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Fin whale (*Balaenoptera physalus*) mitogenomics: A cautionary tale of defining sub-species from mitochondrial sequence monophyly

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**ABSTRACT**

The advent of massive parallel sequencing technologies has resulted in an increase of studies based upon complete mitochondrial genome DNA sequences that revisit the taxonomic status within and among species. Spatially distinct monophyly in such mitogenomic genealogies, i.e., the sharing of a recent common ancestor...
1. Introduction

Genealogies estimated from mitochondrial DNA (mtDNA) sequences have been employed towards resolving inter- and intraspecific taxonomic relationships for more than three decades (Avise, 1989; Ball and Avise, 1992; Burbank et al., 2000). Taxonomic assessments aimed below the nominal species level usually focus on the spatial distinctiveness of monophyletic clades in genealogies estimated from mtDNA sequences, i.e., the presence of phylogeographic structure (Avise et al., 1979, 1987; Ball and Avise, 1992). The presence of spatially confined monophyletic mitochondrial clades has typically been inferred as evidence for a high level of reproductive isolation and consequently some degree of evolutionary distinctiveness. Evolutionary significant units (ESUs) serve as an illustrative example (Ryder, 1986; Bernatchez, 1995). ESUs are generally viewed as distinct components of intraspecific genetic diversity (Ryder, 1986; Bernatchez, 1995). Moritz (1994) proposed that ESUs were defined by their reciprocal monophyly, specifically in genealogies estimated from mtDNA sequences. In addition, allele frequencies at nuclear loci should be “significantly” different between reciprocally monophyletic mtDNA sequence clades. When monophyly in a mtDNA genealogy is employed as the defining criterion, a key question becomes whether this level of phylogeographic structure always equates to isolation and evolutionary distinctiveness. In other words, does the absence of monophyly implies a recent common ancestry and evolutionary indistinguishability? Paetkau (1999) pointed to the fact that the effective population size and the time to the most recent common ancestor (TMRCA) are positively correlated. This fundamental relationship implies that isolated populations at low effective population sizes will become monophyletic faster than populations with large effective population sizes. This difference has immediate ramifications in those cases when mtDNA monophyly is employed as the main, or sole, criterion in defining ESUs (e.g., Banguera-Hinestroza et al., 2002; Archer et al., 2013).

Similarly, low sampling effort may lead to erroneous conclusions when defining ESUs from spatial monophyly in mitogenetic genealogies. Apparent monophyly could be simply a product of insufficient sampling, i.e., an insufficient number of specimens to capture all mtDNA clades (Funk and Omland, 2003). Intraspecific genealogies inferred from mtDNA sequences often contain multiple well-supported clades. However, the relative proportions of such clades typically vary across space. Consequently, insufficient sampling in all, or some regions, may result in failure to sample DNA sequences belonging to uncommon clades, erroneously leading to the conclusion of monophyly (Funk and Omland, 2003).

Initially most phylogeographic studies were based solely upon genealogies inferred from mtDNA sequence variation (Ball and Avise, 1992; Burbank et al., 2000; Pons et al., 2006). The mtDNA genome (mitogenome) was viewed as especially suitable for this kind of assessments due to its haploid, often maternal and clonal inheritance, among con-specific samples collected in the same region has been viewed as evidence for subspecies. Several recent studies in cetaceans have employed this criterion to suggest subsequent intraspecific taxonomic revisions. We reason that employing intra-specific, spatially distinct monophyly at non-recombining, clonally inherited genomes is an unsatisfactory criterion for defining subspecies based upon theoretical (genetic drift) and practical (sampling effort) arguments. This point was illustrated by a re-analysis of a global mitogenomic assessment of fin whales, Balaenoptera physalus spp., published by Archer et al. (2013), which proposed to further subdivide the Northern Hemisphere fin whale subspecies, B. p. physalus. The proposed revision was based upon the detection of spatially distinct monophyly among North Atlantic and North Pacific fin whales in a genealogy based upon complete mitochondrial genome DNA sequences. The extended analysis conducted in this study (1676 mitochondrial control region, 162 complete mitochondrial genome DNA sequences and 20 microsatellite loci genotyped in 380 samples) revealed that the apparent monophyly among North Atlantic fin whales reported by Archer et al. (2013) to be due to low sample sizes. In conclusion, defining sub-species from monophyly (i.e., the absence of para- or polyphyly) can lead to erroneous conclusions due to relatively “trivial” aspects, such as sampling. Basic population genetic processes (i.e., genetic drift and migration) also affect the time to the most recent common ancestor and hence the probability that individuals in a sample are monophyletic.

Among highly derived mammals, which has recently been subjected to several reassessment of species/subspecies status based upon the estimation of intraspecific genealogies from complete mitogenome sequences (Morin et al., 2010; Archer et al., 2013; Meng et al., 2013) remains considerably lower compared to contemporary studies based upon Sanger (1981) DNA sequencing of smaller mtDNA regions and nuclear loci (Pastene et al., 2007; Halbert et al., 2013; Jackson et al., 2014). These two aspects, relying solely on mitogenome sequence data (Zachos et al., 2013) and low sample sizes, implies that the detection of monophyly is prone to the caveats that haunted earlier, similar studies based upon shorter mtDNA sequences, such as the mtDNA control region (CR). Studies based upon complete mitogenome sequences typically yield very high support for the basal nodes, leading to the impression of high accuracy. However, high accuracy in a single-locus genealogy does not necessarily imply that the genealogy accurately reflects the population/subspecies history as has been pointed out by numerous authors in the past (Pamilo and Nei, 1988; Page and Charleston, 1997; Leaché, 2009).

A case in point is Cetacea (whales, dolphins and porpoises), a group of highly derived mammals, which has recently been subjected to several reassessment of species/subspecies status based upon the estimation of intraspecific genealogies from complete mitogenome sequences (Morin et al., 2010; Vilstrup et al., 2011; Archer et al., 2013). The large body sizes, wide ranges and limited availability of osteological specimens in most cetacean species has made it difficult to apply traditional, non-molecular approaches to define intra-specific taxonomic entities and explains the popularity of molecular-based taxonomic assessments in cetaceans. Most baleen whale (Mysticeti) species have global distributions and migrate seasonally between low latitude winter breeding grounds and high latitude summer feeding grounds (Ingebrigtsen, 1929; Dawbin, 1966; Jongsgård, 1966; Katona and Whitehead, 1981). As a result, most baleen whale populations roam across entire ocean basins making it challenging to delineate intra-
specific evolutionary units. Two aspects are generally assumed, a priori, to confine baleen whale distributions and restrict gene flow. The anti-

tropical distribution of most baleen whale species presumably acts as a re-

productive barrier between the two hemispheres, despite the (prox-

imate) low latitude locations of winter breeding grounds, because the 

breeding season for each hemisphere is separated by half a year (Davis

et al., 1998). In addition, most ocean basins are separated by the con-

tinents, which prevent inter-oceanic dispersal as well. Consequently, it 

is generally assumed that gene flow between con-specific baleen whale 

populations in different ocean basins is very limited (Valsecchi et al., 

1997; Bérubé et al., 1998; Pastene et al., 2007; Jackson et al., 2014).

Accordingly, current, recognized baleen whale species and subspecies 

designations typically correspond to ocean basins or hemispheres. For 

instance, the right whales are comprised of Eubalaena glacialis, in the 

North Atlantic; E. australis, in the Southern Hemisphere; and E. japonica, 

in the North Pacific (Rice, 1998; Rosenbaum et al., 2000). Similarly 

Northern Hemisphere blue whales, Balaenoptera musculus, are classified 

as B. m. musculus and Southern Hemisphere blue whales as B. m. in-

termedia, in addition to the pygmy blue whale, B. m. brevicauda (Rice, 

1998).

The fin whale, Balaenoptera physalus spp. (Linnaeus, 1758), is a 

common and globally distributed baleen whale (Gambell, 1985). Fin 

whales in the Northern Hemisphere are classified as belonging to the 

subspecies B. p. physalus and fin whales in the Southern Hemisphere to 

B. p. quayi (Fischer, 1829). The fin whale subspecies designations were 

based upon differences in the vertebrate characteristics (Lönnberg, 

1931) as well as traits correlated with body size (Tomilin 1946 cited by 

Rice, 1998). Employing this classification, North Pacific and North 

Atlantic fin whales both belong to the same subspecies, despite the 

observation that gene flow between the two ocean basins is unlikely, at 

least since the rise of the Panama Isthmus approximately 3.5 million 

years ago (Coates et al., 1992). Recently, Archer et al. (2013) employed

complete mitogenome sequences from North Atlantic, North Pacific and 

Southern Hemisphere fin whale specimens to assess the current sub-

species status of Northern Hemisphere fin whales. Archer et al. (2013) 

concluded that North Atlantic and some North Pacific fin whale cons-

stituted separate subspecies. This conclusion was based upon the ob-

servation of a single monophyletic clade that contained all North 

Atlantic specimens (a sample of 14 specimens), and the presence of 

several monophyletic clades containing solely North Pacific specimens 

(Fig. 3a).

The results of Archer et al.’s (2013) mitogenomic analysis appeared at 

odds with previous phylogeographic assessments by Bérubé et al. 

(1998, 2002). Bérubé and colleagues based their assessments upon DNA 

sequences from the highly variable mtDNA CR. Their study identified 
three mtDNA CR haplotypes in North Atlantic specimens that clustered 

together with mtDNA CR haplotypes identified among North Pacific 

specimens. Bérubé and co-workers inferred this result as evidence for 

recent gene flow between the North Atlantic and North Pacific (Bérubé 

et al., 1998, 2002) likely in a stepping stone manner via the Southern 

Ocean. In order to resolve the discrepancy between the above-men-
tioned studies and the support for the taxonomical revision proposed by 

Archer et al. (2013), this study extended the sample size of North 

Atlantic Ocean (including the Mediterranean Sea) fin whales from the 

34 mtDNA CR sequences analyzed by Archer et al. (2013) to a total of 

828 mtDNA CR sequences. The complete mitogenome was sequenced in 
a subset (n = 6) of North Atlantic specimens, including mtDNA CR 

haplotypes that clustered with mtDNA CR haplotypes detected in spec-

imens sampled outside the North Atlantic. In addition, 20 micro-
satellite loci were genotyped in 358 specimens from the North Atlantic, 

North Pacific and Southern Hemisphere. The re-estimation of the gen-

ealogy based upon the complete mitogenome sequences from this study 
showed all ocean basins to be polyphyletic. In other words, these results 
did not support the current nor the proposed subspecies if monophyly

Fig. 1. Sampling location from the North Atlantic, North Pacific and Southern Hemisphere fin whales. Notes: Approximate sampling locations in the North Atlantic (NATL, red), North Pacific (NPAC, blue) and the Southern Hemisphere (SHEM, green). The numbers represent the total number of mitochondrial control region DNA sequences from each region. Numbers in black denote the sample sizes from the Mediterranean Sea (MED, included in NATL) and the Sea of Cortez (SOC, included in NPAC). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
in a genealogy estimated from mtDNA sequences is employed as the sole or main defining criterion. The basal topology of the genealogies estimated from the mitogenome and mtDNA CR sequences were qualitatively similar as expected given that the mitogenome represents one linked locus. The assignment test based on the genotype of 20 microsatellite loci revealed that the North Atlantic specimens from the two different mitochondrial DNA clades all belonged to the same North Atlantic gene pool.

The findings of this study highlight the implications of insufficient sampling when attempting to identify monophyletic clades from mtDNA sequences. However, the results did not negate the possibility that fin whales from different ocean basins could potentially represent different subspecies, although the analysis from this study revealed recent gene flow between fin whales from different ocean basins and hemispheres.

More generally, employing monophyly in genealogies based upon DNA sequences from non-recombining genomes to classify subspecies ignores fundamental population genetic processes as well as key practical issues. These caveats make the approach less valid than its current widespread use suggests. Although these caveats have been highlighted earlier (Paetkau, 1999; Funk and Omland, 2003), the approach has nevertheless regained momentum given the ease of applying massive parallel sequencing technologies to unparentally inherited, non-recombining genomes, such as the mitogenome.

2. Materials and methods

2.1. Sample collection

Tissue samples were obtained from fin whales in the North Atlantic Ocean basin and the Mediterranean Sea (henceforth referred to collectively as the North Atlantic); the North Pacific Ocean basin and the Sea of Cortez (henceforth referred to collectively as the North Pacific) as well as the Southern Hemisphere between 1982 and 2014 (Fig. 1). Most tissue samples were collected as skin biopsies from free-ranging fin whales as described by Palmsbøll et al. (1991). The tissue samples originating from Iceland and Spain were collected during whaling operations prior to the international moratorium on commercial whaling. Some samples collected in Greenland originated from local subsistence whaling and some samples collected in US waters from dead, beached individuals. All samples were collected in agreement with national and international regulations. Samples were preserved in 5 M NaCl with 20% dimethyl sulfoxide and stored at −20 degrees Celsius (Amos and Hoelzel, 1991).

2.2. Mitochondrial DNA sequence data

The mtDNA sequence data were either generated during this study or obtained from the study by Archer et al. (2013) who deposited the data in the Dryad repository (https://doi.org/10.5061/dryad.084g8). The experimental methods used to generate the published data were described by Archer et al. (2013).

The DNA sequence data generated for this study were obtained in the following manner. Total-cell DNA was extracted from tissue samples either by phenol/chloroform extraction as described by Sambrook and Russell (2001) or using the Qiagen DNAEase™ Blood and Tissue Kit columns (QIAGEN Inc., Valencia, CA, USA) following the manufacturer’s instructions. Samples were sexed using the ZFY/ZFX multiplexing system as described by Bérubé and Palmsbøll (1996b); Bérubé and Palmsbøll (1996a). MtDNA CR DNA sequencing was performed as previously described either by (i) Palmsbøll et al. (1995), but replacing the original reverse primer with BP16071, (Drouet et al., 2004); or (ii) Bérubé et al. (2002). The complete mitogenome was sequenced in eight selected specimens. These specimens were selected from genealogy estimated from all mtDNA CR haplotypes. Six specimens were selected among the 33 North Atlantic specimens with mtDNA CR haplotypes that clustered with specimens sampled in other ocean basins. The remaining two specimens were selected among specimens from the North Atlantic and North Pacific Ocean, respectively, with mtDNA CR haplotypes that clustered with other specimens sampled in the same ocean within monophyletic clades. A total of 35 nested primer pairs were employed (Supplementary Materials, Table S1) to amplify and determine the DNA sequence of the complete fin whale mitogenome from partially overlapping ~500 base pair (bp) fragments. PCR (Mullis and Faloona, 1987) amplifications and DNA sequencing were performed under conditions identical to those described above for the mtDNA CR sequencing albeit at different annealing temperatures (Supplementary Materials, Table S1). Phred quality scores were employed to assess the sequence quality and discard low-quality sequences (phred score below 20). The quality of the previously published data is described by Archer et al. (2013).

2.3. Microsatellite genotyping

The genotype was determined at 20 diploid, autosomal microsatellite loci in samples from four different regions; the North Atlantic (including the Mediterranean Sea) (n = 288), the Eastern North Pacific (n = 25), the Sea of Cortez (n = 46) and the Southern Hemisphere (i.e., the Antarctic, n = 21). The specific microsatellite loci were: AC087, CA234 (Bérubé et al., 2005), EV00, EV037, EV094 (Valacichi and Amos, 1996), GATA028, GATA098, GATA417 (Palmsbøll et al., 1997), GATA25072, GATA43950, GATA5947654, GATA6063318, GATA91083 (Bérubé et al., in prep), GT011 (Bérubé et al., 1998) GT023, GT211, GT271, GT310, GT575, (Bérubé et al., 2000) and TAA023 (Palmsbøll et al., 1997) with tetra-, tri- or di-mer repeat motifs (Supplementary Materials, Table S2). Individual PCR amplifications were performed in 10 μL volumes, each containing ~2–10 ng of extracted DNA, 0.2 μM of each oligo-nucleotide primer (Supplementary Materials, Table S2) and 1X final QIAGEN Microsatellite PCR Multiplex Mix™ (Qiagen Inc.). Thermo-cycling was carried out on a MJ Research PTC-100™ Thermal Cycler (BioRad Inc.). The PCR amplification consisted of an initial step of five minutes at 94 degrees Celsius, followed by 35 cycles; each of 30 s at 95 degrees Celsius, 90 s at 57 degrees Celsius and 30 s at 72 degrees Celsius. The final step was 10 min at 68 degrees Celsius. PCR reactions were diluted 60 times with MilliQ water and then 1 μL of diluted PCR reaction was added to 9 μL of GeneScan-500™ ROX (Applied Biosystems Inc.) and deionized formamide (GeneScan-500™ ROX 1 mL; 70 μL) prior to electrophoresis on an ABI 3730 Genetic Analyzer™ (Applied Biosystems Inc.). The length of each amplification product was determined using GeneMapper™ ver. 4.0 (Applied Biosystems Inc.).

2.4. Assembly and analysis of the mitochondrial DNA sequences

MtDNA sequences were aligned and assembled against the fin whale mitogenome sequence deposited in GenBank™ (accession # NC001321) by Arnason et al. (1991) using SeqMAN™ (ver. 5.05, DNASTAR Inc.) with default parameter settings. All DNA sequences were trimmed to equal length, i.e., 16,423 and 285 bp for the mitogenome and mtDNA CR DNA sequences, respectively.

Estimation of mtDNA haplotype genealogies and divergence times: The genealogies of the mtDNA CR and complete mitogenome haplotypes as well as divergence times, were estimated employing the software BEAST (ver. 1.8.2, Drummond and Rambaut, 2007; Drummond et al., 2012) largely following the approach by Archer et al. (2013). However, in contrast to Archer et al. (2013), only a single copy of each haplotype from each ocean basin (both complete and CR mtDNA sequences) was included in each data set. Insertion and deletions were coded as a fifth character. Genealogies were rooted with the homolog DNA sequence from humpback whale, Megaptera novaeangliae, (GenBank™ accession # NC006927, Sasaki et al. (2005)) using the alignment reported by Archer et al. (2013). The most probable nucleotide
Estimation of genetic diversity and immigration rates: The software MIGRATE-N (ver. 3.6.6, Beerli and Felsenstein, 1999, 2001) was employed to estimate the genetic diversity (θ) and immigration rate scaled by the generational mutation rate (M) per nucleotide site among the North Atlantic, North Pacific and Southern Hemisphere. The prior ranges of θ and M were determined from preliminary estimations with reduced sample sizes and short MCMC chains with the F∞ -based method as starting values. The prior ranges were subsequently adjusted according to the outcomes of these preliminary estimations, i.e., θ (uniform prior, min: 0, max: 0.25, ∂: 0.25) and M (uniform prior, min: 0, max: 250.0, ∂: 25.0). Data sets above 100 DNA sequences randomly sub-sampled (without replacement) at sample sizes of 100 DNA sequences per sample partition. Due to significant levels of intra-ocean population structure in mtDNA DNA sequence variation (Bérubé et al., 2002). Among the 1676 mtDNA CR DNA sequences, 428 DNA sequences were obtained from Archer et al. (2013) and 1248 were generated for this study. A total of 410 mtDNA CR sequences from the 782 North Pacific were collected in the Sea of Cortez, a population with low mtDNA sequence diversity (Bérubé et al., 2002). Among the 828 North Atlantic mtDNA CR sequences, 115 were collected in the Mediterranean Sea (Fig. 1). A total of 161 haplotypes were detected among the 1676 fin whale mtDNA CR sequences, (Table 1) and 147 haplotypes among the 162 complete mitogenome sequences of which 154 were published by Archer et al. (2013) and eight generated during this study (Table 1).

Both genealogies estimated from the complete mitogenome and mtDNA CR haplotypes published by Archer et al. (2013) identified a single monophyletic clade containing all, and only, North Atlantic specimens (denoted NA clade in Figs. 2a and 2a). In contrast, haplotypes detected in North Pacific and Southern Hemisphere specimens were polyphyletic (Figs. 2a and 3a). In contrast, the genealogies estimated from the complete mitogenome and mtDNA CR haplotypes including all data, i.e., both the data generated for this study and those published by Archer et al. (2013), partitioned the North Atlantic specimens in two major clades; one clade (denoted NA clade in Fig. 2b) comprised solely of North Atlantic specimens and another clade comprised DNA sequence haplotypes detected in specimens from the North Pacific and Southern Hemisphere, in addition to the North Atlantic

### Table 1

Mitochondrial DNA control region (mtDNA CR) and complete mitochondrial DNA genome (mitogenome) sequences and haplotypes per ocean basin.

<table>
<thead>
<tr>
<th></th>
<th>North Atlantic</th>
<th>North Pacific*</th>
<th>Southern Hemisphere</th>
<th>Total</th>
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<td>HAP</td>
<td>SEQ</td>
<td>HAP</td>
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<td>13</td>
<td>346</td>
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<td>This study</td>
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<td></td>
<td>Combined data</td>
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<td>80</td>
<td>782</td>
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<tr>
<td>Complete mitogenome</td>
<td>Archer et al. (2013)*</td>
<td>14</td>
<td>12</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>This study</td>
<td>7</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Combined data</td>
<td>21</td>
<td>17</td>
<td>98</td>
</tr>
</tbody>
</table>

* The data was recreated from the sample information file (http://datadryad.org/bitstream/handle/10255/dryad.48318?bgys%20sample%20info.csv?sequence = 1) deposited by Archer et al. (2013) in the Dryad data repository. The file contains GenBank accession numbers for each sample entry (either only mtDNA CR sequence (n = 274), or the mtDNA CR sequence extracted from the complete mitogenome sequence (n = 154). SEQ: number of sequences, HAP: number of unique haplotypes.

* North Atlantic including Mediterranean Sea.

* North Pacific including Sea of Cortez.
Fig. 2. Bayesian genealogy estimated from North Atlantic, North Pacific and Southern Hemisphere fin whale mitochondrial control region (mtDNA CR) haplotypes. Notes: Genealogies were estimated from (a) 82 mtDNA CR haplotypes reported by Archer et al. (2013) and (b) 161 mtDNA CR haplotypes reported by Archer et al. (2013) combined with additional mtDNA CR haplotypes reported in this study. Colors represent the three ocean basins/regions: the Southern Hemisphere (green, denoted SHEM), the North Pacific (blue, denoted NPAC) and the North Atlantic (red, denoted NATL), respectively. Numbers at basic nodes denotes the posterior probability of the specific node (only the support for basic nodes is reported). A humpback whale (Megaptera novaeangliae) mtDNA CR haplotype (Genbank NC_006927) was employed to root the tree (not shown). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Fig. 3. Bayesian genealogy estimated from North Atlantic, North Pacific and Southern Hemisphere fin whale mitochondrial genome (mitogenome) haplotypes. Notes: Genealogies were estimated from (a) 142 mitogenome haplotypes reported by Archer et al. (2013) and (b) 146 mitogenome haplotypes reported by Archer et al. (2013) combined with additional mitogenome haplotypes reported in this study. Colors represent the three ocean basins/regions: the Southern Hemisphere (green, denoted SHEM), the North Pacific (blue, denoted NPAC) and the North Atlantic Ocean (red, denoted NATL), respectively. Numbers at basic nodes denotes the posterior probability of the specific node (only the support for basic nodes is reported). A humpback whale (Megaptera novaeangliae) mitogenome haplotype (Genbank NC_006927) was employed to root the tree (not shown). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
(haplotypes NATL_011 and NATL_012, Fig. 2b). The genealogy estimated from the novel and previously published complete mitogenome haplotypes was similar to the genealogy inferred from the mtDNA CR sequences (Figs. 2b and 3b). In agreement with the genealogy estimated from the mtDNA CR sequences, North Atlantic specimens were partitioned into two different clades: one clade containing solely North Atlantic specimens (NA clade, Fig. 3b) and another clade containing specimens from the North Pacific, Southern Hemisphere and North Atlantic (Fig. 3b). The latter clade contained three haplotypes (haplotypes NATL_011.01, NATL_011.02 and NATL_012.01, Fig. 3b) represented by all six North Atlantic specimens from which complete mitogenome DNA sequences were obtained during this study (Fig. 3b).

The TMRCA estimated from all complete mitogenome haplotypes included in this study (Fig. 3b) was estimated at 1.9 million years and the 95% HPD (highest probability density) interval from 1.1 to 2.8 million years (Table 2). The divergence time of the three North Atlantic complete mitogenome haplotypes, which clustered outside the NA clade (Fig. 3b) detected during this study was estimated at 0.095 million years and the 95% HPD interval from 0.04 to 0.17 million years. The TMRCA for all the complete mitogenome haplotypes detected in the North Atlantic was estimated at 0.99 million years and the 95% HPD from 0.54 to 1.4 million years (Table 2). This estimate was 0.45 million years older than that reported by Archer et al. (2013). The TMRCA for all the mtDNA CR haplotypes included in this study (Fig. 2b) was estimated at 4.3 million years and the 95% HPD interval from 1.97 to 6.8 million years. In the case of the North Atlantic fin whales, the TMRCA was estimated at 4.2 million years and the 95% HPD interval from 1.96 to 6.8 million years.

The population origin of 25 fin whale specimens (22 sampled in the North Atlantic, one in the North Pacific, one in the Sea of Cortez and one in the Southern Hemisphere) was inferred from the assignment tests based upon diploid genotypes at 20 microsatellite loci. Among the 22 fin whale specimens from the North Atlantic, 20 samples had mtDNA CR haplotypes that were assigned to clades that also contained specimens from the North Pacific and Southern Hemisphere (i.e., outside clade “NA”, specifically haplotypes NATL_011 and NATL_012, Fig. 2b). The remaining two North Atlantic specimens had mtDNA CR haplotypes that were assigned to the main North Atlantic clade (clade “NA”, specifically haplotypes NATL_003 and NATL_016, Fig. 2b). All specimens were assigned to the population in which they were sampled with probabilities ≥ 1.0 (Table 4).

The immigration rate (i.e., N_m) estimated from the mtDNA CR sequences from the Southern Hemisphere into the North Pacific was estimated at 0.36 (95% credible interval: 0-3.4, Table 3) which is equivalent to a single immigrant per 2.8 generations. The immigration rates from the Southern Hemisphere into the North Atlantic and from the North Atlantic into the North Pacific were estimated at 0.37 (95% credible interval: 0-2.79, Table 3) and 0.0015 (95% credible interval: 0-1.56, Table 3), respectively. Lastly the immigration rate from the North Pacific into the North Atlantic was estimated at 0.0029 (95% credible interval: 0-1.97, Table 3).

4. Discussion

The initial reason for undertaking this study was the discrepancies between Archer et al. (2013) findings and the earlier work published by Bérubé et al. (2002, 1998). However, there was a more general concern about the recent resurge in mitogenomic-based studies employing monophyly to delineate intraspecific evolutionary distinct units.

Meiotic recombination facilitates the incorporation of population-wide variation into each haploid genome complement (Pamilo and Nei, 1988). Accordingly, population-specific monophyly at recombining loci requires substantial reproductive isolation during a considerable time. How long depends upon the effective population size (see Hudson and Turelli, 2003). The situation is different for a uniparentally inherited, non-recombining genome where each lineage contains only the variation of its own lineage rather than the population at large, which is why non-recombining loci are sensitive to the sampling effects illustrated in this study. This difference highlights the need for a sufficiently exhaustive sampling scheme. It appears that sampling was the cause for the monophyly of North Atlantic fin whales observed by Archer et al. (2013). Archer et al. (2013) included a total of 34 North Atlantic fin whale specimens (including Mediterranean Sea specimens) in their analysis, represented by 13 haplotypes. The extended sample in this study comprised 828 North Atlantic fin whale mtDNA CR sequences representing 80 haplotypes. Among the 828 sequences, 33 (i.e., ~4%) had one of the two haplotypes that clustered outside the main North Atlantic clade (NA clade in Fig. 2b). The relative scarcity of North Atlantic fin whale specimens that carry mtDNA haplotypes clustering outside the North Atlantic could be the result of recent dispersal into the North Atlantic. Consequently, North Atlantic fin whale specimens with these mtDNA haplotypes may simply be immigrants and not actually part of the North Atlantic gene pool per se. However, the analyses of the biparentally inherited microsatellite loci in this study suggested that these individuals were part of the North Atlantic gene pool and unlikely to originate from the North Pacific (both the Sea of Cortez and the eastern North Pacific) nor the Southern Hemisphere. The probability of all these samples’ multi-locus genotypes was higher in the North Atlantic and Mediterranean Sea than in the other ocean basins (Table 4).

The divergence times among the basic nodes estimated during this study, (Table 2) were considerably longer than the estimates reported by Archer et al. (2013). These differences were mainly due to polyphyly detected in the expanded sample from the North Atlantic. As a result, the TMRCA among North Atlantic specimens in this study was much longer. Another consequence of a polyphyletic North Atlantic samples was that the intra-oceanic and global TMRCA were similar. The

Table 2

<table>
<thead>
<tr>
<th></th>
<th>North Atlantic</th>
<th>North Pacific</th>
<th>Southern Hemisphere</th>
<th>All three oceans</th>
<th>Substitution rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TMRCA 95% HPD</td>
<td>TMRCA 95% HPD</td>
<td>TMRCA 95% HPD</td>
<td>TMRCA 95% HPD</td>
<td>mean 95% HPD</td>
</tr>
<tr>
<td>Archer et al. (2013)</td>
<td>2.0 0.7-3.5</td>
<td>3.3 1.4-5.4</td>
<td>3.0 1.2-5.0</td>
<td>3.6 1.6-5.9</td>
<td>0.0075 0.0034-0.0128</td>
</tr>
<tr>
<td>Combined data</td>
<td>4.2 1.9-6.6</td>
<td>3.5 1.5-5.6</td>
<td>3.3 1.3-5.6</td>
<td>4.3 1.9-6.6</td>
<td>0.0087 0.0042-0.0145</td>
</tr>
<tr>
<td>Entire mitochondrial genome</td>
<td>0.45 0.25-0.68</td>
<td>1.9 1.1-2.8</td>
<td>0.87 0.50-1.3</td>
<td>1.9 1.1-2.8</td>
<td>0.003 0.0018-0.0044</td>
</tr>
<tr>
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<td>0.99 0.54-1.4</td>
<td>1.9 1.1-2.8</td>
<td>0.87 0.48-1.3</td>
<td>1.9 1.1-2.8</td>
<td>0.003 0.0018-0.0044</td>
</tr>
</tbody>
</table>

Notes: TMRCA: the time to the most recent common ancestor, 95% HPD: 95% interval of the highest posterior density. Times are in million years, and the substitution rate is in substitution per site per million years.

* North Atlantic excluding Mediterranean Sea.

** North Pacific excluding Sea of Cortez.
emergence of polyphy due to an increased North Atlantic sample size suggests that similar undetected polyphyley may emerge in other ocean basins and regions and hence any inferences made regarding monophyly in these other regions may change as well. This general fundamental sampling issue is problematic in term of defining subspecies from mitogenomic data since the “distinctiveness” may change with the sampling effort. Consequently, such “higher level” intra-specific classifications should not be based solely on uniparentally-inherited, non-recombinating genomes. Perhaps employing measures of evolutionary distinctiveness that do not rely upon the “absence” of contradicting observations, e.g., the absence of poly- or paraphyly. Possible, and likely more robust criteria would include the level of gene flow between baleen whale populations as a monophyly criterion as discussed above.

Rare, but occasional, gene flow between balaen whale populations from different hemispheres is possible and appeared to have occurred in humpback whales (Jackson et al., 2014) and Antarctic minke whales, *Balaenoptera bonaerensis* (Glover et al., 2010). Estimates of inter-oceanic migration rate among fin whales in this study (Table 3) suggested some gene flow between the Southern Hemisphere and the two Northern Hemisphere ocean basins (i.e., the North Atlantic and the North Pacific). However, the wide 95% credible intervals prevented the exclusion of zero migration. The most likely route for recent inter-oceanic divergence time or a combination hereof (Hey and Nielsen, 2004; Jackson et al., 2014) inferred from biparentally inherited recombining genomes. Perhaps employing measures of evolutionary divergence such as a monophyly criterion as discussed above.

The non-trivial nature of defining species and sub-species is, in many instances, hampering implementation of legal protective measures. In response to these practical implications, Taylor et al. (2017) recently proposed employing population genetic statistics (specifically nucleotide divergence) as a means to delineate sub-species in cetaceans. In principle such objective quantitative genetic criteria are desirable, but most many of these statistics are subject to the same sampling and “rate-of-divergence” issues as a monophyly criterion as discussed above.

Table 3
Average estimates of genetic diversity (θ) and number of immigrants per generation (N,m) for the North Atlantic, North Pacific and Southern Hemisphere

<table>
<thead>
<tr>
<th>Parameter</th>
<th>θNA</th>
<th>θNP</th>
<th>θSH</th>
<th>NmNA→SH</th>
<th>NmNP→NA</th>
<th>NmNP→NP</th>
<th>NmSH→NA</th>
<th>NmSH→NP</th>
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<td>mode</td>
<td>0.037</td>
<td>0.018</td>
<td>0.078</td>
<td>0.0029</td>
<td>0.3711</td>
<td>0.0015</td>
<td>0.3565</td>
<td>0.0063</td>
<td>0.0063</td>
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<tr>
<td>95% CI</td>
<td>0.022-0.057</td>
<td>0.008-0.032</td>
<td>0.048-0.139</td>
<td>0.1-1.937</td>
<td>0-2.791</td>
<td>0-1.566</td>
<td>0-3.406</td>
<td>0-3.082</td>
<td>0-18.632</td>
</tr>
</tbody>
</table>

Notes: NA: North Atlantic, NP: North Pacific, SH: Southern Hemisphere, θ: genetic diversity, Nc: effective population size, m: immigration rate per generation, −→ denotes the direction of migration, 95% CI: 95% credible interval. *Estimates were based on the mtDNA CR sequences. Samples from Sea of Cortez and the Mediterranean Sea were excluded from the analysis.

Table 4
Multi-locus microsatellite genotype probability value (p-value) per putative population and assigned population.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>CR haplotype number</th>
<th>North Atlantic (n = 266)</th>
<th>Antarctica (n = 20)</th>
<th>Eastern North Pacific (n = 24)</th>
<th>Sea of Cortez (n = 45)</th>
<th># loci</th>
<th>Missing loci</th>
<th>Assigned population</th>
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<td>0.045</td>
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<td>0.062</td>
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<td>&lt; 0.01</td>
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<td>0.068</td>
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<td>&lt; 0.01</td>
<td>0.076</td>
<td>0.169</td>
<td>20</td>
<td></td>
<td>Sea of Cortez</td>
</tr>
</tbody>
</table>

* The first three letters from the sample ID represent the region where the samples were collected, NAT denotes the North Atlantic Ocean, SHE denotes the Southern Hemisphere, i.e., Antarctic, NPA denotes the Eastern North Pacific Ocean and SOC denotes the Sea of Cortez. The n value in parenthesis represents the sample size for each putative source population. The assigned population was based on the most likely population and its relative score. CR: mtDNA control region.
questioned by Perrin et al. (2009), who suggested that the different latitudinal origin of the holotypes might explain the observed size difference (Perrin et al., 2009). However, this explanation is difficult to evaluate since the holotype that served as the basis for the differences in the vertebral described by Lönnerg (1931) was not collected and hence is unavailable. Alternatively, if the Northern Hemisphere populations were founded from the Southern Hemisphere the observed polyphyly could be due to incomplete lineage sorting (Avise et al., 1984) as suggested by Pastene et al. (2007) in the case of common minke whales, Balaeopetra acutorostra, in the Atlantic Ocean. This appears to be the inference drawn by Archer et al. (2013), who emphasizes that the three well supported North Pacific groups (Fig. 3a) observed in their mitogenome-based genealogy could be due to incomplete lineage sorting. However, whether the patterns observed in the taxon (i.e., monophyletic, polyphyletic or paraphyletic groups) represent sub-species, as opposed to incomplete lineage sorting, population structure and/or incomplete sampling remains unclear.

Unsurprisingly no qualitative differences between the topologies inferred for the basal part of the mitochondrial genealogies were detected when increasing the data from only 285 bp of mtDNA CR sequence to the complete mitogenome DNA sequences. The general support for individual nodes, especially the most recent nodes, increased with the number of base pairs per haplotype and hence node support was substantially higher in genealogy estimated based upon complete mitogenome haplotypes. However, in most cases, the basic nodes are the target of interest in analyses of intraspecific variation aimed at assessing sub-species or ESUs. This observation, together with the obvious need for an increased sampling coverage, suggests that it might be worthwhile to first sequence a limited number of mitogenomes from the extreme parts of the species’ distribution. The mitogenome sequences can then serve as a backbone to identify and subsequently specifically target informative regions, which likely can be sequenced efficiently and at low costs using “standard” Sanger sequencing as proposed by Coulson et al. (2006). Such a strategy, as opposed to next-generation sequencing of the entire mitogenome in all specimens, would facilitate large sample sizes presumably with minimal loss of phylogenetic signal in terms the basic nodes.

In conclusion, the present study found that some of the spatially distinct mtDNA haplotype monophyly in North Atlantic fin whales reported by Archer et al. (2013) was due to an insufficient sample size. Although untested in this study, the same could be the case for some of the monophyly detected in other ocean basins or regions. Since monophyly essentially relies upon “absence of evidence” for poly or paraphyly proving monophyly, especially below the species level is difficult and prone to biases. As pointed out by Crandall et al., 2000 identifying sub-species or ESUs solely from genetic data is an oversimplification and should be complemented with ecological, behavioural and morphological data. In principle, genetic data are well-suited to assess divergence times and the degree of reproductive isolation (when gene low is low) but the choice of suitable statistics and appropriate threshold values is no simple task.

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Contributions to the paper

PJF, MB, JP AH and AAC conceived and designed the study. AA, SGB, SB, DB, AB, HAC, LDR, PG, SL, FL, VM, SM, NO, CP, SP, RP, CR, JR, CR, RS, MAS, JU, GV, FWW provided data or sample material, MB, JP AH, CPD, WH, VR-L conducted laboratory analyses. AAC conducted the data analysis with contributions from JP AH and inputs from ES. TO conducted the Web of Science review. AAC, JP AH, PJF and MB drafted the manuscript. All authors read, edited, commented on and approved the final manuscript.

Declaration of interest

None.

Data accessibility

Mitochondrial DNA sequence data, microsatellite genotypes and input data files for the analyses conducted in this manuscript have been deposited in Datadryad.org under accession: https://doi.org/10.5061/dryad.qf528n0.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1146/j.ympev.2019.02.003.

References


