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Examining metrics and magnitudes of molecular genetic differentiation used to delimit cetacean subspecies based on mitochondrial DNA control region sequences

This is the fourth of six papers forming a special issue of Marine Mammal Science (Vol. 33, Special Issue) on delimiting cetacean subspecies using primarily genetic data. An introduction to the special issue and brief summaries of all papers it contains is presented in Taylor et al. (2017a). Together, these papers lead to a proposed set of guidelines that identify informational needs and quantitative standards (Taylor et al. 2017b) intended to promote consistency, objectivity, and transparency in the classification of cetaceans. The guidelines are broadly applicable across data types. The quantitative standards are based on the marker currently available across a sufficiently broad number of cetacean taxa: mitochondrial DNA control region sequence data. They are intended as "living" standards that should be revised as new types of data (particularly nuclear data) become available.

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Abstract

Cetacean taxonomy continues to be in flux and molecular genetic analyses examining alpha taxonomy in cetaceans have relied heavily on the mitochondrial DNA control region. However, there has been little consistency across studies; a variety of metrics and levels of divergence have been invoked when delimiting new cetacean species and subspecies. Using control region sequences, we explored, across pairs of well-recognized cetacean populations, subspecies, and species, a suite of metrics measuring molecular genetic differentiation to examine which metrics best categorize

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these taxonomic units. Nei's estimate of net divergence (d_A) and percent diagnosability performed best. All but a single, recently diverged species were unambiguously identified using these metrics. Many subspecies were found at intermediate values as expected, allowing separation from both populations and species, but several had levels of divergence equivalent to populations, resulting in underclassification errors using this single marker. Coupling d_A with additional measures, such as percent diagnosability, examining appropriate nuclear genetic markers, and interpreting results in a broader biological context will improve taxonomic investigations in cetaceans.

Key words: cetacean taxonomy, genetic divergence, subspecies delimitation, d_A.

Species delimitation has a long and contentious history. Historically, morphological characters have been used to infer reproductive isolation and species boundaries (Mayr 1942, Simpson 1951). However, convergence of morphological characters as well as the cryptic nature of some species can cause problems for morphology-based species delimitation. The advent of molecular and genomic data sets has expanded the field of species delimitation into a new, though often no less contentious realm (Groves 2013, Zachos *et al.* 2013), that relies on molecular genetic data to identify and delimit species boundaries. A variety of methods to delimit species based on molecular data have been developed (Hebert *et al.* 2003, Pons *et al.* 2006, Yang and Rannala 2010), and more recently there have been calls for a more integrative taxonomic process that utilizes theory and lines of evidence drawn from multiple disciplines (*e.g.*, Padial *et al.* 2010, Fujita *et al.* 2012) with the development of methods to do so (*e.g.*, Edwards and Knowles 2014, Solís-Lemus *et al.* 2015).

In contrast, methods for subspecies delimitation have attracted less attention and, in fact, the merit of subspecies as a taxonomic unit has experienced considerable debate (*e.g.*, Wilson and Brown 1953, Starrett 1958, Zink 2004, Fitzpatrick 2010, Remsen 2010, Winker 2010). Subspecies are the smallest recognized taxonomic unit. They lie between populations and species along the continuum of evolutionary diversification. Traditional definitions of subspecies commonly included a component of allopatry (Mayr 1969). Here, we follow definitions by Reeves *et al*. (2004) and Taylor *et al.* (2017*b*) for species, subspecies, and populations. "A *species* is a separately evolving lineage comprised of a population or collection of populations; a *subspecies* is a population, or collection of populations, that appears to be a separately evolving lineage with discontinuities resulting from geography, ecological specialization, or other forces that restrict gene flow to the point that the population or collection of populations is diagnosably distinct."[2] Finally, the definition of population encompasses a sympatric group of individuals whose dynamics are more a consequence of births and deaths within the group (internal dynamics) than of immigration or emigration (external dynamics) (Taylor 2005) through to Evolutionarily Significant Units (ESUs) as defined in Waples (1991).[3]

Taylor *et al.* (2017*b*) suggested that as many as 34% of the currently recognized cetacean taxa may suffer from underclassification errors, primarily a result of both the difficulties of working with such inaccessible and often rare taxa and the fact that

[2]Diagnosability implies a high probability (but not necessarily a 100% probability) of identifying an individual as belonging to the taxon.

[3]Waples (1991) defined an ESU as: a population (or group of populations) that (1) is substantially reproductively isolated from other conspecific population units, and (2) represents an important component in the evolutionary legacy of the species.

some groups have arisen *via* recent and rapid radiation events. It is likely that many cetacean subspecies and some species remain unrecognized today, resulting in the prospect of taxa with little or no protection against human impacts. The many current anthropogenic threats faced by cetaceans (Reeves *et al.* 2013, Van Der Hoop *et al.* 2013, Baulch and Perry 2014) and the fact that conservation actions are often targeted below the species level (Haig *et al.* 2006, Haig and D'Elia 2010) argue for exploration of methods to delimit cetacean subspecies using molecular genetic data, particularly as sampling for molecular work can provide much larger sample sizes than for classical morphological analyses (Taylor *et al.* 2017*b*). Providing accurate and consistent subspecies (and species) delimitation resulting in a stable taxonomy is critically important for determining and developing appropriate and successful conservation actions. In a recent review, Rosel *et al.* (2017) found that the mitochondrial DNA (mtDNA) control region has been by far the marker of choice for species and subspecies delimitation in cetaceans, but that these data are analyzed in a variety of different ways, making comparisons across studies difficult. Furthermore, Rosel *et al.* (2017) found no consensus among authors as to the type and level of molecular genetic differentiation that constitute subspecies or species level differences for cetaceans.

In this paper, we used mtDNA control region sequences to calculate a suite of commonly used metrics and systematically examined their ability to successfully classify a selected set of undisputed population, subspecies, and species pairs. MtDNA is considered because it has been the most commonly applied molecular marker in genetic studies of cetacean taxonomy and it is the only marker with sufficient data available across many populations, subspecies, and species (Rosel *et al.* 2017) with which to conduct the empirical comparisons implemented here. We sought both metrics and potential thresholds, for possible use with this marker, that performed well at distinguishing populations from subspecies and subspecies from species. This empirical evaluation is similar to previous studies (Bradley and Baker 2001, Tobias *et al.* 2010) with an added focus on subspecies. Note that we are not advocating that mtDNA is sufficient for all cases (see below for examples where it is insufficient) nor that other lines of evidence are not often critical. MtDNA is a matrilineal marker and does not provide information on male-biased dispersal. Evidence of a lack of male-mediated gene flow would be a requirement for delimiting a species, but at the subspecies level, some gene flow (male- or female-mediated) is allowed. We also included some well-accepted taxa that we expected, *a priori*, would likely fail for any metric due either to social structure or large population sizes (see below) to illustrate some of the factors that must be considered when undertaking a study of alpha taxonomy in cetaceans.

In addition, because subspecies lie on a continuum of genetic divergence between populations and species, identifying a threshold indicative of population/subspecies and subspecies/species "boundaries" may be difficult. However, classifying the levels of differentiation among biological groupings is valuable for understanding biological processes and invaluable for working towards specific conservation objectives (Taylor *et al.* 2017*b*, Milinkovitch *et al.* 2001). Thus, the objective of exploring metrics and identifying potential empirical values that researchers could apply to make consistent arguments of taxonomic status would be of great benefit. As suggested by Zachos *et al.* (2013), comparison of new data against empirical thresholds identified here for cetaceans can serve to support taxonomic hypotheses. Taylor *et al.* (2017*a*) expand on the empirical results presented here and suggest a suite of guidelines and standards to promote consistency in taxonomy for cetaceans when using mtDNA control region sequence data.

Materials and Methods

Choosing Taxa

Our choice of population, subspecies, and species pairs to use in the analysis was deliberate (not random). We chose pairs of taxa that had adequate control region sequence data and for which we felt there was no taxonomic uncertainty. Thus, control region sequences needed to be available from multiple individuals and reasonably cover the geographic range of the species, subspecies, or population in question. In addition, taxa had to be recognized and well-accepted members of their respective category (*i.e.,* subspecies, species). For this criterion, we relied on the list of marine mammal species and subspecies compiled by the Society for Marine Mammalogy's Committee on Taxonomy on 3 December 2013 (Committee on Taxonomy 2013). This Committee is composed of 16 experts in the field and produced the first official Society for Marine Mammalogy list of marine mammal species and subspecies in 2010. The Committee reviews the list annually by evaluating all new peer-reviewed information on marine mammal taxonomy published in the previous year. The original classification is based on Rice (1998), with modifications since then reflecting new findings.

However, even here we were conservative and did not consider species for which the taxonomic status might soon be changed given that the chosen comparisons were to be used as ground truths for identifying thresholds that might be used for future taxonomic decisions. For example, fin whales in the Northern Hemisphere are listed as a single nominate subspecies (*Balaenoptera physalus physalus)* encompassing the North Atlantic and North Pacific. Recent analyses have shown that fin whales in the North Atlantic and North Pacific are likely to be at least different subspecies (Archer *et al.* 2013), but a formal description has not yet been proposed. Thus, if there was any taxonomic uncertainty such as this, a pairwise comparison was not included in our analysis. Details of pairs are given in Table 1 and GenBank accession numbers for sequences used in alignments are in Table S1. For subspecies, we included all pairs without taxonomic uncertainty and with adequate data. For species and population comparisons we considered a wide range of taxa but concentrated on cases thought to be closer to the subspecies boundaries, *i.e.,* recently diverged sister species, and populations thought to be evolutionarily significant units (ESUs) *sensu* Waples (1991). Therefore, we did not, at the species level, for example, tabulate metrics for a comparison of sperm whales (*Physeter macrocephalus*) to blue whales (*Balaenoptera musculus*), two species from different families. These are well-recognized species with a deep evolutionary split and would provide no information on the amount of divergence of incipient species. Similarly, we generally did not include two populations of a given species that experience moderate levels of gene flow based on F_{ST} estimates; for example, harbor porpoise (*Phocoena phocoena*) populations in Morro Bay and Monterey Bay, California, were not compared.

We also considered effective population size (N_e) and social structure in our choice of taxa. Effective population size plays an important role in both the amount of genetic variation that can be maintained in a population and the amount of genetic divergence observed between two populations, due largely to the effect of random genetic drift. Genetic drift is a stochastic micro-evolutionary process that results in random changes in allele frequencies over time. The force of genetic drift is felt more strongly on groups with a small N_e than those with a large N_e. As a result, over the same time frame and with the same amount of relative gene flow, two groups with a

Table 1. Taxa used in paired comparisons of cetacean populations (Pop), subspecies (Subspp) and species (Spp) to estimate metrics of genetic divergence based on mtDNA control region sequences. GenBank accession numbers available in Table S1.

Type	Species	Strata (sample size)	Sequence length (bp)	Source
Pop	*Balaena mysticetus*	Atlantic (279) *vs.* Beaufort/Chukchi/ Bering Sea (343)	349	LeDuc *et al.* 2005, Postma *et al.* 2005, LeDuc *et al.* 2008, Alter *et al.* 2012
Pop	*B. mysticetus*	Beaufort/Chukchi/Bering Sea (343) *vs.* Sea of Okhotsk (20)	397	LeDuc *et al.* 2005, Postma *et al.* 2005, LeDuc *et al.* 2008
Pop	*Balaenoptera physalus*	Gulf of California (33) *vs.* eastern North Pacific (313)	411	Archer *et al.* 2013
Pop	*Megaptera novaeangliae*	Hawaii (59) *vs.* eastern North Pacific (60)	454	Baker *et al.* 2013
Pop	*M. novaeangliae*	Northern Gulf of Alaska (59) *vs.* Southeast Alaska (60)	455	Baker *et al.* 2013
Pop	*Physeter macrocephalus*	California Current (52) *vs.* Eastern Tropical Pacific (114)	399	Engelhaupt *et al.* 2009, Mesnick *et al.* 2011
Pop	*P. macrocephalus*	North Atlantic (293) *vs.* North Pacific (194)	399	Engelhaupt *et al.* 2009, Mesnick *et al.* 2011
Pop	*Pseudorca crassidens*	Hawaii insular (96) *vs.* Pacific pelagic (69)	945	Martien *et al.* 2014
Pop	*Orcinus orca*	eastern Aleutians and Trinity Islands (65) *vs.* western Aleutians and Russia (95)	919	Hoelzel *et al.* 2002
Pop	*O. orca*	Northern Resident (13) *vs.* Southern Resident (25)	919	Hoelzel *et al.* 2002
Pop	*Stenella longirostris*	Maui (59) *vs.* Oahu (39)	415	Andrews *et al.* 2010
Pop	*S. attenuata*	Central America (36) *vs.* Costa Rica (32)	421	Escorza-Treviño *et al.* 2005
Pop	*S. attenuata*	Ecuador (33) *vs.* Northern Mexico (34)	421	Escorza-Treviño *et al.* 2005
Pop	*S. attenuata*	Hawai'i-four islands (27) *vs.* Oahu (27)	535	Courbis *et al.* 2014

(Continued)

Table 1. (Continued)

Type	Species	Strata (sample size)	Sequence length (bp)	Source
Pop	*Tursiops truncatus*	Northwest Atlantic (100) *vs.* northern Gulf of Mexico (72)	354	Sellas *et al.* 2005, Rosel *et al.* 2009
Pop	*T. truncatus*	Kauai (41) *vs.* Hawai'i Offshore (69)	402	Perrin *et al.* 2011, Martien *et al.* 2012
Pop	*T. truncatus*	Hawai'i - four islands (26) *vs.* Oahu (30)	401	Martien *et al.* 2012
Pop	*Phocoena phocoena*	Gulf of Maine (80) *vs.* Newfoundland (42)	342	Rosel *et al.* 1999, Tolley *et al.* 2001
Pop	*Phocoenoides dalli*	eastern North Pacific (33) *vs.* Pacific gyre (15)	379	Escorza-Treviño and Dizon 2000
Subspp	*B. physalus*	North Atlantic (33) *vs.* Southern Hemisphere (48)	410	Archer *et al.* 2013
Subspp	*Globicephala melas*	*G. m. edwardii* (573) *vs. G. m. melas* (70)	345	Oremus *et al.* 2009
Subspp	*Cephalorhynchus commersonii*	*C. c. commersonii* (196) *vs. C. c. kerguelensis* (11)	423	Pichler *et al.* 2001, Pimper *et al.* 2010
Subspp	*C. hectori*	*C. h. hectori* (318) *vs. C. h. maui* (70)	340	Pichler *et al.* 1998, Pichler and Baker 2000, Pichler 2002, Hamner *et al.* 2012
Subspp	*Lagenorhynchus obscurus*	*L. o. fitzroyi* (14) *vs. L. o. obscurus* (21)	591	Cassens *et al.* 2005*a*
Subspp	*L. obscurus*	*L. o. fitzroyi* (14) *vs. L. o. posidonia* (118)	591	Cassens *et al.* 2005*a*
Subspp	*L. obscurus*	*L. o. obscurus* (21) *vs. L. o. posidonia* (118)	591	Cassens *et al.* 2005*a*
Subspp	*S. attenuata*	*S. a graffmani* (135) *vs. S. a. attenuata* (90)	421	Escorza-Treviño *et al.* 2005
Subspp	*S. longirostris*	*S. l. longirostris* (116) *vs. S. l. orientialis* (87)	400	Galver 2002, Leslie 2016
Subspp	*T. truncatus*	*T. t. ponticus* (43) *vs. T. t. truncatus* (eastern Atlantic and eastern Mediterranean) (88)	415	Viaud-Martinez *et al.* 2008
Subspp	*P. phocoena*	Black Sea (113) *vs.* North Atlantic (560)	338	Rosel *et al.* 1995, Rosel *et al.* 1999, Tolley *et al.* 2001, Tolley and Rosel 2006, Viaud-Martínez *et al.* 2007
Spp	*B. mysticetus vs. Eubalaena glacialis*	*B. mysticetus* (642) *vs. E. glacialis* (430)	388	Malik *et al.* 2000, LeDuc *et al.* 2005, Postma *et al.* 2005, LeDuc *et al.* 2008, Alter *et al.* 2012

(Continued)

Table 1. (Continued)

Type	Species	Strata (sample size)	Sequence length (bp)	Source
Spp	*E. australis vs. E. japonica*	*E. australis* (637) *vs. E. japonica* (23)	380	Carroll *et al.* 2011, LeDuc *et al.* 2012
Spp	*E. australis vs. E. glacialis*	*E. australis* (637) *vs. E. glacialis* (430)	426	Malik *et al.* 2000, Carroll *et al.* 2011
Spp	*E. glacialis vs. E. japonica*	*E. glacialis* (430) *vs. E. japonica* (23)	399	Malik *et al.* 2000, Carroll *et al.* 2011, LeDuc *et al.* 2012
Spp	*B. physalus vs. M. novaeangliae*	*B. physalus* (427) *vs. M. novaeangl*iae (1424)	388	Olavarría *et al.* 2007, Archer *et al.* 2013, Baker *et al.* 2013
Spp	*B. musculus vs. B. physalus*	*B. musculus* (295) *vs. B. physalus* (427)	347	LeDuc *et al.* 2007; Archer *et al.* 2013; Torres-Florez *et al.* 2014
Spp	*B. physalus vs. Eschrichtius robustus*	*B. physalus* (427) *vs. E. robustus* (262)	417	LeDuc *et al.* 2002, Archer *et al.* 2013, Lang *et al.* 2014
Spp	*E. robustus vs. M. novaeangliae*	*E. robustus* (262) *vs. M. novaeangliae* (1424)	543	LeDuc *et al.* 2002, Olavarría *et al.* 2007, Jackson *et al.* 2009, Baker *et al.* 2013, Lang *et al.* 2014
Spp	*Kogia breviceps vs. K. sima*	*K. breviceps* (258) *vs. K. sima* (90)	402	Chivers *et al.* 2005, Viricel 2012
Spp	*G. macrorhynchus vs. G. melas*	*G. macrorhynchus* (891) *vs. G. melas* (232)	304	Oremus *et al.* 2009; Van Cise *et al.* 2016; Rosel *et al.*, unpublished data
Spp	*G. macrorhynchus vs. Pepenocephala electra*	*G. macrorhynchus* (891) *vs. P. electr*a (177)	963	Oremus *et al.* 2009; Van Cise *et al.* 2016; Rosel *et al.*, unpublished data; Martien *et al.*, unpublished data
Spp	*Feresa attenuata vs. P. electra*	*F. attenuata* (54) *vs. P. electra* (177)	961	Hanser *et al.*, unpublished data

(Continued)

Table 1. (Continued)

Type	Species	Strata (sample size)	Sequence length (bp)	Source
Spp	*F. attenuata vs. P. crassidens*	*F. attenuata* (54) *vs. P. crassidens* (202)	955	Martien *et al.* 2014; Hanser *et al.*, unpublished data
Spp	*C. commersonii vs. C. hectori*	*C. commersonii* (207) *vs. C. hectori* (388)	340	Pichler *et al.* 1998, Pichler and Baker 2000, Pichler *et al.* 2001, Pichler 2002, Pimper *et al.* 2010, Hamner *et al.* 2012
Spp	*Sotalia fluviatilis vs. S. guianensis*	*S. fluviatilis* (21) *vs. S. guianensis* (55)	280	Caballero *et al.* 2007
Spp	*L. obliquidens vs. L. obscurus*	*L obliquidens* (59) *vs. L. obscurus* (153)	520	Hayano *et al.* 2004, Cassens *et al.* 2005*b*
Spp	*S. attenuata vs. S. longirostris*	*S. attenuata* (325) *vs. S. longirostris* (735)	390	Galver 2002, Escorza-Treviño *et al.* 2005, Andrews *et al.* 2010, Courbis *et al.* 2014
Spp	*Orcaella brevirostris vs. O. heinsohni*	*O. brevirostris* (46) *vs. O. heinsohni* (14)	403	Beasley *et al.* 2005, Palmer *et al.* 2011, Vilstrup *et al.* 2011
Spp	*Delphinapterus leucus vs. Monodon monoceros*	*D. leucus* (122) *vs. M. monoceros* (421)	291	Palsbøll *et al.* 1997, O'Corry-Crowe *et al.* 2010
Spp	*P. sinus vs. P. spinipinnis*	*P. sinus* (43) *vs. P. spinipinnis* (29)	398	Rosel and Rojas-Bracho 1999, Rosa *et al.* 2005
Spp	*Neophocaena asiaeorientalis vs. N. phocaenoides*	*N. asiaeorientalis* (10) *vs. N. phocaenoides* (27)	294	Wang *et al.* 2008

small N_e may show moderate levels of neutral genetic divergence due to drift, while two groups with a large N_e would not. Thus, groups with large N_e may be vulnerable to underclassification errors (*i.e.*, genetic divergence would remain small, though the groups had not experienced gene flow over an extended period), while groups with small effective population sizes may result in overclassification errors. Social structure that results in either nonrandom mating, sex-biased dispersal, or site fidelity may also pose a challenge to the goal of finding a divergence threshold because it may strongly impact the level of mtDNA variability (Whitehead 1998, Oremus *et al.* 2009, Moura *et al.* 2014). Recognizing these potential sources of error, we specifically included some comparisons that we expected could prove problematic, *e.g.*, spinner (*Stenella longirostris*) and pantropical spotted (*Stenella attenuata*) dolphins (because of high abundance), and killer (*Orcinus orca*) and false killer (*Pseudorca crassidens*) whales (because of social structure), knowing *a priori* they might be outliers or result in classification errors. We also suspected that the recently described species of finless porpoise (*Neophocaena phocaenoides*; Wang *et al.* 2008) would prove problematic as the authors based the description primarily on morphological data and suggested that the shared haplotypes of the mtDNA control region resulted from the recent divergence of these two species.

To summarize the rationale for pairwise comparisons: pairs of taxa were deliberately chosen so that the analysis could focus on the upper and lower boundaries delimiting subspecies to reveal potential molecular genetic metric thresholds. Because the pairwise comparisons were not chosen randomly, results should not be used to calculate overall classification error rates for the three taxonomic levels. Instead, errors reveal the relative performance of the different genetic metrics and the potential pitfalls to be expected when relying on a single neutral marker.

DNA Sequence Data Sets

Control region sequence data have been and continue to be the most commonly used molecular marker for addressing many taxonomic questions in cetaceans (Rosel *et al.* 2017). We compiled a set of control region sequence alignments for population, subspecies, and species pairs from which to calculate divergence metrics. Published mtDNA control region sequences, available through 2012, were downloaded from GenBank, while some unpublished sequences and/or haplotype frequency data were provided directly by researchers. In total, we evaluated 51 alignments; 19 alignments at the population level, 11 at the subspecies level, and 21 at the species level. The alignments involved 36 species: 8 mysticetes and 28 odontocetes (Table 1). Sequences were chosen to best represent the geographic range of each taxon. The *a priori* assignment of individuals to strata was based on the original authors' designations for published data sets or based on geography for unpublished data sets. Alignments were also visually examined for sequences that looked significantly different from the others and BLAST (Johnson *et al.* 2008) and GenBank were used to verify the species identification. Sequences with 100% identity over the length of the sequence to a different species than expected were removed from the alignment.

Sequences for each pairwise comparison were aligned using the "–auto" option, gap opening penalty = 3, and offset value = 0.123 in MAFFT v. 6.951b (Katoh *et al.* 2002). Each alignment was then checked and refined by eye. Any flanking tRNA sequence was trimmed from the alignments. We enforced a minimum alignment length for the control region sequences and focused alignments on the 5′ hypervariable region of the control region because this is the most commonly generated

sequence for cetacean studies and was available for the broadest suite of taxa. The shortest alignment used was 280 bp, three were less than 300 bp in length, while the longest was 961 bp. Eighty-four percent of the alignments were ≤500 bp in length. The longest sequences were used in four species: killer and false killer whales, pygmy killer whales (*Feresa attenuata*), and melon-headed whales (*Pepenocephala electra*) where social structure may limit genetic variability. Sample sizes for each stratum (*i.e.*, each of the taxa in a pairwise comparison) were highly variable. We generally sought larger sample sizes for the population level comparisons and accepted smaller sample sizes at the higher taxonomic levels. A minimum of 20 samples was an initial target for all comparisons, however, for some pairs (13.7%) one of the members was represented by fewer than 20 samples (Table 1).

Genetic Metrics

For each pairwise comparison, the alignment was used to calculate a set of commonly used measures summarizing the level of genetic divergence between strata: (1) Nei's frequency-based divergence, D_A, (Nei *et al.* 1983 eq. 7), which corrects for unequal sample size; (2) net nucleotide divergence, d_A (Nei 1987 eq. 10.21); (3) the number of fixed differences between members of each stratum (sequential indels were considered as a single substitution); (4) F_{ST} (Weir and Cockerham 1984); (5) Φ_{ST} (Excoffier *et al.* 1992). For measures involving genetic distance (*e.g.*, d_A and Φ_{ST}), we applied the Tamura-Nei substitution model (Tamura and Nei 1993). Pairwise deletion of sites with indels was applied for all calculations. Note that Nei's D_A is a simple frequency-based estimate of genetic distance and different from Nei's d_A, which measures net nucleotide divergence between two groups, correcting for within-group genetic diversity and, as such, is expected to be a better metric for evolutionary divergence. The number of fixed differences between two groups is commonly examined in species-level studies, while F_{ST} and Φ_{ST} are metrics most commonly applied to population level studies, though they have frequently been utilized in publications examining species level questions in cetaceans (see Rosel *et al.* 2017). All calculations were run using the *strataG* package (Archer *et al.* 2017*a*) available at https://cran.r-project.org/web/packages/strataG/index.html in R v3.0.0 (R Core Team 2013).

Once metrics were calculated, the degree of overlap among the population, subspecies, and species pairs was visualized by creating box plots to show mean and 1st and 3rd quartiles. To compare the number of over- and underclassification errors for each metric, we chose cut-off values that minimized overlap in value and misclassification errors for each metric. Taxa pairs that fell below/above the expected value were considered misclassified. We assumed that populations should not exhibit fixed differences and species should. Subspecies do not necessarily require fixed differences, as low levels of gene flow are accepted between subspecies.

In classical taxonomy using morphological data, subspecies delimitation is often performed based on diagnosability, *i.e.*, the ability to classify specimens to the correct taxon based on a set of distinguishing characters (Archer *et al.* 2017*b*). Amadon (1949) described the 75% rule for diagnosing subspecies: to qualify as a subspecies 75% of one population must be separable from 99% of the members of the other, or equivalently 97% of the distribution of a character trait for one group lies outside 97% of the distribution of the second group (Amadon 1949). As described in Archer *et al.* (2017*b*), methods for applying diagnosability criteria to subspecies delimitation using MtDNA data are generally lacking (although see Austerlitz *et al.* 2009) and

the authors describe the application of Random Forests (Breiman 2001) for this purpose. Here we use the Random Forests (RF) analysis implemented in Archer *et al.* (2017*b*) to calculate a measure of diagnosability for each taxonomic comparison. Briefly, this analysis uses the variable sites in the mitochondrial sequences to create a model that classifies samples from a pairwise comparison to strata based on a suite of decision trees (the forest). See Archer *et al.* (2017*b*) for details on the methodology. The key to appropriate diagnosability is quantification of the overall diagnosability of a population (Patten and Unitt 2002). With this information in hand, the probability of correct assignment of unknown individuals can be obtained. Generally, species are expected to have diagnosabilities of 100%, while for subspecies, some error is acceptable, thus diagnosability can be less. Here, we recorded misclassifications based on the recommendation from Archer *et al.* (2017*b*) in which species should be 100% diagnosable while the minimum diagnosability threshold for subspecies was 80%.

Results

The genetic metrics we examined exhibited varying levels of success for distinguishing between the voucher pairs of populations, subspecies, and species (Fig. 1, Table S2). Of the metrics examined, Nei's D_A and F_{ST} exhibited broad overlap across the three taxonomic classes. For D_A, many population and subspecies pairs are overclassified (as species) with D_A values at or near 1, leaving little power to distinguish among populations, subspecies, and species (Fig. 1). F_{ST} also exhibited cases with populations fixed at $F_{ST} = 1$, while some accepted subspecies and species pairs exhibited values lower than many of the population comparisons (Table S2).

The number of fixed differences, while performing better than D_A and F_{ST}, was also not particularly useful at identifying subspecies, though it was better identifying a species boundary. Given our data set, all but one species exhibited >4 fixed differences in the portion of the control region we examined. The species pair *Neophocaena phocaenoides*/*N. asiaeorientalis* was the outlier with no fixed differences—an underclassification error under the assumption that species should exhibit fixed differences (but see Discussion). However, two of the population pairs also exhibited a fixed difference—both comparisons involving killer whales. For subspecies, 8 of the 11 subspecies comparisons displayed no fixed differences between them, equivalent to most population comparisons. Therefore, fixed differences are not a useful metric to distinguish between populations and subspecies. Two pairs exhibited a single fixed difference, and one pair exhibited two. Under an assumption that subspecies do not need to exhibit fixed differences but species do, there were three subspecies overclassification errors.

Nei's (1987) measure of net genetic divergence (d_A) and Φ_{ST} returned the best results, exhibiting the least amount of overlap for the estimates among population, subspecies, and species pairs, but both also had outliers. For Φ_{ST}, population comparisons ranged from 0.02 to 1.0 with five comparisons significantly higher than the other 15, including three comparisons that fell within the range of the pairwise comparisons of species. Subspecies values ranged from 0.01 to 0.56 and species comparisons ranged from 0.61 to 0.98. Based on this data set, threshold values of $\Phi_{ST} = 0.2$ for subspecies and $\Phi_{ST} = 0.6$ for species provided the fewest classification errors and resulted in five population overclassification errors and three subspecies underclassification errors. These included the population comparisons for North Atlantic and

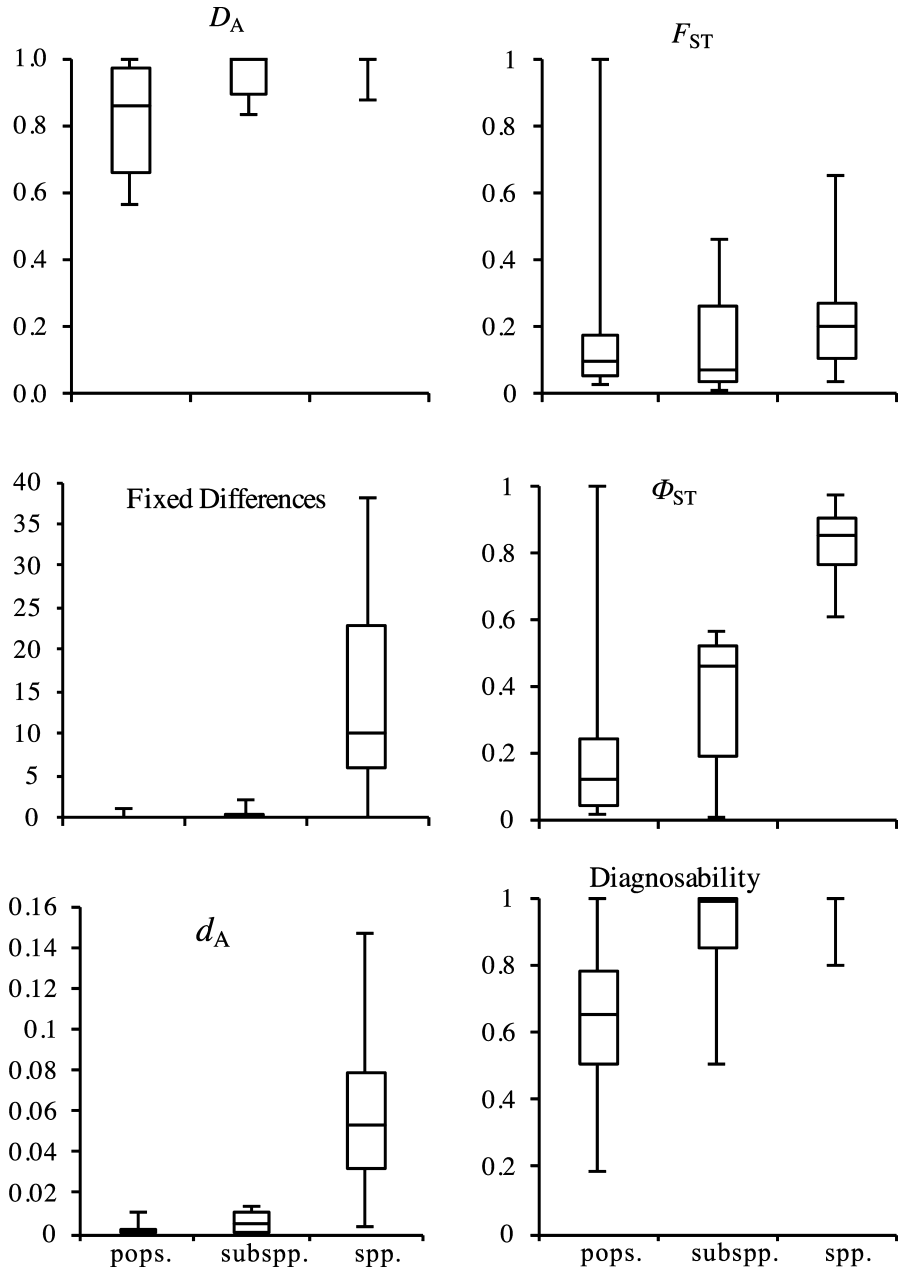

Figure 1. Box and whisker plots showing median and 1st and 3rd quartiles, and minimum and maximum values for six metrics of genetic divergence among cetacean population, subspecies, and species pairs estimated using mitochondrial DNA control region sequence data.

North Pacific *P. macrocephalus*, western Atlantic and Gulf of Mexico *Tursiops*, Hawaii insular and Pacific pelagic *Feresa*, and the two killer whale population comparisons, and the subspecies comparisons for both *Stenella* species, as well as one *L. obscurus* subspecies pair.

For d_A, population comparisons ranged between 0.00007 and 0.0039 with one outlier significantly above this range (d_A = 0.0108) for the comparison of coastal populations of common bottlenose dolphins in the western North Atlantic and Gulf of Mexico. The majority of the subspecies comparisons ranged from 0.0048 to 0.014, but four outliers fell between 0.00015 and 0.0013, more indicative of population-level comparisons. Species comparisons ranged between 0.029 and 0.147 with one outlier (0.0033 for the comparison of *Neophocaena phocaenoides* to *N. asiaeorientalis*). After examining the data, we chose threshold values of 0.004 and 0.02 for subspecies and species, respectively. Overall, Nei's d_A exhibited the fewest classification errors, and all but one (populations of common bottlenose dolphins in the western North Atlantic) were underclassification errors. These included subspecies of *S. attenuata*, *S. longirostris*, *L. obscurus*, and *G. melas*, and the *Neophocaena* species comparison.

In the Random Forests analysis, 5 of 11 subspecies pairs were "overclassified" (100% diagnosability) and two were underclassified, *i.e.*, exhibited diagnosabilities <80% (*S. longirostris* and *S. attenuata* subspecies). Thus, all but two of the subspecies pairs were distinguishable from populations using this metric and threshold, but percent diagnosable was not a suitable metric to distinguish subspecies from species. All species comparisons except one (*Neophocaena* sp.) exhibited 100% diagnosability.

Discussion

Comparing genetic divergence estimates to identify whether a pair of taxa has crossed a threshold worthy of a higher taxonomic status is not a new endeavor (see for example, Bradley and Baker 2001). For marine mammals, conclusions about species status have been drawn after comparing the estimate of genetic sequence divergence for the study organisms to estimates from the literature for accepted species (*e.g.*, Caballero *et al.* 2007, Frère *et al.* 2008). The DNA barcoding initiative also used this basic concept for assigning specimens to species and for identifying new species (Hebert *et al.* 2003, 2004), promoting the use of a universal "barcoding gap" in the mtDNA cytochrome oxidase I gene (*cox1*) to distinguish populations from species. As more *cox1* data were collected and more taxa were examined, it became clear that neither a single gene nor a single, universal threshold was going to be sufficient for barcoding in all eukaryotes, cetaceans included (Amaral *et al.* 2007, Viricel and Rosel 2012). Furthermore, in order for such a comparative method to have broad value, the data and metric used and the method used to calculate the metric all need to be consistent across studies, yet Rosel *et al.* (2017) found that such consistency was generally lacking in the cetacean literature. By compiling this data set of cetacean mtDNA control region sequences, we are able to make analogous comparisons of different divergence metrics and of the ability of those metrics to distinguish among populations, subspecies, and species of cetaceans. Overall, we found that most commonly used metrics were not informative, but that Nei's d_A performed well under the conditions we explored here.

Divergence Metrics That Performed Poorly

Of the metrics tested, Nei's D_A, F_{ST}, and number of fixed differences performed poorly in terms of providing clear thresholds for the population-subspecies and subspecies-species boundaries. Nei's D_A has the characteristic that if there are no shared haplotypes between strata, D_A will be one even if there are no fixed differences between the strata. Thus, many D_A values in our comparisons were quite high, producing significant overlap among all comparisons (Fig. 1) and rendering this metric impractical for our purpose.

It is also not surprising that F_{ST} does not serve as a good proxy for net divergence between two taxa at the higher taxonomic levels we considered here. It has been shown that correlations between F_{ST} and gene flow (Nm) and between F_{ST} and other measures of evolutionary divergence are relatively poor (Martien *et al.* 2017, Lessios and Robertson 2006, Hey and Pinho 2012, Marko and Hart 2012). Hey and Pinho (2012) examined values of F_{ST}, $2Nm$ (the population migration rate), and τ (the time since divergence) across a broad taxonomic range of population and species pairs (they lumped subspecies pairs in with the species pairs) of mammals, birds, invertebrates, and plants. They found significant overlap in F_{ST} values for these comparisons. Lessios and Robertson (2006) examined differentiation between 20 pairs of tropical fish populations on either side of the eastern Pacific. Many population pairs exhibited the expected coupling of low differentiation (F_{ST}) and high gene flow and *vice versa* (Lessios and Robertson 2006). However, several pairs exhibited conflicting estimates: high levels of divergence and high levels of gene flow, or little or no differentiation coupled with low estimates of gene flow. These patterns suggest the expected correlation of high divergence with low gene flow may not always be met. A common issue is that the underlying assumption in estimating F_{ST}—that of mutation-drift equilibrium following a Wright island model—is often violated in taxa that are diverging and may be experiencing different selective pressures. Marko and Hart (2012) concluded that variation in isolation time and in effective population size can be more important than gene flow for explaining patterns of population differentiation and therefore inferences based on gene flow, and hence F_{ST}, may be misleading. Even though our pairwise comparisons involved a focused taxonomic group (cetaceans) with similar life history characteristics, we also saw broad overlap in F_{ST} estimates for population, subspecies, and species pairs. Thus, while an important metric for examining and understanding population structure, F_{ST} did not prove useful for delimiting cetacean subspecies or species.

Fixed differences are often recommended as evidence for species-level differences because fixed differences among haplotypes in two groups suggest a lack of gene flow, particularly in parapatry and sympatry (Milinkovitch *et al.* 2001). Fixed differences provide a means to diagnose a species and are, in fact, the underlying basis for the phylogenetic species concept (Eldredge and Cracraft 1980). With one exception, cetacean species pairs exhibited 4–38 fixed differences between them, and this is an expected result as most recognized species are likely to be well along independent evolutionary pathways and thus exhibit fixed differences in their DNA sequences. The one exception involved the *Neophocaena* species pair, which exhibited no fixed differences. These species are thought to be of recent origin, having diverged at the last glacial period ~18,000 yr ago (Wang *et al.* 2008) and they share a control region haplotype. However, there are morphological traits that separate the two species and

Wang *et al.* (2008) concluded that the recent divergence time has resulted in incomplete lineage sorting among mtDNA haplotypes between the two species. Given the shared haplotypes and low level of genetic divergence, all metrics incorrectly classified this pair.

The difficulty inherent in using fixed differences arises at the lower taxonomic boundary. Subspecies lie along a continuum of genetic divergence between populations and species and do not have to be reproductively isolated from other subspecies of that species (Patten 2010), *i.e.*, gene flow is allowed, though not mandatory, among subspecies (Taylor *et al.* 2017*b*). Thus, there should not be an expectation nor requirement of fixed differences between subspecies, making separation of populations, which are also not expected to exhibit fixed differences, and subspecies difficult using this metric. Comparisons among cetacean taxa support this conclusion as no fixed differences were seen among almost all population and many subspecies comparisons. At the population level, the two killer whale population pairs each exhibited one fixed difference between them. These populations are relatively small and killer whales exhibit strong matrilineal-based social structure. These two characteristics can exert a strong force on lineage sorting and lead quickly to fixed differences in sequence data, particularly in the maternally inherited mitochondrial genome, even in the presence of genetic exchange between populations. The killer whale population comparisons provide a good example highlighting the impacts that demographic histories, in this case social structure, can have on genetic diversity and divergence. It should also be noted that several well-accepted subspecies pairs did exhibit 1–2 fixed differences between them, suggesting that the presence of one or two fixed differences alone may not make a strong enough case for species designation in cetaceans.

Φ_{ST} performed better at correctly identifying taxonomic levels than the previous three metrics. As a distance-based metric, it captures divergence and not simply differentiation based on frequency differences. However, where it performed poorly it primarily produced overclassification errors, classifying some populations and subspecies as species (Fig. 2). This is an undesirable characteristic as it can result in taxonomic inflation. As an analog of F_{ST}, it is constrained by the same underlying assumption of mutation-drift equilibrium and the possibility that the interplay of effective population size, gene flow, and drift could produce similar estimates under disparate evolutionary scenarios. Unlike d_A (discussed below), Φ_{ST} is also constrained to an upper limit of one. This upper limit is reached when groups are fixed for different haplotypes, such as in the case of the killer whale population pairs. In cases where two groups do not share haplotypes but there is within group polymorphism, the upper limit of Φ_{ST} will be something less than one and correlated to the level of haplotypic diversity within the groups.

Net Nucleotide Divergence (d_A) *and Percent Diagnosability*

Within cetaceans, d_A worked remarkably well in distinguishing among the three taxonomic levels when applied to mtDNA control region sequence data. For d_A and the threshold values chosen for this study, there were six classification errors. As discussed earlier, the *Neophocaena* species pair failed at all metrics (always underclassified). At the subspecies level, d_A exhibited four underclassification errors where pairs exhibited d_A values of a magnitude seen within population pair comparisons (Fig. 2) and two of these pairs also did not meet the diagnosability criterion of 80% as suggested by Archer *et al.* (2017). Three of these misclassified pairs involved comparisons that we had included because we expected *a priori* they might perform poorly,

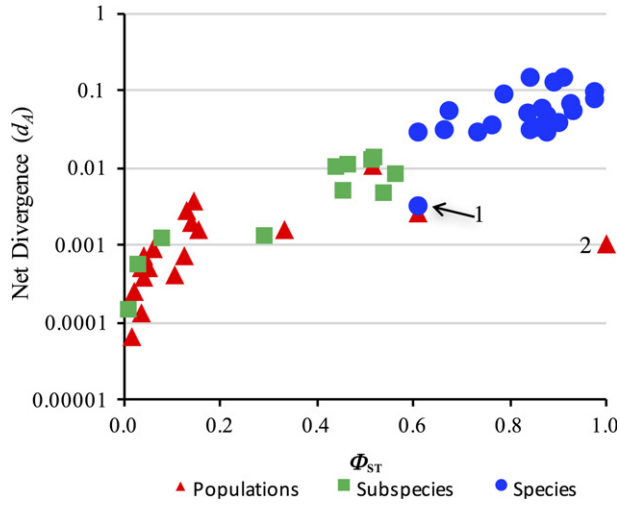

Figure 2. Relationship between Φ_{ST} and Nei's estimate of net divergence (d_A) among cetacean population, subspecies, and species pairs estimated using mitochondrial DNA control region sequence data. Specific values mentioned in the text are numbered: 1 = *Neophocaena* species; 2 = killer whale populations. The three green squares in the left-hand side of the figure ($\Phi_{ST} < 0.07$) represent, from bottom to top, the subspecies comparisons for *S. attenuata*, *S. longirostris*, and *L. obscurus*, respectively.

namely comparisons involving taxa with large N_e and a comparison within a highly social species. The subspecies of *S. attenuata* and *S. longirostris* have been well studied and exhibit diagnosable morphological differences between them (Perrin *et al.* 1987; Perrin 1990, 2009*a, b*). Abundance estimates for each of these two species in the Pacific are greater than one million (Gerrodette *et al.* 2008). Haplotype diversity estimates from our data sets for these species are also quite high, 0.97–0.99, reflecting the large population sizes. As described earlier (see also Martien *et al.* 2017), genetic drift acts much more slowly on large populations than small populations and fewer migrants are needed to counteract its effects in large populations. As a result, genetic divergence between large populations *via* genetic drift is a much slower process and will be more difficult to detect using a strictly divergence-based metric and the underclassification errors observed for the *Stenella* species are not unexpected. Interestingly, the diagnosability measure was more successful with these taxa.

The long-finned pilot whale subspecies met the 80% diagnosability threshold, but were incorrectly classified using d_A. Social structure in pilot whales may play a role in this as it results in stable pods that are matrilineally directed. Genetic diversity has been found to be extremely low in pilot whales and other social species like killer whales (*Orcinus orca*), particularly for mitochondrial DNA because it is maternally inherited (Oremus *et al.* 2009). Haplotype diversity for *G. melas* overall was 0.025. Only two other strata had lower estimates—the critically endangered vaquita, (*Phocoena sinus*) and Maui's dolphin (*C. hectori maui*). The low haplotypic diversity, coupled with haplotypes that exhibit little differentiation, will minimize d_A values and could thereby cause classification errors.

One of the three *Lagenorhynchus obscurus* subspecies comparisons (*L. o. fitzroyi vs. L. o. obscurus*) also exhibited a net d_A more indicative of a population than a subspecies

pair. To our knowledge, this species does not exhibit the kind of social structure seen in killer whales or pilot whales, nor do we expect it to have large abundance, although no worldwide abundance estimate is available. Furthermore, one would expect social structure or large abundance to affect all three pairwise comparisons involving *L. obscurus* subspecies. This particular pair has some of the smallest sample sizes in our data set, which may cause this result, reinforcing the need for adequate sampling in these studies. If small sample size was the cause for the low d_A value, it did not have as much of an effect on diagnosability, as this subspecies pair exhibited an 86% correct classification rate. Further examination of this subspecies pair is warranted.

Percent diagnosable performed well to separate subspecies from populations in all but a few cases at our threshold of 80%. The two subspecies cases that failed were again the two *Stenella* species, both of which, as described above, have relatively large N_e. Interestingly, the cases of populations being incorrectly overclassified (killer whales and false killer whales) have values for d_A that would correctly classify them as populations. In contrast, the two subspecies pairs with percent diagnosable at values consistent with population levels also had values of d_A indicative of populations. Therefore, applying percent diagnosability to mtDNA control region data and using that as a single line of evidence could result in underclassification errors but the empirical comparisons examined here did not reveal cases of overclassification. The number of cases that fail, will of course, depend on the threshold chosen (see further discussion in Taylor *et al.* 2017*a*). The definition of subspecies requires evidence that the putative subspecies is diagnosable *and* is a separately evolving lineage. Percent diagnosability pertains to the former but is not a suitable metric for the latter since both subspecies and species can be 100% diagnosable.

Conclusion

As illustrated by the considerable body of literature on DNA barcoding, attempting to identify a genetic distance threshold to use for determining whether two groups belong to separate species is not without controversy. Ferguson (2002) argued that genetic divergence should not be used for this purpose because its use is tied to the biological species concept (Mayr 1942), making it dependent on underlying assumptions of speciation *via* reproductive isolation and because a single value cannot be applied consistently across a wide range of taxa. Fregin *et al.* (2012) described additional mechanical problems associated with identifying a universal threshold using genetic distance data from the literature. Differences in choice of locus, sequence length, and choice of substitution model to correct genetic distance estimates (although Collins *et al.* (2012) suggest the latter is less of a problem) render comparisons across studies suspect. These are all valid arguments against use of a common genetic divergence threshold. However, in these cases the search has been for a threshold value that could be universally applied broadly across taxa, as is the goal in the widely accepted field of DNA barcoding. Our study has the advantage in that it is much more circumscribed; we examined metrics and thresholds and promote our results only in the context of cetaceans, the Tamura-Nei substitution model, and the first 300–500 bp of the mtDNA control region. This latter point is critical. Increased sequence length should result in detection of more variable positions between groups, but this increase is not expected to be linear. Instead, it will depend on underlying substitution rates, which are not constant across the control region. As a result, estimating d_A using full control region sequences (~900–1,000

bp) can result in a different range of values from those calculated here. This fact must be taken into consideration when making comparisons across studies.

Our analysis illustrates that several biological features seen in some cetacean taxa, including recent divergence, large effective population size, and social structure, can result in errors using control region sequence data regardless of what metric is used. Nevertheless, Nei's (1987) measure of net genetic divergence, d_A, worked well for distinguishing among taxonomic levels, particularly in separating species from subspecies and populations, based on the metrics we used in this empirical evaluation using the control region in cetaceans. Thus, it may serve as a useful metric, under the conditions explored here, for hypotheses about the taxonomic status of a group. However, robust taxonomic arguments will require more than a simple estimate of d_A based on mtDNA control region sequences (or any single gene for that matter). Following a more integrative approach to taxonomy and coupling the information on d_A presented here with a measure of diagnosability and demographic information, Taylor *et al.* (2017*a*) suggest a set of guidelines and standards that may be used to create a robust argument for cetacean subspecies delimitation, including a decision tree that utilizes d_A. As described in Reeves *et al*. (2004) species delimitation requires additional evidence of independence among evolutionary lineages, for example nuDNA, morphological data, *etc.*; mtDNA data alone are insufficient.

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Supporting Information

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Table S1. Accession numbers for sequences used in alignments.

Table S2. Alignment information.