higher quality tissue samples using PCR conditions following Caballero et al. (2007) with a negative control included with each batch of 15 samples. Each reaction consisted of 15-20 ng of DNA, 1 × Platinum Taq buffer (Invitrogen), 0.4 µM each primer, 20 mM dNTPs, 1 U Platinum Taq polymerase, and 1 U of BSA to reduce inhibition of PCR, with a final volume of 20 µl. For Actin-1, 1.5 mM $MgCl_2$ was used. For all other introns, 2.0 mM of $MgCl_2$ was used. Reactions were carried out in a 25 µl final volume. For Actin-1, CAT, GBA and IFN-1, the temperature profile consisted of an initial denaturing step of 3 min at 94°C, followed by 35 cycles of 94°C for 30 s, 55°C for 45 s and 72°C for 30 s followed by an extension at 72°C for 10 min. For CHRNA1 and DBY7, touchdown temperature protocols were used. CHRNA1 had an initial denaturation at 94°C for 2 min, followed by 10 cycles at 94°C for 20 s, 64°C-55°C (decreased by 1°C per cycle) for 20 s, and 72°C for 40 s. This touchdown was followed by 30 cycles at 94°C for 20 s, 55°C for 20 s and 72°C for 40 s. DBY7 started with a denaturation at 94°C for 2 min, followed by 20 cycles at 94°C for 30 s, 60°C -50°C (decreased by 0.5°C per cycle) for 1 min and 72°C for 1.5 min. This was followed by 10

cycles at 94°C for 30 s, 55°C for 1 min and 72°C for 1.5 min. A final extension at 72°C for 10 min was performed for both touchdown reactions.

2.5 Sanger sequencing

PCR products were purified in preparation for Sanger sequencing with SAPEX (Amersham). The sequencing reaction was carried out with BigDye v3.1 (Applied Biosystems, Inc.) with post-sequencing reaction clean-up using Agencourt CleanSEQ Kit (Beckman Coulter). Products were then run on an ABI 3730 Genetic Analyzer (Applied Biosystems, Inc.). Sequences were aligned and quality control carried out using Sequencher 4.6 (Gene Codes Corporation). MtDNA sequences were sequenced in the forward direction and trimmed to a length of 319 bp of the control region. As quality control, sequences were required to have a minimum average *Phred* score of >30 (Ewing et al., 1998), and were re-sequenced if they fell below this threshold. If they failed again, they were removed from the data set. In addition, any variable sites with *Phred* <40 were visually confirmed. If a haplotype was represented by only one sample, the identity of the haplotype was confirmed by sequencing in both directions. Variable sites and unique haplotypes were identified using Sequencher 4.6 and then MacClade, Version 4.0 (Maddison and Maddison, 2000).

Nuclear introns were sequenced for each individual in both the forward and reverse direction, to ensure the sequencing of the entire fragment, using the same primers as for PCR amplification. Potential heterozygote sites were identified using a 25% secondary peak threshold in Sequencher, followed by visual confirmation (Hare and Palumbi, 1999). Heterozygote sites were considered valid if a decline in *Phred* score values at a specific site was observed, accompanied by a secondary peak with a height ≥ 30% of the height of the primary peak (Lento et al., 2003). After identifying heterozygote sites, introns were phased using *Phase v2.1.1.* (Stephens et al., 2001). Similar to Caballero et al. (2007), the resulting alleles were concatenated, combining the sequences

of every gene fragment for each individual in MacClade (Maddison and Maddison 2000). This approach has been used successfully (Caballero et al. 2007, Weisrock et al. 2012) and simulation studies found this concatenated approach yielded accurate results (Gadagkar et al. 2005).

2.6 Long-range amplification and Illumina MiSeq sequencing of mitogenomes

We attempted to generate mitogenome sequences from a subset of 24 individuals available from the Pacific (n=12), Atlantic (n=8), and Indian (n=4) Oceans via long-range PCR and Illumina MiSeq sequencing. Samples for the mitogenome analysis were chosen based on DNA quality and sample locality. Mitogenomes were amplified using nine overlapping long-range fragments ranging in size from 1,473 to 3,874 bp (Table S3) adapted from Alexander et al. (2013). PCR reactions consisted of 0.2 U High Fidelity Phusion® Polymerase (New England Biolabs, USA), 1 × Phusion HF (1.5mM $MgCl_2$) Buffer (NEB, USA); 0.5 µM of each primer; 2% DMSO (NEB, USA); 15-30 ng of template DNA, 20 mM dNTP (Promega, USA) and 1 U BSA with a final volume of 20 µl. Thermocycle profiles began with an initial denaturation of 98°C for 30 sec, followed by 35 cycles of 98 °C for 8 s, T_A for 30 s (as specified in Table S3) and 72°C for 1min 15 s, followed by a final extension of 72 °C for 10 min. Further details are provided for each fragment in Table S3.

PCR fragments were combined in an equimolar fashion for each individual. Excess primers and nucleotides were removed using a Qiagen QIAQuick PCR and gel purification kit (Qiagen). Products were individually barcoded and prepared for sequencing using a Nextera XT DNA Sample Preparation Kit (Illumina). Individuals were pooled and sequenced on three Illumina MiSeq runs (two at 250 bp paired end, one at 75 bp paired end). Reads were trimmed to remove poor quality sequence and adaptor sequence using default settings in *Trim Galore! v0.2.8* (Babranham Bioinformatics 2013), and then assembled to a rough-toothed dolphin mitogenome reference (GenBank Accession no. JF339982.1; Vilstrup et al., 2011) using *BWA v0.7.4* (Li and Durbin, 2009). The consensus sequence from the BWA assembly was obtained with *Samtools v0.1.19* (Li et al.,

2009). For quality control purposes, any putatively variable site across individuals with a read depth <10 was resequenced using Sanger sequencing and verified for the correct base. In addition, base calls supported by fewer than 70% of reads were reviewed for possible heteroplasmy/indels/pseudogene incorporation, following Alexander et al. (2013).

Each assembled mitogenome was examined for nuclear mitochondrial DNA (numt) pseudogenes by ensuring overlap in fragments and a lack of frameshift/premature stop codon coding sequence in the protein-coding region. We used the concatenated protein-coding regions (Figure S1) in downstream analysis excluding ND6 due to its location on the opposing strand and therefore potential for distinct patterns of evolution (Alexander et al., 2013; Ho and Lanfear, 2010). For each individual, overlapping regions of protein-coding genes in *GENEIOUS* (Biomatters LTD) were represented in the concatenated data set only once. The start of the first codon position for each gene was identified in *GENEIOUS* and then verified in *MEGA X* (Kumar et al., 2018). Saturation of the third codon position was evaluated with *DAMBE* (Xia, 2013) in order to assess the accuracy of our estimates of sequence divergence.

2.7 Mitochondrial DNA phylogenies and estimation of divergence time

A maximum likelihood (ML) phylogeny of the 319 bp mtDNA CR data set was reconstructed in RAxML (Stamatakis, 2014) using the Cyberinfrastructure for Phylogenetic Research (CIPRES) Portal Gateway (Miller et al., 2010) and the GTR + GAMMA substitution model following Abadi et al. (2019). The heuristic search conditions for ML used starting trees obtained by stepwise addition with ten random sequence addition replicates and tree-bisection-reconnection branch swapping. We used rapid bootstrapping and 1,000 iterations. The tree was rooted to *Orcinus orca,* as a representative taxon located outside of the subfamily.

To date phylogeographic events through a molecular clock analysis, a Bayesian phylogeny was reconstructed using the protein-coding mitogenomes (hereafter referred to as mitogenomes) in *BEAST v1.7* (Bouckaert et al., 2014) rooted to *Orcaella brevirostris*, a proposed subfamily taxa (Caballero et al 2008, McGowen 2011), and *Orcinus orca* as an outgroup outside the subfamily. To determine a specific substitution rate for rough-toothed dolphins we first reconstructed the phylogenetic relationship for 46 cetacean species, including a randomly chosen rough-toothed dolphin sequence from this study (Accession number OL461802), using the fossil calibrations and a minimum age constraint for Delphinoidea discussed in Steeman et al. (2009) (e.g., Table 2, Crown group Delphinoidea; minimum constraint 10.0; age 11-10 Ma). The second phylogenetic reconstruction used the substitution rate derived in the first analysis specific for rough-toothed dolphins with the rough-toothed dolphin mitogenome only.

Two independent chains were generated in *BEAST v1.7* for each analysis using a burn-in period of 100,000 and 90,000,000 Markov Chain Monte Carlo (MCMC) steps. The multispecies analysis used the parameters discussed in Alexander et al. (2013) Supplementary Material 6A, including different site models for each of the three partitions (codon position concatenated across the protein-coding genes), an uncorrelated lognormal relaxed clock, and a linked Yule tree prior across the partitions. For each partition we used the nucleotide substitution model GTR, as supported by jModeltest2 (Darriba et al., 2012). In the second analysis specific to rough-toothed dolphins, a strict molecular clock was used (with the rough-toothed dolphin specific rate of 0.009776 substitutions per site per Myr established in the first analysis), since lineages within a species are not expected to show rate variation (Ho and Lanfear, 2010).

For each analysis, log files generated from each of the two runs were evaluated for convergence using *Tracer v1.6* (Rambaut et al., 2018). A combined log and combined tree file (across the two runs) were produced using *LogCombiner.* Following the confirmation that each

parameter had an Effective Sample Size (ESS) of >500 in *Tracer v1.6,* a maximum clade credibility tree was produced in *TreeAnnotator* file and visualized in *Figtree v1.4.4* (Rambaut, 2018).

The three ocean basins (Indian, Pacific, and Atlantic) were traced on the mitogenome phylogeny as an ancestral history using *Mesquite v3.01* (Maddison and Maddison, 2000). We used a likelihood calculation and a likelihood reconstruction to estimate ancestral states.

2.8 Genetic diversity and population structure

Standard measures of population structure and genetic diversity were estimated among the three broad sampling regions (Indian/West Pacific, Central/East Pacific, and Atlantic) for the mitogenome and the mtDNA CR data sets. Haplotype diversity, number of alleles, proportion of variable sites, and nucleotide diversity were calculated in *Arlequin* v3.5 (Excoffier and Lischer, 2010). As mentioned above, we used the program jModelTest2 (Darriba et al. 2012) to select the model of nucleotide substitution that best fit our data for both data sets. Pairwise Φ_{ST} estimates of differentiation were measured between each pair of the broad sampling regions and the finer-scale subregions for the mtDNA CR data set. Due to limited sample size in the mitogenome data set, only the Atlantic and the Indian/Pacific Ocean sampling regions were compared. All Φ_{ST} estimates were conducted using 50,000 permutations in *Arlequin* v3.5. Differentiation was measured by Φ_{ST} rather than traditional F_{ST} because the former includes scaling of nucleotide distances (Meirmans and Hedrick 2011). We also calculated Nei's net nucleotide divergence d_A (Nei, 1987) for the mtDNA CR data set using the equation

$$(d_A = d_{XY} - (d_X + d_Y)/2)$$

where d_{XY} is the average genetic distance between regions X and Y, and d_X and d_Y are the mean within region genetic distances. This net nucleotide divergence was calculated as a metric for assessing subspecies status (Taylor et al., 2017a) using *MEGA X* (Kumar et al., 2018) with the Tamura-Nei substitution model (Tamura and Nei, 1993). A median-joining haplotype network was

constructed using the mtDNA CR data set in Population Analysis with Reticulate Trees (PopART) (Bandelt et al., 1999) using the default settings.

For the intron data set, we used only the three broader sampling regions (Atlantic, Indian/West Pacific, and Central/East Pacific) to investigate whether patterns found in the mtDNA data sets were also found for nuclear DNA. F_{ST} and G''_{ST} were estimated for phased alleles in Genodive (Meirmans and Van Tienderen, 2004). For each locus, observed heterozygosity was calculated on a per-locus individual basis, dividing the total number of sampled heterozygote individuals by the total number of individuals sequenced.

2.9 Diagnosability

Following the methods described in Archer et al. (2017), we estimated the diagnosability of the mtDNA CR data set with a Random Forest model as implemented in the *randomForest* package in R (Liaw and Wiener, 2002). The model was initially constructed to classify the three *a-priori* designated biogeographic regions (Atlantic, Indian/West Pacific, and Central/East Pacific) using the individual base pairs for each variable site in the mtDNA CR sequence as independent predictors. However, in this initial model, we found only weak evidence for differentiation between the Indian and Pacific Oceans, so for the purpose of evaluating possible subspecies delimitation for the Atlantic, we combined the samples from the Indian and Pacific Oceans samples.

Given that this was a two-strata model, individuals were assigned to the stratum for which more than 50% of the trees voted for them. The percent of individuals diagnosable (correctly assigned) is thus referred to as PD_{50} (Archer et al., 2017). A total of 10,000 trees were created for the forest. To avoid classification bias due to uneven sample sizes, the number of samples selected to build each tree in the forest was set to half of the smallest sample size in both strata (Archer et al., 2017; Berk, 2006). Samples for each tree were randomly selected without replacement. All other *randomForest* parameters were left at their default settings. In order to apply the guidelines for

423 species/subspecies delineation using diagnosability (Archer et al., 2017), the class-specific correct
424 classification estimate is reported. Central 95% confidence intervals for PD_{50} were calculated using
425 a binomial distribution.
426
427 **3 RESULTS**
428 The availability of the mtDNA CR, mitogenomes, and nuclear loci for rough-toothed dolphins varied
429 across the three broad oceanic regions, Atlantic, Indian/West Pacific, and Central/East Pacific due
430 to sample quality. A total of 360 individuals (n=324 tissue and n=36 teeth) were sequenced
431 successfully for 319 bp of mtDNA CR. Of these, 35 individuals were sequenced for the six nuclear
432 loci with a total combined length of 2,510 bp (Table 1). The protein-coding regions of the
433 mitochondrial genome (length 10,810 bp) were concatenated for a subset of the individuals used
434 for the intron and 319 bp CR data sets representing the three oceanic regions. Of the 24 individuals
435 for which we attempted to generate mitogenome sequences (all of which were tissue samples), 19
436 were successful (Table S4). These 19 had an average mapping quality exceeding 35 (BWA: PHRED
437 quality) and the median number of missing bases in a sequence was 12. The five sequences of the
438 mitogenome that did not meet these criteria (mapping quality below 20) were considered poor
439 quality and were deleted from the data set.

Table 1. Basic diversity estimates of nuclear and mtDNA sequences of rough-toothed dolphins. DBY-7 is not shown (no variation across samples). 'Intron Allele Total' shows number the of alleles summed over all concatenated introns. Parentheses represent private alleles to each ocean region. For the mtDNA data sets the numbers in the eighth and ninth columns represent the number of haplotypes with the number of haplotypes unique to that region in parentheses. The number of individuals with data for each region is given by 'n'. MtDNA mitogenome refers to the concatenated mtDNA protein-coding gene sequences (10,810 bp), and mtDNA CR refers to the mtDNA control region sequence (319 bp). Observed heterozygosity for introns and haplotype diversity for mtDNA sequences are reported in the Observed heterozygosity row along with the standard deviation in parentheses.

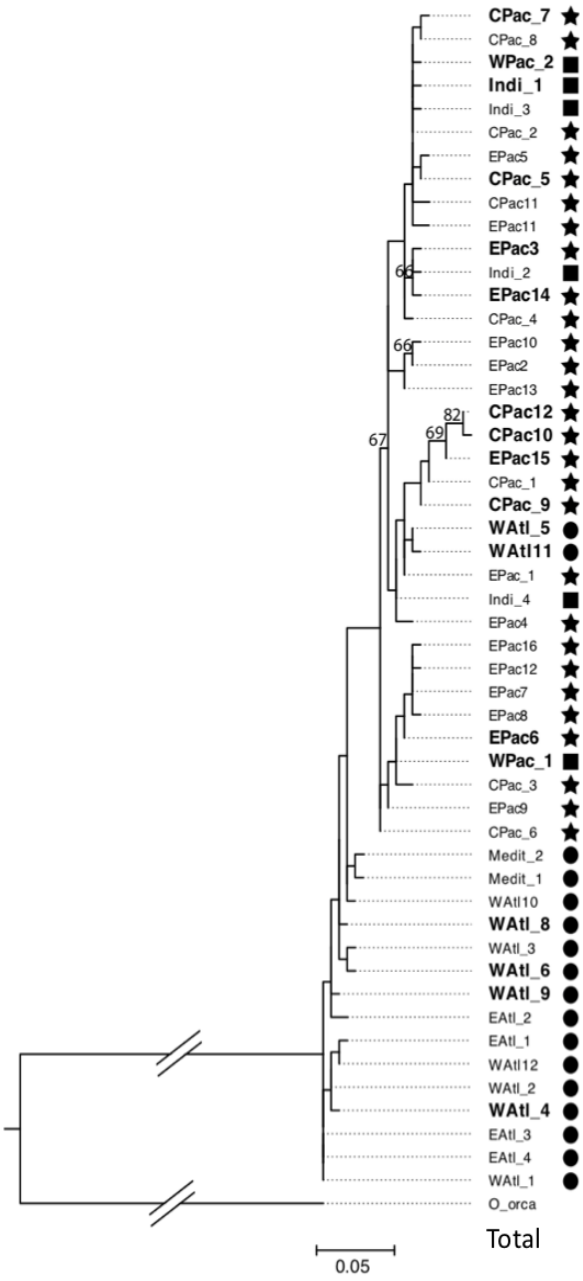
	ACT-1	CAT	GBA	CHRNA1	IFN1	Intron Allele Total	mtDNA Mitogenome [a]	mtDNA CR 319 bp [a]
Length (bp)	980	520	310	360	340	2,510	10,810	319
No. of individuals	32	35	34	35	35	35	19	360
No. of variable sites	8	2	1	1	4	16	386	27
Atlantic Intron n=10 Mitogenome n=7 mtDNA CR n=44	5(4)	1(1)	1(1)	1(1)	5(1)	13(13)	7(7)	17(17)
Indian/ West Pacific Intron n=7 Mitogenome n=3 mtDNA CR=20	4(1)	3(0)	1(0)	1(0)	5(0)	12(1)	3(3)	5(4)
Central/ East Pacific Intron n=18 Mitogenome n=9 mtDNA CR=296	7(2)	3(0)	1(1)	2(1)	8(0)	21(4)	9(9)	29(25)
Observed Heterozygosity	0.400 (0.082)	0.200 (0.072)	0.028 (0.033)	0.057 (0.133)	0.911 0.147)	0.155 (0.148)	1 (0.0006)	0.942 (0.0041)

π	0.0041 (0.0019)	0.0028 (0.0016)	0.0002 (0.0001)	0.0001 (0.0001)	0.0022 (0.0009)	0.0036 (0.0017)	0.0126 (0.0094)	0.0165 (0.0104)

447

448 **3.1 Mitochondrial DNA phylogenies and divergence time**

449 Within the mtDNA CR, we identified 51 haplotypes (Figure 2) and 27 variable sites (Table 1) across
450 the 360 individuals sequenced (Table S4). The Indian/West Pacific and Central/East Pacific regions
451 shared five haplotypes (Figure 2). No haplotypes identified in the Atlantic were shared with
452 another region. However, there were no fixed substitutions unique to the Atlantic or any other
453 region. Within the Atlantic region there was one haplotype shared between the western Atlantic
454 and eastern Atlantic. The phylogenetic tree identified a paraphyletic Atlantic group (Figure 2).
455 Within this larger Atlantic group, a small clade made up of two sequences from the Mediterranean
456 Sea (Medit_1 and Medit_2) was clearly clustered with the Atlantic sequences. We found no further
457 segregation of the Atlantic Ocean. A notable feature in the tree is the two Atlantic haplotypes
458 (sequences WAtl_5 and WAtl11 in Figure 2), collected from both the North and South Atlantic, that
459 were nested within a clade containing haplotypes from Indian/West Pacific and Central/East
460 Pacific regions.



	Western Atlantic	Eastern Atlantic	Indian	Western Pacific	Central Pacific	Eastern Pacific
CPac_7 ★	0	0	0	0	32	0
CPac_8 ★	0	0	0	0	1	0
WPac_2 ■	0	0	0	2	32	0
Indi_1 ■	0	0	2	0	0	0
Indi_3 ■	0	0	1	1	0	0
CPac_2 ★	0	0	2	2	8	0
EPac5 ★	0	0	0	0	0	1
CPac_5 ★	0	0	0	0	37	0
CPac11 ★	0	0	0	0	2	0
EPac11 ★	0	0	0	0	0	2
EPac3 ★	0	0	0	2	1	3
Indi_2 ■	0	0	1	0	0	0
EPac14 ★	0	0	0	0	2	2
CPac_4 ★	0	0	0	0	14	0
EPac10 ★	0	0	0	0	0	3
EPac2 ★	0	0	0	0	11	1
EPac13 ★	0	0	0	0	2	4
CPac12 ★	0	0	0	3	35	1
CPac10 ★	0	0	0	0	16	11
EPac15 ★	0	0	0	0	1	2
CPac_1 ★	0	0	0	0	2	0
CPac_9 ★	0	0	0	0	29	1
WAtl_5 ●	1	0	0	0	0	0
WAtl11 ●	2	0	0	0	0	0
EPac_1 ★	0	0	0	0	0	1
Indi_4 ■	0	0	1	0	0	0
EPao4 ★	0	0	0	0	0	4
EPac16 ★	0	0	0	0	0	1
EPac12 ★	0	0	0	2	0	12
EPac7 ★	0	0	0	0	0	4
EPac8 ★	0	0	0	0	0	4
EPac6 ★	0	0	0	0	0	2
WPac_1 ■	0	0	0	1	0	0
CPac_3 ★	0	0	0	0	0	4
EPac9 ★	0	0	0	0	0	2
CPac_6 ★	0	0	0	0	6	0
Medit_2 ●	0	2	0	0	0	0
Medit_1 ●	0	1	0	0	0	0
WAtl10 ●	1	0	0	0	0	0
WAtl_8 ●	2	0	0	0	0	0
WAtl_3 ●	5	0	0	0	0	0
WAtl_6 ●	1	0	0	0	0	0
WAtl_9 ●	15	0	0	0	0	0
EAtl_2 ●	0	2	0	0	0	0
EAtl_1 ●	0	1	0	0	0	0
WAtl12 ●	1	0	0	0	0	0
WAtl_2 ●	2	0	0	0	0	0
WAtl_4 ●	3	0	0	0	0	0
EAtl_3 ●	0	1	0	0	0	0
EAtl_4 ●	0	1	0	0	0	0
WAtl_1 ●	2	1	0	0	0	0
O_orca						
Total	35	9	7	13	199	65

Figure 2. A maximum-likelihood reconstruction of 319 bp mtDNA CR haplotypes from rough-toothed dolphin. Bootstrap values above 60% are shown. The tree is rooted to the killer whale (*Orcinus orca*). The number of individuals from each region with the haplotype are shown in the table to the right, and shared haplotypes between oceanic subregions are shaded in yellow. **Bolded** sequences were used in the mitogenome data set. The Mediterranean sequences (Medit_1 and Medit_2) are considered part of the Atlantic region. The ocean region the sequences originated from (where samples were taken) are shown as symbols for the Atlantic (circle), Indian (square), and Pacific (star).

Each individual in the mitogenome Bayesian phylogeny generated from rough-toothed dolphin sequences had a unique haplotype as defined over 386 variable sites (Figure 3). There were four main clades, one from the Indian/West Pacific (Figure 3, Clade A), one from the Central/East Pacific (Figure 3, Clade B), one from the Atlantic (Figure 3, Clade D), and a cosmopolitan clade consisting of haplotypes from all three ocean regions (Figure 3, Clade C). In general, posterior probabilities in the rough-toothed dolphin mitogenome tree were above 0.95 for all nodes with the exception of a single node within Clade C where the posterior probability was 0.79 (Figure 3). The Indian/West Pacific (Clade A), Central/East Pacific (Clade B), and the cosmopolitan clade (Clade C) were observed in the mtDNA CR phylogeny but were not well supported (bootstrap value <68). There is one additional haplotype in the mitogenome tree in Clade C compared to the mtDNA CR tree due to two of the North Atlantic haplotypes collapsing at 319 bp. A private Atlantic clade was present and well supported in the mitogenome tree (Clade D). However, despite the support for this private Atlantic clade, the phylogenetic reconstruction does not show a pattern of reciprocal monophyly for haplotypes from the Atlantic. Instead, three of the Atlantic haplotypes are nested within the cosmopolitan clade along with Indian/West Pacific and Central/East Pacific haplotypes (Figure 3, Clade C).

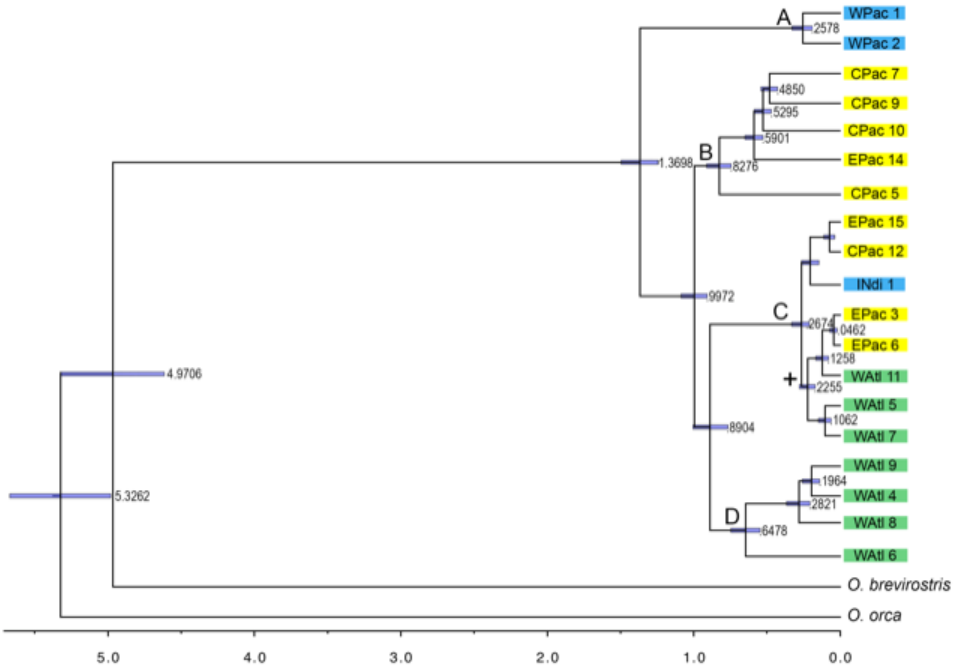


Figure 3. Bayesian reconstruction of the rough-toothed dolphin phylogeny based on concatenated protein-coding genes of the mitogenome rooted by *Orcaella brevirostris* and *Orcinus orca* shown in black. Bayesian posterior probabilities were all above 0.98, with the exception of a single clade shown by **+** where the posterior probability was 0.79. Individuals are color coded according to the region where they were sampled. Blue represents the Indian and Western Pacific Oceans, yellow represents the Central and Eastern Pacific Ocean, and green represents the Atlantic Ocean. Each letter designates a main clade discussed in the text. The time scale is in millions of years, and the error bars on the nodes indicate uncertainty around divergence time estimates.

Based on the interspecies phylogeny, the substitution rate calculated for the rough-toothed dolphin was 0.0098/site/Myr, with a 95% highest posterior density (HPD) of 0.0073-0.012 (Table S5). This value is well within the range of estimates from Steeman et al. (2009), McGowen et al. (2009), and Alexander et al. (2013) for delphinid substitution rates using the protein-coding regions of the mitogenome (median value across those studies 0.0059-0.0123, 95% HPD = 0.0039-0.0199). Based on the rate calculated for rough-toothed dolphins, the initial divergence of the Indian/West Pacific Clade (A) from the remainder of the samples occurred ~1.37 Mya (95% HPD = 1.12 - 1.49 Mya). The divergence between the Central/East Pacific Clade B and the remaining clades

occurred ~ 0.997 Mya (95% HPD = 0.0724 – 1.297 Mya). The divergence of the Cosmopolitan Clade C and the Atlantic only Clade D occurred around 0.890 Mya (95% HPD = 0.0629 – 1.161 Mya).

The ancestral state reconstruction (Figure 4) suggests that rough-toothed dolphins originated in the Pacific Ocean. The presence of equivocal nodes after this event suggest it is not possible to determine the directionality of the next migration events between the Pacific and the Atlantic. There could have been three migration events into the Atlantic from the Indo/Pacific region. Equally likely from the mitogenome phylogeny and ancestral state reconstruction, there could have been a migration into the Atlantic and two returns to the Pacific, or two Atlantic migrations and one return to the Pacific.

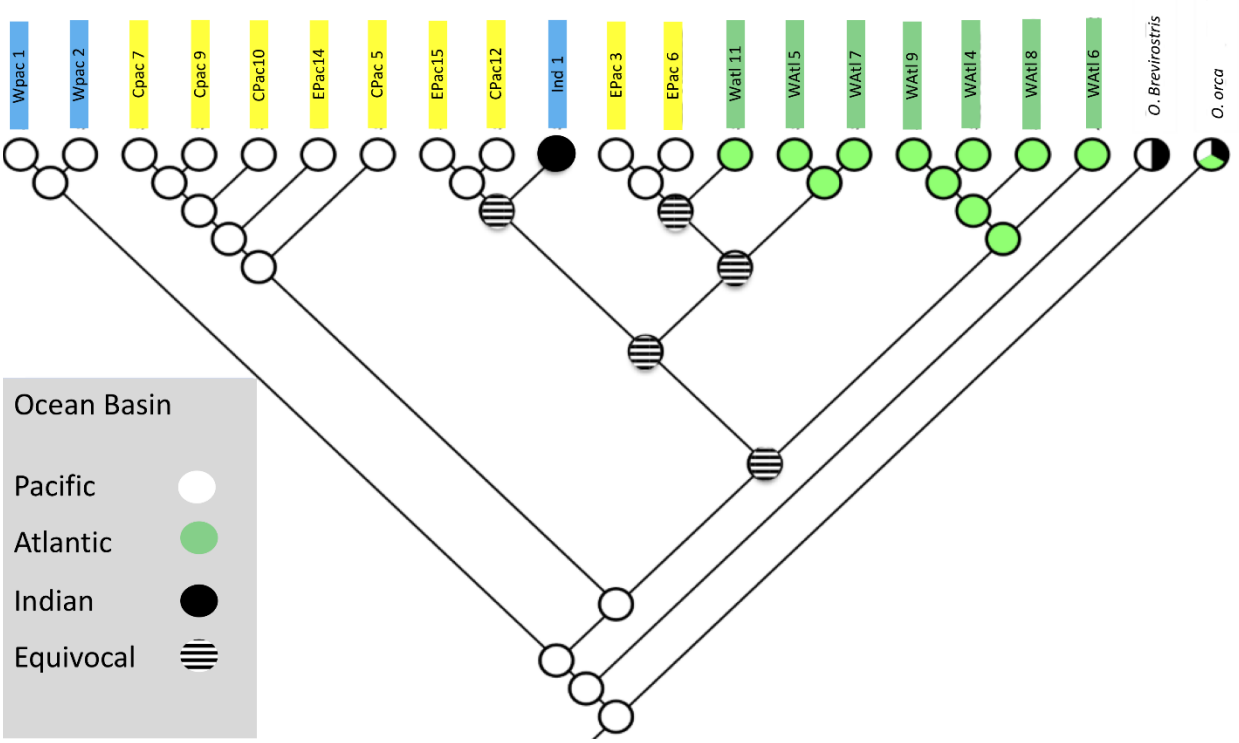


Figure 4. Likelihood ancestral character state reconstruction traced onto the rough-toothed dolphin (*Steno bredanensis*) phylogeny shown in Figure 3. The name of the haplotype is color coded according to where the sequence was sampled: blue represents the Indian Ocean, yellow represents the Pacific Ocean, and green represents the Atlantic Ocean.

3.2 mtDNA genetic diversity and differentiation

For both mtDNA CR and mitogenome data sets, the highest genetic differentiation was between the Atlantic and other regions for Φ_{ST} (Tables 2 and 3). This was also true when the three oceanic regions were further divided into six regions (western Atlantic, eastern Atlantic, Indian, and western, central, and eastern Pacific Oceans, Table 4). All pairwise comparisons were significant except between the Indian and the western Pacific subregions. Nucleotide diversity (π) was 0.0165 for the mtDNA CR and 0.0126 for the mitogenome across the total data set. Nei's net nucleotide divergence (d_A) for the mtDNA CR of the Atlantic and Indian/Pacific regions was 0.02.

Table 2. Inter-ocean genetic differentiation of rough-toothed dolphins Φ_{ST} (and associated p-value) as calculated in *Arlequin* using mtDNA CR 319 bp sequences. Sample totals (n) for each region are given in parentheses.

	Atlantic (n=44)	Indian/West Pacific (n=20)
Indian/ West Pacific (n=20)	0.554 (<0.001)	
Central/ East Pacific (n=296)	0.557 (<0.001)	0.020 (0.017)

Table 3. Inter-ocean genetic differentiation of rough-toothed dolphins Φ_{ST} (and associated p-value) as calculated in *Arlequin* using protein-coding mitogenome sequences. Sample totals (n) for each region are given in parentheses.

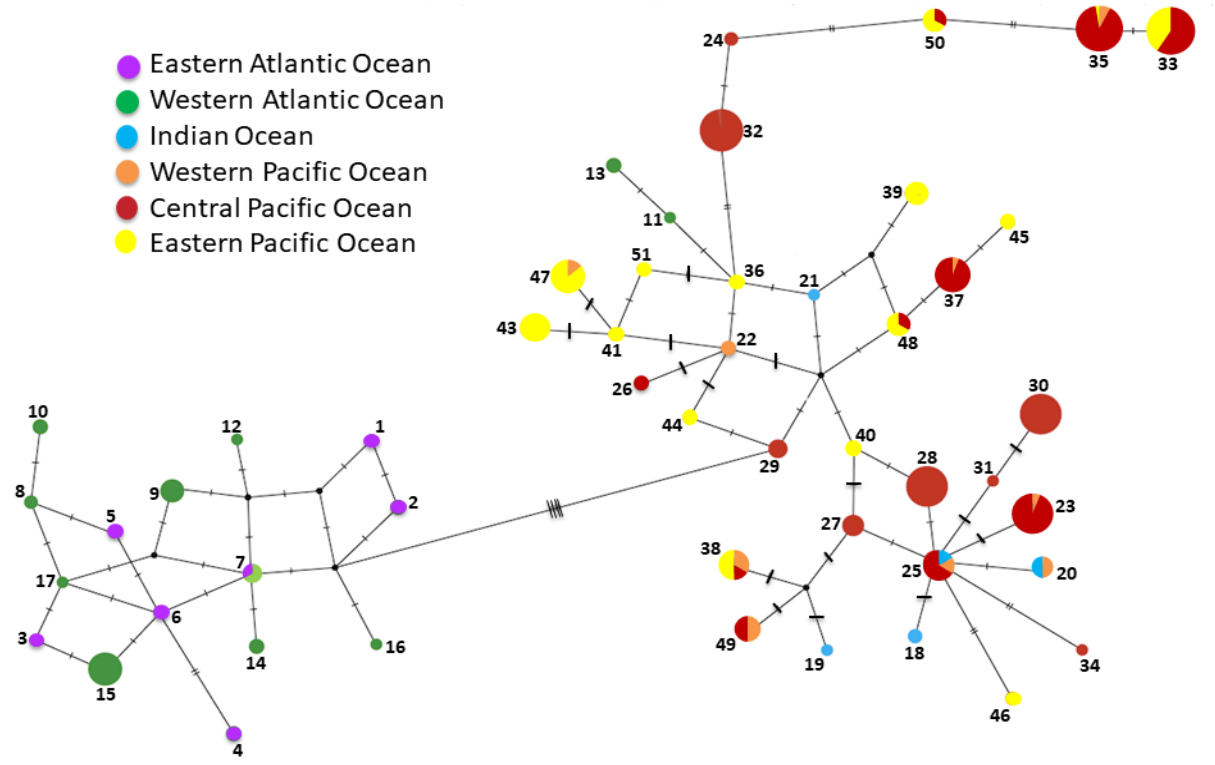
	Indian/Pacific (n=13)
Atlantic (n=7)	0.255 (<0.001)

Table 4. Genetic differentiation for six subregions of rough-toothed dolphins using mtDNA CR; Φ_{ST} (and associated p-value) as calculated through *Arlequin*. Sample totals for each region are given in parentheses (n).

	Western Atlantic (n=35)	Eastern Atlantic (n=9)	Indian (n=7)	Western Pacific (n=13)	Central Pacific (n=231)	Eastern Pacific (n=65)
Eastern Atlantic	0.0793 (0.010)					

Indian	0.6215 (<0.001)	0.6742 (<0.001)				
Western Pacific	0.5602 (<0.001)	0.5764 (<0.001)	0.0229 (0.025)			
Central Pacific	0.5870 (<0.001)	0.6233 (<0.001)	0.0662 (0.014)	0.0830 (0.010)		
Eastern Pacific	0.4921 (<0.001)	0.4989 (<0.001)	0.2296 (<0.001)	0.0702 (0.010)	0.1707 (0.001)	

The median-joining network (Figure 5) illustrates the relationship among haplotypes and their frequencies from the six finer-scale subregions (western Atlantic, eastern Atlantic, Indian, western Pacific, central Pacific, and eastern Pacific Oceans). The network identified the private Atlantic cluster (green and purple, left side, Figure 5) as at least five mutational steps away from all the other haplotypes.



548 Figure 5. Network using 319 bp CR haplotypes of the rough-toothed dolphin (*Steno bredanensis*).
549 Size of circles is proportional to the number of samples for that haplotype. Branch lengths are
550 proportional to the number of mutations. Colors illustrate where the haplotypes were sampled.
551 Black dots represent inferred node haplotypes not found in the data set. Tick marks represent
552 mutational steps. The numbers reference the haplotype number (see Table S4). The haplotype
553 originating from the East Coast of South Africa in the Indian Ocean is haplotype 21. The haplotype
554 originating from Taiwan is haplotype 22, and the haplotype composed of samples from the Indian
555 and Pacific Oceans is haplotype 25. Due to missing data in some haplotypes (e.g., EAtl_4, Medit_2) at
556 one of the variable sites in the alignment (site 277), this site was not utilized in constructing the
557 haplotype network. Therefore, haplotype EPac 7 is not displayed, but is separated from EPac6 by a
558 single substitution at site 277.

559

560 Similar to the phylogenetic trees (Figures 2 and 3), within this Atlantic cluster there was no clear
561 pattern of northern versus southern hemisphere or eastern versus western Atlantic haplotypes.
562 Also similar to the phylogenetic trees, two Atlantic haplotypes: one North Atlantic and one South
563 Atlantic, were clustered together, but with haplotypes from other regions rather than the larger
564 Atlantic cluster (Figure 5). An Indian Ocean haplotype from eastern South Africa (Figure 5,
565 haplotype 21) lies two steps away from these two Atlantic haplotypes with an eastern Pacific
566 haplotype between them. On the other side of this eastern Pacific haplotype is a western Pacific
567 haplotype from Taiwan (Figure 5, haplotype 22). In general, Indian and western Pacific haplotypes
568 are interspersed among central and eastern Pacific haplotypes, indicating no clear phylogeographic
569 pattern for these regions. The one shared haplotype between the Indian, western, and central
570 Pacific oceans (identified in individuals from Taiwan, Japan, French Polynesia, Samoa, and the
571 Arabian Sea; Figure 5, haplotype 25) appeared central to multiple private Indian, and
572 Central/Eastern Pacific haplotypes.

573

574 **3.3 Nuclear diversity and differentiation**

575 From a total of 2,510 bp of the six concatenated introns there were 16 variable sites across 35
576 individuals. Phasing indicated between 2 and 9 alleles for each intron (Table 1). The Y-linked DBY7
577 was invariant. Although private alleles were found in some introns in some oceanic regions, there

were no fixed differences between regions for any intron (Table S6). Nucleotide diversity (π) ranged across loci from 0.01% (CHRNA-1) to 0.41% (Actin-1) (Table 1). Significant genetic differentiation was found between the Atlantic and the other two regions, but not between Indian/West Pacific and Central/East Pacific regions (Table 5).

Table 5. Inter-ocean genetic differentiation of the rough-toothed dolphin using concatenated nuclear intron alleles as calculated in *Genodive* for F_{ST} (below diagonal) and G''_{ST} (above diagonal) each with associated p-value in parentheses.

	Atlantic (n=10)	Indian/ Western Pacific (n=7)	Central/Eastern Pacific (n=18)
Atlantic		0.177 (<0.001)	0.146 (0.001)
Indian/ Western Pacific	0.150 (0.001)		0.0091 (0.079)
Central/ Eastern Pacific	0.135 (0.001)	0.018 (0.067)	

3.4 Diagnosability

As with other analyses discussed above, we found weak evidence for the differentiation between the Indian and Pacific Oceans. For the purpose of evaluating subspecies delimitation for the Atlantic region we combined the Indian and Pacific Ocean regions to evaluate diagnosability. The Random Forest models built on the mtDNA CR sequences were able to correctly classify 100% of the Indian/Pacific and 98% of the Atlantic samples (Table 6). The distribution of individual classification probabilities as measured by the fraction of trees in the forest voting for each region showed that most Atlantic individuals were classified with high certainty (Figure 6). The proximity plot (Figure S2) illustrates that the Atlantic haplotypes occupy a separate space in the Random Forest from the Indian/Pacific samples.

Table 6. Confusion matrices from Random Forest analyses for the mtDNA CR data set of rough-toothed dolphins (*Steno bredanensis*) from the Atlantic and Indian/Pacific oceans. The first column gives the original strata, followed by the two predicted strata. The last column is the proportion diagnosable (PD) with assignment probabilities and 95% Confidence Intervals (CI) from the binomial distribution given in parentheses.

	Predicted		
Original	Atlantic	Indian/Pacific	PD (CI)
Atlantic	43	1	98 (88-100)
Indian/ Pacific	0	316	100 (99-100)
Overall			100 (98-100)

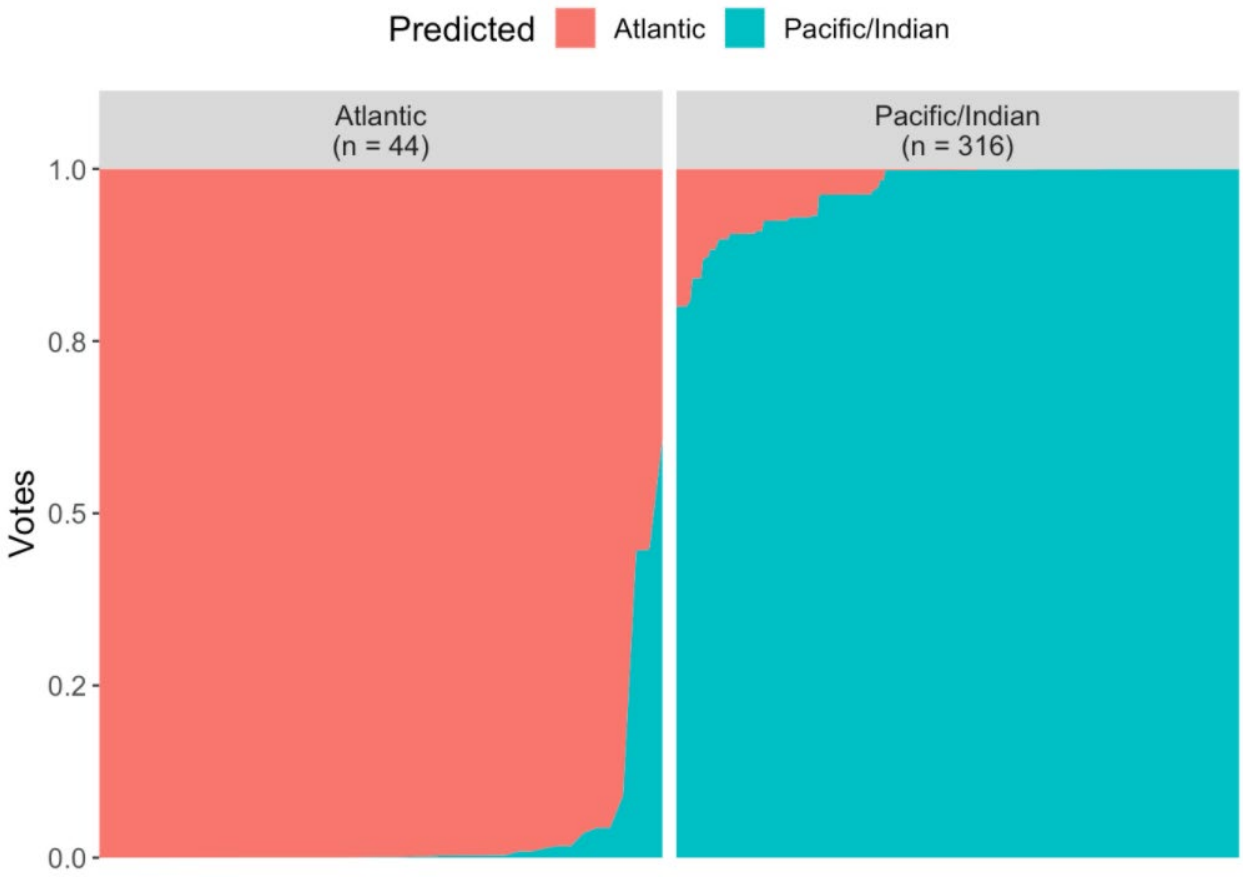


Figure 6 – Distribution of classification probabilities for individual rough-toothed dolphins (*Steno bredanensis*) in two oceanic regions from Random Forest models on the mtDNA CR data set. Within each region individual samples are sequentially arranged along the x-axis. Sample sizes are in parentheses.

4 DISCUSSION

Taxonomic delimitation recognizes that time and space can set populations on different evolutionary trajectories due to local adaptation (Ayala, 1976; De Queiroz, 2007). Biogeographic barriers contribute to species diversification and are often concordant with significant differences in gene frequencies (Bowen et al., 2016). The South African Species Gate is a biogeographic barrier, essentially acting as a one-way gate (Indian into South Atlantic Ocean), for many pelagic species and has affected the dispersal and population structure of fauna with worldwide distributions (Daly-Engel et al., 2012b; Perrin, 2007; Rocha et al., 2007). Our results for the rough-toothed dolphin largely reflect this pattern originally described by Davies (1963) for cetaceans. We found significant genetic differentiation among the three broader scale regions (Atlantic, Indian/West Pacific, and Central/East Pacific) and almost all the finer-scale subregions (western and eastern Atlantic, Indian, and western, central, eastern Pacific ocean regions) for mtDNA. This supports the existence of local populations, and rejects the assumption that rough-toothed dolphins are panmictic across their range. Using the nuclear data set we also found significant genetic differentiation between the Atlantic and each of the other broader scale biogeographic regions. Furthermore, using mtDNA to evaluate the broader scale regions, we found sufficient evidence from d_A and diagnosability to support further investigation of subspecies delimitation of the rough-toothed dolphin in the Indian and Pacific oceans with respect to the Atlantic. However, we did not find monophyly for regions in either the mtDNA or fixed differences in nuclear loci; nor did we find fixed differences with geographic concordance in either marker, suggesting a lack of species level divergences between the regions sampled here.

4.1 Evidence of subspecies delimitation within *Steno*

633 In the guidelines for delimiting cetacean subspecies using mtDNA, Taylor et al. (2017a) outlined
634 two criteria to separate subspecies from populations and species. The first criterion for subspecies
635 is that Nei's net divergence (d_A) values fall within the range of 0.004 - 0.020 for control region
636 sequences. This helps support the requirement for species to be on separate evolutionary
637 trajectories (Archer et al. 2017; Rosel et al. 2017a; Taylor et al. 2017a). The d_A values for rough-
638 toothed dolphins from the Atlantic with the combined Indian/Pacific regions was 0.02, within the
639 threshold (upper range) for subspecies. The second criterion is diagnosability. According to
640 recommendations of Taylor et al. (2017a), the threshold value for subspecies delimitation should
641 be 95%. Diagnosability for Atlantic rough-toothed dolphins for the mtDNA CR was 98% (CI 88%-
642 100%). As Archer et al. (2019) found for fin whales (*Balaenoptera physalus*), diagnosability can be
643 helpful in delimiting subspecies when there are no fixed differences in mtDNA lineages due to
644 polyphyly, paraphyly, or uncertainty in tree topology. We define polyphyly here from Funk and
645 Omland (2003) to include both paraphyly, where haplotypes of one taxon are nested within the
646 haplotypes of one or more separate taxa, and polyphyly where haplotypes from different taxa are
647 phylogenetically interspersed with one another. With the possibility of polyphyly in the tree
648 topologies here, d_A and diagnosability serve as evidence that Atlantic rough-toothed dolphins are on
649 a separate evolutionary trajectory from rough-toothed dolphins in the Indian and Pacific Oceans.
650 Moreover, Da Silva et al. (2015), investigated the molecular taxonomy of rough-toothed dolphins
651 using multiple mtDNA markers, and identified strong intraspecific differentiation between the
652 Atlantic and Indo/Pacific regions. These analyses also suggested oceanic subspecies, but lacked the
653 framework of analytical criteria used here to support subspecies delimitation.
654 The nuclear introns provided further evidence to support isolation of Atlantic rough-
655 toothed dolphins. The introns showed significant genetic differentiation between the Atlantic
656 region and the other regions, but not between the Indian/West Pacific and Central/East Pacific
657 regions. This suggests that male-mediated gene flow is unlikely to be occurring in these regions,

something that cannot be tested using only mtDNA. Martien et al. (2017a) highlight the importance of using multiple lines of evidence (e.g., mtDNA, nuclear markers, morphology) especially in species that may exhibit strong matri-focal social structure or if social structure is unknown. Social structure of rough-toothed dolphins has not been extensively studied, although photo-identification studies indicate preferred associations (Baird et al., 2008; Kuczaj II and Yeater, 2007; Oremus et al., 2012; Ritter, 2005) and enduring mother-offspring bonds (Mahaffy and Baird, 2019). Therefore, the use of nuclear markers for this purpose provides additional evidence of a separate evolutionary trajectory for Atlantic rough-toothed dolphins.

4.2 Phylogeographic patterns and population subdivision recommendations

Although the limited sampling of the Indian Ocean does not fully represent the region, it is useful for a preliminary description of phylogeographic patterns. The shared mtDNA CR haplotypes and no significant genetic differentiation in the nuclear data set between the Indian/West Pacific and the Central/East Pacific regions indicate recent divergence or low levels of continued gene flow. It is clear that rough-toothed dolphins form insular populations exhibiting site fidelity, yet they are also observed far offshore (Ballance and Pitman, 1998; Gannier and West, 2005; Wade and Gerrodette, 1993). Oceanic individuals are underrepresented in our data set due to logistical constraints in field sampling. Additional oceanic sampling could illuminate levels of exchange between oceanic and neritic insular populations of rough-toothed dolphins. Within areas like the ETP, insular populations of similar species maintain connectivity through occasional long-distance dispersal or gene flow with oceanic populations (Andrews et al., 2013; Caballero et al., 2013; Martien et al., et al., 2017b; Mignucci-Giannoni, 1998; Tezanos-Pinto et al., 2009). Moreover, there may be seasonal shifts in oceanic populations. Kerem et al. (2016) observed a temporal pattern of oceanic rough-toothed dolphin sightings in the Mediterranean Sea. The dolphins were found in deep water during the months of May to November, while nearshore sightings and strandings were

most common between February and June. The authors suggest the offshore dolphins may move nearshore seasonally following preferred prey species. Gannier and West (2005) also found a seasonal pattern in the Society Islands with the lowest offshore sightings during the winter months, although both studies cite survey effort was not uniform throughout the year. Future studies should make a concerted effort to sample both offshore and nearshore dolphins across their range to evaluate this question on a finer scale than was possible here with the current sampling.

The mtDNA CR data set showed significant Φ_{ST} values, not only among the three major regions but also between most of the pairwise comparisons for the six subregions. The exception was the pairwise comparison between the Indian Ocean and West Pacific Ocean regions. This may be due to the small sample sizes, giving us lower power to detect differentiation of a similar scale among these regions. The highest Φ_{ST} values were between the Atlantic with other subregions, illustrating that haplotypes from the Atlantic are largely divergent from those of the Indian and Pacific oceans. Da Silva et al. (2015) also found large Φ_{ST} values that were significant between the Atlantic and Pacific oceans using mtDNA CR sequences.

Within the subregions, discrete populations have been identified in the Central Pacific (Albertson et al., 2017; Oremus et al. 2012) and the South Atlantic (da Silva et al., 2015). Oremus et al. (2012) and Albertson et al. (2017) found large F_{ST} and Φ_{ST} values even between islands in relatively close proximity (190 km) within the Society Islands archipelago in French Polynesia. Albertson et al. (2017) found a similar pattern in the main Hawaiian Islands. The authors in both studies concluded that these results suggest high site fidelity, which has been confirmed with photo-identification in the Society, Hawaiian, Samoan, and Canary Islands (Baird, 2016; Baird et al., 2008; Johnston et al., 2008; Ritter, 2005). The large values of these metrics in this study may be a reflection of some insular population structure within the larger oceanic regions. As additional sampling becomes available it is likely other populations within these regions will be recognized.

4.3 Population structure within and among the Atlantic region

We would expect haplotypes from the eastern North Atlantic to be significantly different from the western North Atlantic due to the Mid-Atlantic Barrier Ridge. Rocha et al. (2007) suggest the Mid-Atlantic Barrier Ridge is responsible for the phylogeographic structure between the eastern and western Atlantic observed in reef fish. Daly-Engel et al. (2012a) note that for scalloped hammerhead sharks (*Sphyrna lewini*), estimates of gene flow across the North Atlantic were lower than across the Indo-Pacific, and Caballero et al. (2013) found genetic differentiation for Atlantic spotted dolphins between the western and eastern Atlantic populations despite shared haplotypes. We identified just one shared haplotype and significant Φ_{ST} values between the western and eastern Atlantic samples, although the number of eastern Atlantic samples was very limited (n=9). In the phylogenetic trees there was not a clear phylogeographic pattern of further division of western and eastern Atlantic regions. Using some of the same sequences but extending the mtDNA CR to 450 base pairs, Kerem et al. (2016) generated a phylogenetic tree with a similar topology to our trees. Worth noting from the Kerem et al. (2016) study is the well-supported divergence of the Atlantic and Indo-Pacific haplotypes (bootstrap value >95). Moreover, Kerem et al. (2016) identified the same two western Atlantic haplotypes nested within the Indo-Pacific clade shown in this study. Da Silva et al. (2015) also identified a deep divergence between the Atlantic and Pacific/Indian Oceans and a western Atlantic haplotype nested within the Indo-Pacific clade in their mtDNA Control Region sequences. The Random Forest proximity plots illustrate the isolation of the Atlantic haplotypes compared to the other regions (Figure S2). Therefore, it is clear that the eastern Atlantic haplotypes are more closely related to western Atlantic haplotypes than to the Indo-Pacific haplotypes, further supporting the subspecies delimitation of rough-toothed dolphins.

4.4 Colonization into or out of the Atlantic Ocean?

733 Implementing the molecular clock with the *Steno* substitution rate and acknowledging the
734 incomplete geographical coverage of our samples, we were able to trace back within-species
735 radiation events during the last one million years. Based on the estimated divergence dates of
736 Figure 3 and the inferred ancestral node in Figure 4, it would seem that rough-toothed dolphins
737 inhabited the western Pacific early in this period. That was followed by subsequent radiation
738 events where rough-toothed dolphins have been distributed across the Atlantic and Indo-Pacific
739 Ocean regions for at least the last 647,800 years. However, the direction of these subsequent
740 radiation events is ambiguous and may have been either from the Atlantic into the Pacific, or from
741 the Pacific into the Atlantic. Note that during the period in question, dispersal events between the
742 Atlantic and Pacific, in either direction, could only occur via the Indian Ocean due to the closure of
743 the Isthmus of Panama at least 3 Mya (Steeman et al., 2009). Ancestral state reconstruction alone
744 suggests that either direction of migration is plausible (Figure 4). However, the "Agulhas leakage"
745 described as occasional warm and salty water flowing out of the Indian Ocean and into the eastern
746 South Atlantic, could enhance travel of fauna in this direction (Peeters et al., 2004). This Species
747 Gate would episodically "open" allowing cetaceans and other pelagic predators into the Atlantic.
748 Perrin (2007) suggests dispersal from Indian to Atlantic would be an easier direction of travel.
749 Indeed, the timing of the Agulhas leakage coincides with colonization or recolonization into the
750 Atlantic of other pelagic predator species, e.g., white sharks (*Carcharodon carcharias*) (Gubili et al.,
751 2011) and killer whales (*Orcinus orca*) (Foote et al., 2011). According to Peeters (2004), there was a
752 higher probability of Agulhas leakage into the Atlantic that coincides with the two most recent
753 radiation events we identified for rough-toothed dolphins (0.226 – 0.126 Mya). Therefore, this
754 biogeographic barrier supports the option of three separate migrations into the Atlantic as more
755 likely than either two Atlantic migrations and one return to the Pacific or one migration into the
756 Atlantic followed by two returns to the Pacific. However, the limited number of samples from the
757 Indian and Atlantic Oceans does not allow us to resolve one scenario over another.

758

759 **4.5 Conservation considerations**

760 Correct delimitation of subspecies is important in conservation in order to accurately apportion
761 anthropogenic and ecological impacts to the specific evolutionary units within a species. This is
762 essential for seemingly pelagic species like the rough-toothed dolphin, which are challenging to
763 study, yet also inhabit coastal areas where anthropogenic threats are greater. For the rough-
764 toothed dolphin these threats are particularly prevalent on the coastlines of the western North and
765 South Atlantic Oceans where mass strandings, pollution, and fishery interactions occur (Donato et
766 al., 2019; Ewing et al., 2020; Lailson-Brito et al., 2012; Lemos et al., 2013; Lodi and Maricato, 2020;
767 Meirelles and Barros, 2007; Monteiro-Neto et al., 2000). Off the coast of Brazil in particular, rough-
768 toothed dolphins have one of the highest rates of fishery bycatch of any small cetacean (Donato et
769 al., 2019). The phylogeographic pattern for rough-toothed dolphins that we identified supports
770 previous studies (da Silva et al., 2015; Kerem et al., 2016) and illustrates significant divergence
771 between the Atlantic and other regions. Separate management considerations for rough-toothed
772 dolphins in the Atlantic are crucial for the future of the species.

773

774 **4.6 Taxonomic considerations**

775 The type locality of *Steno bredanensis* Lesson 1828 is the mouth of the River Scheldt, Netherlands
776 (Smeenk, 2018). If future investigation outside of the North Atlantic provides additional support for
777 delimitation of rough-toothed dolphin subspecies, the North Atlantic form would be *Steno*
778 *bredanensis bredanensis*. Then two nominal species considered synonyms of *S. bredanensis* would
779 need to be examined for consideration as possible names for subspecies outside of the North
780 Atlantic. The first, *Delphinus reinwardtii* (Schlegel, 1841) from Java, has two co-type skulls housed
781 in the Leiden Museum (Jentink, 1887). The second, *Delphinus (Steno) perspicillatus* (Peters 1877) is
782 from the eastern South Atlantic (32°29'S, 02°1'W) off South Africa and the type specimen is

preserved in the Berlin Museum. Notably, there are no nominal species in the synonymy of *Steno bredanensis* from the northern Indian Ocean region or North Pacific Ocean (Smeenk 2018). Future work should include sequencing of mitogenomes and morphological analyses of the available type specimens and comparisons with the collection of additional samples from a broader area within the tropical and subtropical waters of the Indian Ocean and western North and South Pacific Oceans.

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1972. Oregon State University's Institutional Animal Care and Use Committee approved a protocol for the collection of biopsy samples (Animal Care and Use Protocol 4285). Samples in French Polynesia were collected under permit issued to M. Michael Poole by the Ministry of the Environment. The samples originating from outside U.S. jurisdiction were imported under the Convention on International Trade in Endangered Species (CITES) import permit numbers US774223, US689420 and US799055. The Southwest Fisheries Science Center and Oregon State University are Registered Scientific Institutions under CITES.

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G. Renee Albertson: Conceptualization, sequencing and analyses of mtDNA and intron data set, writing original draft, and editing

Alana Alexander: Sequencing of mitogenomes, analyses, writing review, and editing

Eric Archer: random forest analyses, taxonomic review, editing

Karen Martien: data curation and editing

Lenaïg G. Hemery: phylogenetic analysis assistance and editing

Robin W. Baird: sample collection, data curation, and editing

Susana Caballero: assisted in intron development and long-range fragment primer design specific to *Steno bredanensis*, editing

M. Michael Poole: sample collection, data curation, and editing

Marc Oremus: sample collection, mtDNA sequencing of some French Polynesia samples

Debbie Duffield: western North Atlantic sample loan, editing

Robert L. Brownell Jr: taxonomic review and editing

Dan Kerem: mtDNA sequencing of eastern Atlantic samples and editing

Antonio A. Mignucci-Giannoni: Caribbean sample loan, editing

C. Scott Baker: Conceptualization, data curation, review, and editing

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