Phylogenetic relationships in southern African Bryde's whales inferred from mitochondrial DNA: further support for subspecies delineation between the two allopatric populations.

Gwenith S Penry[1,2,3*], Philip S. Hammond[2], Vic Cockcroft[1], Peter B Best[3], Meredith Thornton[3], Jeff A. Graves[2]

1. Department of Zoology, Nelson Mandela University, PO Box 77000 - Port Elizabeth, 6031, South Africa.
2. Sea Mammal Research Unit, Scottish Oceans Institute, University of St Andrews, St Andrews KY16 8LB, Fife, UK.
3. Mammal Research Institute, Department of Zoology and Entomology, University of Pretoria, Private Bag X20, Hatfield, 0028, South Africa.

We dedicate this manuscript to Dr Peter B Best who established an impressive foundation of information on the two forms of Bryde's whale occurring off southern Africa. We are pleased to have molecular support for what he suspected nearly 40 years ago and are eternally grateful for his dedication to South African marine mammal science.

*corresponding author: gwenpenry@gmail.com

ABSTRACT

Bryde's whales (*Balaenoptera edeni*) are medium-sized balaenopterids with tropical and subtropical distribution. There is confusion about the number of species, subspecies and populations of Bryde's whale found globally. Two eco-types occur off South Africa, the inshore and offshore forms, but with unknown relationship between them. Using the mtDNA control region we investigated the phylogenetic relationship of these populations to each other and other Bryde's whale populations. Skin, baleen and bone samples were collected from biopsy-sampled individuals, strandings and museum collections. 97 sequences of 674 base pair (bp) length were compared with published sequences of Bryde's whales (n=6) and two similar species, Omura's (*B.omurai)* and sei (*B.borealis)* whales (n=3). We found eight haplotypes from the study samples: H1- H4 formed a distinct, sister clade to pelagic populations of Bryde's whales (*B.brydei*) from the South Pacific, North Pacific and Eastern Indian Ocean. H5 - H8 were included in the pelagic clade. H1 – H4 represented samples from within the distributional range of the inshore form. Pairwise comparisons of the percentage of nucleotide differences between sequences revealed that inshore haplotypes differed from published sequences of *B.edeni* by 4.7-5.5% and from *B.brydei* by 1.8-2.1%. Ten fixed differences between inshore and offshore sequences supported 100% diagnosability as subspecies. Phylogenetic analyses grouped the South African populations within the Bryde's-sei whale clade and excluded *B.edeni*. Our data, combined with morphological and ecological evidence from previous studies, support subspecific classification of both South African forms under *B.brydei* and complete separation from *B.edeni*.

Keywords: Bryde's whale, *Balaenoptera edeni*, *Balaenoptera brydei,* Southern Africa, mtDNA control region, phylogenetics.

INTRODUCTION

The Bryde's whale (*Balaenoptera edeni*) is one of 14 currently accepted species of mysticete whale and one of eight recognised species in the family Balaenopteridae (Committee on Taxonomy, 2017). Consensus on the number of species and subspecies of *Balaenoptera* has not been agreed due to insufficient information (Bannister, 2002; Rice, 1998). The recent classification of Omura's whale (*Balaenoptera omurai)* as a distinct species excluded from the sei-Bryde's whale complex has clarified some of the confusion surrounding the taxonomy of medium-sized balaenopterid whales, which includes Bryde's, sei and Omura's whales (Wada et al. 2003; Sasaki et al. 2006; Cerchio et al. 2015). Bryde's whales closely resemble sei whales in size and shape and the two species were often confused by commercial whalers, resulting in inaccurate catch statistics and an inability to estimate past population sizes (Best, 1977; Ohsumi, 1977; Kato, 2002; Yamada *et al*., 2008). However, several unique morphological characteristics distinguish Bryde's whales from other balaenopterids, most notably three prominent rostral ridges that extend from the tip of the rostrum to anterior to the blowholes (Omura, 1962; Best, 1977; Kato, 2002). Bryde's whales are found in tropical and temperate waters and have been recorded in the North and South Pacific, Indian, and Atlantic Oceans, approximately between 40° N and 40° S (Kato, 2002).

Since they were first described at the end of the 19th century Bryde's whales have often been referred to as 'little known', with much confusion over their taxonomic position and the global number and distribution of populations. *B.edeni* was first described by Anderson in 1878 from a stranded specimen in Burma and was named Eden's whale, after Sir Ashley Eden, the British High Commissioner to Burma at the time. In 1912, during a visit to South Africa, Ørjan Olsen described a new species of mysticete whale, which had previously been confused with the sei whale. Olsen named this new species *Balaenoptera brydei* after Johan Bryde, the Norwegian consul to South Africa, who set up the first whaling station in Durban (Kato, 2002). *B.edeni* and *B.brydei* were

72 subsequently synonymised based on skeletal comparisons (Junge, 1950). It was later agreed they
73 were conspecific (Junge, 1950; Best, 1960), which led to the use of *B.edeni* as the specific name
74 and Bryde's whale the popular name. Recent findings suggest that this synonymization was
75 premature and that there are a number of geographic, morphological, osteological, behavioural and
76 genetic differences amongst the various populations of Bryde's whales worldwide that may warrant
77 subspecies or species designations (Omura, 1981; Best, 1977; Perrin *et al*., 1996; Pastene *et al*.,
78 1997; Yoshida and Kato, 1999; Wada et al. 2003; Sasaki et al. 2005,2006; Kanda et al. 2007;
79 Kershaw et al. 2013; Rosel and Wilcox, 2014; Luksenburg, 2015).
80
81 Despite the growing number of studies on the topic, Bryde's whale taxonomy remains unresolved
82 and several publications recommend that molecular studies should be combined with knowledge
83 of the external morphology and ecology of each regional population before consensus is reached
84 on the number of species, subspecies and their respective nomenclature (Bannister 2002, Rice 1998,
85 Yamada et al. 2008). It is generally accepted that at least two species exist (*B.edeni* Anderson, 1878
86 and *B.brydei* Olsen, 1913), however a type specimen for *B.brydei* was never defined and the genetic
87 identity of the *B.edeni* holotype (Anderson, 1878) has not been verified. Therefore, all Bryde's
88 whales currently remain classified as a single species, *Balaenoptera edeni*, by the Society for
89 Marine Mammalogy (Committee on Taxonomy, 2011, 2014, 2017). Reference was made, but not
90 listed, to possible subspecific level distinction between small-form coastal Bryde's whales of the
91 western Pacific and Indian oceans (*B.edeni*) and the larger, globally distributed oceanic form
92 (*B.brydei*) (Committee on Taxonomy, 2011). In 2014, the Committee updated the listing of these
93 provisional subspecies to *B.edeni edeni* and *B.edeni brydei* to which the small-coastal form and
94 larger, oceanic form have respectively been referred (as in Kershaw et al. 2013 and Rosel and
95 Wilcox, 2014). This provisional nomenclature may not be suitable for all geographic locations and
96 the possibility that *B.edeni* and *B.brydei* are separate species, with subspecies level separation
97 within each of them, should be explored further.

98

99 To complicate matters further, Best's (1977) description of two allopatric forms of Bryde's whale
100 off South Africa has led to the realisation that Olsen's (1913) description of *B.brydei* was not
101 correctly specified and included features from both the inshore and offshore forms (Best 2001;
102 Kanda et al. 2007; Yamada et al. 2008). Table 1 summarises the differences in body size, scarring,
103 reproductive cycles, diet, migrations, and a lack of distributional overlap between the two ecotypes
104 (Best 1977). Contrary to the provisional subspecies designation of *B.edeni edeni* and *B.edeni brydei*
105 (Committee on Taxonomy 2017), here we propose subspecific level separation of the inshore and
106 offshore South African ecotypes under *B.brydei* and their complete separation from *B.edeni edeni*.
107
108 According to Taylor et al. (2017), a subspecies can be defined as "… a population, or collection of
109 populations, that appears to be a separately evolving lineage with discontinuities resulting from
110 geography, ecological specialisation, or other forces that restrict gene flow to the point that the
111 population or collection of populations is diagnosably distinct". It is therefore necessary to base
112 subspecies classification on proven genetic differences between suspected subspecies in the
113 Bryde's-sei whale complex using the diagnosable criteria set out in Archer et al. (2017).
114
115 Previous studies using the complete mitochondrial DNA (mtDNA) control region (901bp) found
116 that the number of nucleotide differences between *B. edeni* (coastal Japan) and *B. brydei* (pelagic
117 North Pacific) was greater than that between *B. brydei* and the sei whale (*B. borealis)* (Wada et al.
118 2003). The same study also separated *B. edeni* from the *borealis/brydei* group. This was further
119 supported in a later study using complete mtDNA sequences and short interspersed nuclear
120 elements (SINE) insertion patterns (Sasaki et al. 2006).
121
122 The effective population size (N_e) of the inshore population was estimated at 582 (+- 184) for the
123 entire population in 1982 (Best et al 1984) and between 158 (SE = 17) and 248 (SE = 93) for the

124 eastern section of their range thirty years later (Penry 2010). Survey design and spatial limitations
125 to data collection considered, the population is small, certainly less than 1000 individuals. The
126 offshore (SE Atlantic) population has never been assessed and therefore the estimated N_e is not
127 available.

128
129 Insert Table 1.

130 Within the southern African sub-region, a third population similar in body size to the South African
131 inshore form, but differing in prey type, was found in the south west Indian Ocean (SWIO), south
132 and east of Madagascar (Fig 1, Best 2001). Available information suggests that the distribution of
133 this latter population does not extend as far south as Durban, South Africa (Fig. 1) and is likely to
134 be geographically isolated from the South African populations (Best 2001). The degree of genetic
135 differentiation between the three putative populations is needed, however molecular data is lacking,
136 with only one mtDNA sequence for a South African Bryde's whale available prior to this study
137 ((Genbank X72196) Árnason and Best 1991).

138 Insert Figure 1

139 The aims of this study were to determine the molecular taxonomic position of southern African
140 Bryde's whales in the Bryde's-sei whale complex, to determine the degree of genetic separation
141 between the two ecotypes off South Africa, and to identify whether mtDNA control region
142 sequences position the inshore form with *B. edeni* or *B. brydei.* This would enable the determination
143 of subspecies classification in southern African waters. The molecular identity of extra-limital
144 samples of Bryde's whales from Namibia and the south western Indian Ocean (Fig. 2) is discussed
145 in relation to the known distributional limits of the South African inshore population.
146
147 Hereafter the South African inshore population will be referred to as 'inshore' and the SE Atlantic
148 pelagic population as 'offshore'. Although the use of the name *B.brydei* has not been formally

149 accepted, here we use it to refer to the larger, offshore or pelagic form of Bryde's whales in several
150 different geographic regions.
151
152 **METHODS**
153 Samples from 111 Bryde's whales were available for this study. These included skin biopsies from
154 free-ranging animals (n=78), soft tissue from stranded animals (n=23), and bone (n=5) and baleen
155 (n=5) from museum collections (Fig 2A). One biopsy from the NE Atlantic (#35) and one from the
156 SWIO (#36), east of the Madagascar Plateau, (28.4° S, 48.2° E) were collected during delivery of
157 the Research Vessel *Whale Song* (RVWS) from the Mediterranean to Australia (Jenner and Jenner
158 2011) (Fig. 2B).
159
160 Insert Figure 2
161 A summary of the samples used in this study is given in Table 2.
162
163 Insert Table 2
164
165 Biopsy samples were collected using a compound crossbow and modified biopsy darts (n=76
166 samples) or a Larsen gun (Larsen, 1998) on loan from the International Whaling Commission
167 (IWC) (n=2 samples). Biopsy tips were sterilized in 5% hydrogen peroxide prior to use. Thirty-
168 three sub-samples of Bryde's whale tissue specimens (skin, bone, baleen) were obtained from the
169 Port Elizabeth (PEM) and Iziko South African (ISAM) museums, the Department of Environmental
170 Affairs (DEA) and the Namibian Dolphin Project (NDP). One of these samples (#11) was from the
171 same individual analysed by Árnason and Best (1991), (Genbank Accession X72196). The origin
172 of samples #37 and #38 is unclear; both are thought to originate from the SE Atlantic (offshore)
173 population based on information associated with the samples on where and when they were
174 collected (Appendix 2). The two samples from Namibia collected by the Namibian Dolphin Project

175 (NDP) were from a dead stranded adult (#43) and a live stranded juvenile (#44). The museum
176 skeletal and baleen remains were cleaned and prepared prior to and during drilling to reduce the
177 possibility of contamination (Pichler et al. 2001).
178
179 Samples were processed and sequenced over a period of c. 5 years in different laboratories and
180 amplification conditions, equipment, primers and sequencing methods varied slightly between
181 laboratories. DNA was extracted from skin and muscle tissue using either the Puregene isolation
182 method (Centra systems) or the Qiagen™ DNeasy Blood and Tissue kit. For samples with a low
183 yield of DNA, the Invisorb® Forensic kit 1 or the QIAampTM DNA microkit was used. We
184 followed the protocol for each kit for the extraction of animal blood and tissue. Some specimens
185 also required secondary cleaning of the extracted DNA using phenol-chloroform (Sambrook et al.
186 1989).
187
188 DNA extraction from bone and baleen samples were conducted in a sterile LaminAir flow cabinet
189 isolated from the main laboratory. The flow cabinet, equipment and solutions were exposed to ultra
190 violet (UV) light between individual extractions to prevent cross-contamination. Bone drillings
191 were manually pulverised into a fine powder and DNA extracted following the protocol for 'ancient
192 bones' set out according to the specifications of the Invisorb® Forensic kit 1. The pre-treatment and
193 extraction procedures for baleen followed those used in Rosenbaum et al. (1997). After the DNA
194 was re-suspended in ultrapure Milli-Q water, the concentration was measured on a Nanodrop (ND-
195 1000 Spectrophotometer, Thermo Fisher Scientific, USA) and diluted to 20 ng DNA/µl. The primer
196 pairs M13DLp1.5 and Dlp8 G; (Dalebout et al. 2005) and ProL-He and DLH-He (Seddon et al.
197 2001) were used to amplify approximately 700bp and 400bp overlapping portions of the
198 mitochondrial DNA control region respectively.
199

The older museum specimens contained degraded DNA and amplification required targeting shorter segments of the control region (~250bp). Seven internal primers were designed (Table 3) using PRIMER3 (Rozen and Skaletsky, 2000) to amplify four consecutive sections of the control region (a total of approximately 750bp). These primers amplified the same section of the control region that was amplified for the non-degraded samples. Sufficient overlap was allowed between each short section to ensure accurate readings of the entire sequence. BeIP1f was modified from the forward primer M13Dlp 1.5. where the non-specific nucleotide 'R' was replaced by 'G' in the sequences amplified using the internal primers. This ensured that the sequence was more specific to the Bryde's whale. BeIP3 and BeIP4 were used to extend the shorter 400bp sequences amplified using ProL_He and DLH-He to ~700bp.

Insert Table 3

Polymerase Chain Reaction (PCR) reaction mixes for primer M13DPp1.5 and Dlp8G were as follows: 1x PCR buffer (Bioline), 1.5mM magnesium chloride ($MgCl_2$), 0.5 unit *Taq* DNA polymerase (Bioline), 0.24mM deoxyribonucleotide triphosphates (dNTP's), 0.2 pmol of each primer, and ~40 ng genomic DNA in a 10 µl reaction. The PCR was conducted in a G-Storm Thermal Cycler (Gene Technologies), and the cycling profile was 94°C for 2 minutes, 30 cycles of: 30s at 94°C, 30s at 58°C and 40s at 72°C, and a final 5 minutes at 72°C. Amplification conditions for primers ProL-He and DLH-He were as in Seddon et al. (2001). Products of all amplifications were manually checked for length and single bands on a 2% Agarose gel using Ethidium bromide and UV transillumination.

The amplified products were outsourced (Macrogen, Korea) for sequencing on an automatic sequencer (ABI 3730 xl DNA Analyzer) using BigDye™ Terminator version 3.1 cycling conditions

225 (Applied Biosystems). All successfully amplified sequences were trimmed to equal lengths (674bp)
226 and aligned using ClustalW, available in MEGA version 6.0 (Tamura et al. 2013). Alignments were
227 checked and confirmed by eye (GSP) and any uncertainties were checked by JAG. The number of
228 haplotypes, haplotype frequencies, number of polymorphic sites, transitions, transversions and
229 nucleotide composition, were calculated in ARLEQUIN version 3.5 (Excoffier et al. 2005).
230 Haplotypic diversity and nucleotide diversity were calculated in DNASP version 5 (Librado and
231 Rozas, 2009). Two samples, #37 and #38, were excluded from the above analyses due to the large
232 amount of missing sequence data.
233
234 Phylogenetic trees were constructed using the mtDNA sequences from this study and published
235 sequences from GenBank that included *B.edeni, B.brydei, B.borealis* and *B.omurai*. The humpback
236 whale (*Megaptera novaeangliae*) and fin whale (*B. physalus*) were included as outgroups (Table
237 4). Pairwise comparisons of 18 haplotypes were conducted using the Maximum Composite
238 Likelihood method (sum of log-likelihoods for all pairwise distances in a distance matrix, using the
239 Tamura-Nei model (Tamura and Nei 1993)) available in MEGA version 6 (Tamura et al. 2013).
240 This assumes an equal substitution pattern among lineages and of substitution rates among sites
241 and was chosen as the best fit to the sequences based on the model assumptions. All positions
242 containing alignment gaps and missing data were eliminated in the pairwise sequence comparisons
243 (pairwise deletion option). Samples #37 and #38 were not included in pairwise comparisons.
244
245 Insert Table 4
246 The sequences were loaded into SeaView version 4 (Gouy et al. 2010) and the resulting multiple
247 alignment was loaded into IQ tree (Trifinopoulos et al. 2016) which uses ModelFinder
248 Kalyaanamoorthy et al. 2017) to determine the best model for phylogenetic estimates. The results
249 were sorted by corrected Akaike's Information Criterion (AICc) scores and the HKY+F model
250 (Hasegawa et al. 1985) was the best model and was used in an heuristic Maximum Likelihood

phylogenetic search. There were 674 positions in the dataset. To map the origin of samples #37 and #38, a second ML phylogenetic analysis was conducted in which all positions containing alignment gaps and missing data were eliminated in sequence comparisons (complete deletion option) resulting in a total of 379 positions.

To determine genetic differentiation, the number of nucleotide changes and pairwise distances between the individual sequences were calculated in MEGA version 6 (Tamura et al. 2013). This enabled quantification of the variation between the two populations of Bryde's whales off southern Africa. Comparisons with other closely related species were made to investigate the number of differences between the inshore haplotypes and *B. edeni* as a relative measure of their level of relationship (population, sub species or species). The level of differentiation between the inshore and offshore types was measured using the PhiST (ΦST) scores calculated using strataG (Archer et al. 2016). Of the eight haplotypes (4 inshore and 4 offshore) identified in the study only seven were used for this comparison because Haplotype 6 (samples 37 and 38) had a large amount of missing data.

RESULTS

From a total of 111 samples, a 674bp region of the mitochondrial control region was successfully sequenced for 87% (97) of individuals. Partial sequences were obtained for the samples #37 and #38 where only the internal primers BeIP2 and BeIP4 amplified. The analyses that included these two samples used sequences trimmed to 379bp to account for the large amount of missing data. Table 5 gives details on the number of haplotypes, polymorphic sites, haplotypic diversity (Hd), nucleotide diversity and pairwise differences for the inshore and offshore populations.

Insert Table 5

Of the eight haplotypes identified, H1 was the haplotype for 86 (93%) of the inshore samples (Table 6), H2 for four individuals, H6 for samples #37 and #38, and the other five haplotypes were only present in one individual each. H5 (#12) and H7 (#43) represent two stranded individuals and H8 represents the single North Atlantic specimen. The SWIO (#36) and second Namibian (#44) samples (outside the known distributional limits for the inshore form) were identical to H1, the haplotype found in the majority of biopsy samples collected in inshore waters. There were 10 fixed differences between the samples that formed a clade with pelagic populations of *B.brydei* and those representing whales sampled in inshore waters (SA inshore) (Table 6). Sequences were submitted to GenBank as *B.edeni* under the accession numbers GU085094 – GU085099.

Insert Table 6

Nucleotide diversity amongst the inshore samples (n=92) was low (0.0003; SD = 0.0004); despite the much larger sample size this is considerably lower than amongst the 5 offshore samples (0.005; SD = 0.004). Haplotypes 2, 3 and 4 differed from H1 by only one indel (Table 6). H5 and H7 (SA offshore) differed from the inshore samples (H1) by 12 and 11 base changes respectively. The North Atlantic sample (H8) differed from the SE Atlantic (SA offshore) haplotypes by 4-5 base changes. The SWIO sample that was expected to differ greatly from the two South African populations due to the large geographical separation, had an identical haplotype to the inshore animals (H1). Given the available literature on this population, this result questions whether the population found south and east of Madagascar is isolated from the South African forms as was proposed by Best (2001).

The number of nucleotide changes and pairwise differences (percentage difference) was higher between the inshore haplotypes and the *B. edeni* sequences (4.5 -5.7%) than between SA inshore and pelagic Bryde's whale populations (*B.brydei)* (1.7-2.3%). The inshore haplotypes also had a higher number of differences from *B.edeni* than they did from the Antarctic sei whale (4%)

(Table 7). Haplotypes 5, 7 and 8 were most similar to the pelagic Bryde's whale (*B.brydei*) samples from the North and South Pacific and Indian oceans. The six samples collected for this study that grouped with other offshore (*B.brydei*) populations differed from each other by one to eight base changes (0.1-1.2%). This is similar to the number of differences between the two *B.edeni* specimens from Japan and Malaysia (1.1%).

Insert Table 7

310 *Phylogenetic Analysis*

311 Figure 3 shows the Maximum likelihood (ML) bootstrap phylogenetic tree and bootstrap support
312 values. Haplotypes 5,7 (offshore) and 8 (N Atlantic) are in a sister group to haplotypes 1-4 (inshore)
313 and appear to conform to *B.brydei,* forming a clade with other pelagic/offshore Bryde's whale types
314 from three different oceanic regions (South Pacific, Eastern Indian Ocean and North Pacific). There
315 is a large separation between the inshore haplotypes and the *B.edeni* specimens from coastal Japan
316 and Malaysia (Fig. 3).

317

318 Insert Figure 3

319 The clade containing haplotypes 1-4 had strong bootstrap support (96%) as did its
320 separation from a sister group containing haplotypes 5, 7 and 8 and the other *B. brydei* haplotypes
321 (93%). The relatively low bootstrap probability (77%) for the six South African offshore Bryde's
322 whale specimens is most likely due to the few differences between their control regions (0.1%-
323 1.2%). Although there was strong support (81%) for the separation of the *B.edeni* group from the
324 sei-Bryde's clade, the bootstrap support for the sei-Bryde's clade was low (49%) and a larger
325 sample size from the offshore Bryde's population is needed to fully understand the relationship of
326 the two clades.

327
328 When samples 37 and 38 (H6) were included in the analysis and alignment gaps and
329 missing data were deleted, a total of 379bp were available. These two samples formed a clade with
330 other *B.brydei* populations from different oceanic regions, offering strong support that these two
331 samples of unknown origin belong to the SE Atlantic (offshore) population as was predicted by
332 PBB (Fig. 4, Appendix 2).

333

334 Insert Figure 4

335 *Genetic Differentiation*

In total, 674 usable bases were available for distance computation with the allowed level of missing data at 0.05. There were no shared haplotypes between the two populations (inshore and offshore) with an average Phi-statistic over all loci of $\Phi ST = 0.984$ ($p < 0.001$). The high ΦST score indicates complete separation between the inshore and offshore populations, with little or no gene flow between them.

DISCUSSION

The aims of this study were primarily to identify the phylogenetic relationship between the two forms of Bryde's whales found off South Africa, and to demonstrate the separation between *B.edeni* and the South African populations. Since the two allopatric forms of South African Bryde's whales were described by Best (1977) genetic confirmation of the degree of separation between these two types has been largely anticipated (Kershaw et al. 2013).

The mtDNA control region has been shown to be a suitable marker choice for cetacean taxonomic clarification, and in particular for subspecies delineation due to its high mutation rate (Rosel et al. 2017). The differentiation of populations into subspecies can occur over relatively short evolutionary timescales, especially in small populations that do not have high historical abundance or haplotypic diversity (Rosel et al. 2017). The present study detected low haplotypic diversity for the inshore population and despite unreliable catch records for the species due to confusion with the sei whale, the species is not thought to have ever had a substantially higher abundance than at present (+- 600 individuals) (Best et al 1984; Penry 2010).

Previous information on the inshore population summarised earlier addresses many of the diagnosable characteristics defined in Taylor et al. (2017). In this study, high diagnosability was provided by the 10 fixed differences in the mtDNA control region sequences between the inshore and offshore samples. This characteristic is indicative of at least subspecies-level separation (Taylor et al. 2017, Archer et al. 2017).

Taylor et al. (2017) also provided guidelines for the recommended data and analyses required to make conclusive recommendations for taxonomic separation and subspecies or species identification. We acknowledge that several of the guidelines were not addressed by this study and

367 therefore we refrain from making complete taxonomic revision recommendations until such time
368 as the following additional data is available; nuclear DNA data to detect limitations to gene flow
369 and the calculation of divergence times, effective population size estimates for the offshore
370 population, and sufficient genetic sample sizes for the offshore population and other Bryde's
371 whales found globally.
372
373 Molecular evidence of genetic divergence at higher than the population level is important to local
374 conservation initiatives and for global conservation status assessments. Of particular conservation
375 concern is the status of the inshore population that numbers only a few hundred animals and was
376 recently reassessed as Vulnerable in the National Red List Assessment (Best et al. 1984; Penry
377 2010, Penry et al. 2016). This small population faces several perceived threats such as competition
378 with fisheries for commercially important fish stocks, entanglement in coastal fishing gear (6
379 fatalities in 3 years) and disturbance from commercial marine tourism. Another predator that relies
380 on the same prey and habitat as the inshore Bryde's whale, the African penguin, *Spheniscus*
381 *demersus*, has shown a significant decline in numbers and a negative change in conservation status
382 at both national and global level (Birdlife International 2016, Crawford et al. 2011). Clarification
383 of the delineation of the inshore population is therefore critically important to encourage and
384 support global and local conservation efforts.
385
386 The status of the offshore (SE Atlantic) population is harder to assess because of the logistical and
387 financial constraints to sampling in offshore waters and therefore this population remains classified
388 as Data Deficient (DD) both nationally and globally (Reilly et al. 2008; Penry et al. 2016). The
389 samples found to represent this population were all from strandings or museum collections and
390 their source population was unknown prior to analysis. This highlights the importance of museum
391 collections, and of accurate labelling and well-maintained records pertaining to each specimen.

392

393 Below we discuss the findings of our study in relation to available knowledge of these populations
394 and the distributional ranges that were identified from commercial catch data. It is possible that the
395 historical distributional ranges identified in Best (1977, 2001) were underestimated because they
396 were limited to areas where commercial whaling fleets operated. This study identified two samples
397 as inshore Bryde's whales that were collected well outside the boundaries (by several hundred
398 kilometres) of the inshore form described in Best (2001). This result, although represented by only
399 two samples, does offer some evidence of a larger distributional range for the inshore population;
400 high individual resighting rates detected in photo-identification studies (Penry 2010) and
401 subsequent unpublished fieldwork do not however suggest any substantial change in the small
402 population size estimate for the inshore form.

403 *Identifying the specimens*

404 **South African inshore population**: One of the main aims of this study was to determine
405 the identity of the South African inshore population within the Bryde's-sei whale complex. Most
406 coastal or small-form Bryde's whales are thought to conform to *B.edeni* (Anderson 1878).
407 However, morphological investigations of animals caught in South African waters showed that the
408 smaller, inshore form differed from *B.edeni* in several morphometric measurements (Best 1977).
409

410 The majority of samples used in this study were collected from live Bryde's whales occurring in
411 shallow, coastal bays along the South African coast and were therefore expected to be from the
412 inshore population. Extremely low haplotypic variation is present within the population and is
413 consistent with limited variation for coastal populations of Bryde's whales occurring off the coasts
414 of Bangladesh and Oman, and in the Gulf of Mexico (Kershaw et al. 2013, Rosel and Wilcox 2014).
415 The genetic diversity found in this study and that of Kershaw et al. (2013) is unusually low for
416 baleen whales. Although the South African inshore form is currently referred to as *B.edeni* by the

417 Society for Marine Mammalogy, maximum likelihood analyses show that it groups more closely
418 with *B.brydei* (pelagic populations) than with either of two *B.edeni* populations (coastal Japan and
419 Malaysia) used for comparison in this study. Excluding the outgroups used here, the South African
420 inshore form differed most from *Balaenoptera edeni.* This is supported by the higher number of
421 differences in pairwise comparisons between the inshore haplotypes and *B.edeni* than between the
422 inshore haplotypes and both *B.borealis* and *B.brydei*.
423
424 Our results support that the inshore form could be a subspecies of *B.brydei* (offshore form) but we
425 acknowledge that additional molecular markers and a larger sample size from the offshore
426 population and other geographic areas is needed for confirmation of this. Our data do however
427 show that the two populations are genetically divergent and that the inshore form is not synonymous
428 with *B.edeni*. When combined with morphological, reproductive, behavioural, and distributional
429 characteristics, taxonomic separation between the inshore and offshore populations at the
430 subspecific or specific level should be considered. Previous studies have reported similar findings
431 (Wada and Numachi, 1991; Arnason et al. 1993; Wada et al. 2003).
432
433 **Offshore (Southeast Atlantic) population**: Four individuals were identified as
434 *Balaenoptera brydei* (offshore form). The presence of *Isistius sp* (cookie-cutter shark) scars on the
435 body of sample #12 (Fig. 5) and # 43 support the offshore origins of these individuals, as does the
436 account by PBB (Appendix 2) for samples #37 and #38. As predicted, the assumed offshore
437 specimens identified in this study form a clade with *B.brydei* in the South Pacific, North Pacific
438 and Eastern Indian Ocean. *B.brydei* from South Africa only differs from its conspecific in the South
439 Pacific (Omura et al. 1981) by ~0.5%. Together with published information on the morphology,
440 distribution, feeding, breeding and migrations of the South African offshore form, the results of the
441 molecular analyses do provide support for their identity as *B.brydei*, the pelagic/offshore form.

442 Insert Figure 5

443

444 **South West Indian Ocean (Madagascar Ridge):** The single sample from the South
445 Western Indian Ocean surprisingly had an identical haplotype to the South African inshore animals
446 (H1). Discussion of this result is made cautiously because it represents only one individual and
447 further samples from this area are needed to confirm the findings. However, based on the
448 information provided by Best (2001), available data on the population off the south and east of
449 Madagascar (from commercial catches) showed it to be morphologically smaller than the SA
450 inshore form and differing in prey type. We therefore expected any animals sampled here to have
451 a different genetic identity. It is possible that there may be several different populations of medium-
452 sized balaenopterid whales in this region, as was recently shown with the discovery of Omura's
453 (*Balaenoptera omurai*) whale off Madagascar (Cerchio et al. 2015). We did consider that the
454 whaling records and measurements discussed in Best (2001) may therefore actually refer to *B.*
455 *omurai*, however the distributions do not overlap (Best, 2001, Cerchio et al. 2015). It is also
456 possible that the collection of this sample is due to range extension of the inshore form due to
457 climate change, inaccurate distributional range definition due to limited coverage by commercial
458 whaling vessels, or simply that this area has never been properly surveyed before. More samples
459 from this area are needed before any conclusions can be made, but due to the result found for one
460 of the stranded individuals in Namibia (discussed below), it may be the case that the distribution of
461 the SA inshore form extends further up both the east and west coasts of southern Africa than was
462 previously thought (see Best PB 2001).

463 **Walvis Bay, Namibia**: Both samples from stranded Bryde's whales in Namibia were
464 expected to belong to the offshore population due to the presence of *Isistius* scars on the bodies and
465 the published distributional range of this population on the west coast of Southern Africa.
466 Additionally, the range of the inshore population is not known to extend as far up the west coast as

Walvis Bay. However, the results confirmed the identity of one individual (#43) as an offshore type (*B.brydei)* and the other (#44) as an inshore animal (H1), making it the first confirmed record of the SA inshore form occurring further north than Saldhana Bay, the western limit from catch data (Fig. 2A).

Photographs of this animal (#44) show at least five fresh *Isistius* scars on the body and head. When the known distribution of the inshore population is considered, the occurrence of this animal in Walvis Bay (outside the known range by > 800 km) could be explained by it being young animal (juvenile at 5.6m) that became caught in the strong Benguela current system and swept out of range. However, the continental shelf off Walvis Bay is extremely wide, with the 100m isobath situated around 30km offshore, making the habitat conditions in terms of bathymetry similar to those for the known range of the inshore population (Best et al. 1984). The presence of *Isistius* scars on this individual was unexpected.

The South African inshore and offshore forms differ from each other by far less than they would if the inshore form had fallen within the *B.edeni* clade, supporting the suggestion by Best (1977) that the two forms could both be *B.brydei.* Best (1977) summarised the descriptions and identifications of *B.edeni* and *B.brydei* (Anderson, 1878; Olsen, 1913; Junge, 1950; Soot-Ryan, 1961) and based on these sources it appears that *B.edeni* (as described by Anderson, 1878) is smaller than the inshore form off South Africa. It was however recommended that the inshore and offshore South African forms should be kept separate, and referred to as *B.edeni* and *B.brydei* respectively, pending further and specifically genetic investigations (Best, 1977). The mtDNA control region data used in this study separates the inshore form from *B.edeni* and supports its recognition as a subspecies of *B.brydei* through the diagnosable feature of 10 fixed differences between the inshore and offshore populations

492 Molecular comparisons with other Bryde's whales in adjacent waters (west Africa; Namibia,
493 Angola, Gabon and east Africa; Mozambique, Madagascar and Northern Indian Ocean) are needed
494 to clarify their taxonomic status in the Bryde's whale complex and to determine the distributional
495 limits, and environmental and geographical boundaries for each species, subspecies or population.
496 Of note are the findings of Yoshida and Kato (1999) who identified complete separation between
497 offshore Bryde's whales in the Western North Pacific and a coastal population in the East China
498 Sea. In this region the Kuroshio Current appears to act as a physical barrier between the two
499 populations. It is possible that the Agulhas and Benguela currents have a similar influence over the
500 two allopatric forms found off southern Africa.

501 *Conclusions and Future work*

502 A number of molecular studies on Bryde's whales in different geographic regions have now been
503 completed (Luksenburg 2015; Rosel and Wilcox 2014; Kershaw et al. 2013; Pastene et al. 1997;
504 Yoshida and Kato, 1999; Wada et al. 2003; Sasaki et al. 2005, 2006; Kanda et al. 2007). Several
505 have recommended subspecific level separation between coastal and pelagic forms and the general
506 consensus is that these molecular studies should be combined with further investigations on
507 morphology, behaviour, ecology (prey type, distribution, migrations) and biology (reproductive
508 patterns) before recommendations can be made on species designation and nomenclature.
509 Limitations considered, this study further supports that there are numerous discrete populations of
510 Bryde's whales that must be considered separately for conservation purposes, particularly the
511 coastal populations which appear to be inherently small, a reflection of their apparent restricted
512 distributions. Regardless of the current recommended nomenclature, until all available genetic data
513 are included in a single global analysis, we will continue to debate the suggestions for species or
514 subspecies recognition based on area specific studies.
515

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695

LIST OF FIGURES AND TABLES

731 **Table 5** Differences between the South African inshore and offshore ecotypes: Number of sequences for
732 each population (N_S), number of haplotypes identified (N_H) and the number of usable sites (sites) for each
733 population are shown. Differences are represented by the haplotype diversity (HD), polymorphic site
734 composition (number (No., Transitions (Ts), Transversions (Tv) and Indels (In)), nucleotide diversity (ND)
735 and number of pairwise differences (PDs).

736 **Table 6.** The unique haplotypes identified in this study (H1- H8). The numbers in brackets refer to the number
737 of individuals represented by each haplotype.

738 **Table 7**. Number (above diagonal) and percentage (below diagonal) of pairwise differences in control region
739 sequence substitutions. H1-H8 refer to haplotypes identified from the study samples. H6 was excluded due
740 to large amount of missing data. Abbreviations for Genbank sequences are as follows: *B. edeni* from Malaysia
741 and Coastal Japan (BedM, BedJ); *B. brydei* from South Pacific, Eastern Indian Ocean and Northwest Pacific
742 (BbrSP, BbrEIO, BbrNP); *B.borealis* from the Antarctic Ocean and Icelandic waters (BborA and BborI);
743 *Balaenoptera omurai* (Bomu); *Balaenoptera physalus* (Bphy) and *Megaptera novaeangliae* (Mnov).

Appendix 1. Specimen number, source, type of material, date of collection and location where the sample was collected are given. DEA = Department of Environmental Affairs, ISAM = South African Museum, Cape Town, PEM= Port Elizabeth Museum, RVWS-Research Vessel Whale Song.

No. (#)	*Source*	*Museum/Biopsy No.*	*Material*	*Date*	*Location*	*Latitude*	*Long*
1	Wild		Skin biopsy	31/08/2007	Plettenberg Bay	34.16913	23.41558
2	PEM	PE 3337	Skin, blubber and muscle	24/02/2008	The Willows,PE		
3	Wild		Skin biopsy	16/04/08	Plettenberg Bay	34.16913	23.41558
4	Wild		Skin biopsy	16/04/08	Plettenberg Bay	34.16913	23.41558
5	Wild		Skin biopsy	21/04/08	Plettenberg Bay	34.16913	23.41558
6	Wild		Skin biopsy	24/04/08	Plettenberg Bay	34.16913	23.41558
7	Wild		Skin biopsy	07/05/08	Plettenberg Bay	34.16913	23.41558
8	Wild		Skin biopsy	07/05/08	Plettenberg Bay	34.16913	23.41558
9	Wild		Skin biopsy	23/05/08	Plettenberg Bay	34.16913	23.41558
10	Wild		Skin biopsy	05/06/08	Plettenberg Bay	34.16913	23.41558
11	ISAM	84/20	Skin and blubber	10/07/84	Asfontein		
12	ISAM	84/28	Skin and blubber	11/09/84	St Helena Bay		
13	ISAM	88/4	Blubber	15/02/88	Die Dam		
14	ISAM	90/37	Skin and blubber	1/12/90	Blouberg Beach		
15	ISAM	91/16	Blubber	03/09/91	Scarborough		
16	ISAM	ZM 12962	Bone-L mandible	1913	Saldanha Bay		
17	PEM	70	Bone-skull	15/03/69	Cape St Francis		
18	PEM	72	Bone-T.bulla	01/07/69	The Willows, PE		
19	PEM	413	Bone-T.bulla	06/07/79	Sundays River mouth		
20	PEM	758	Baleen	23/07/81	Maitland River mouth		

21	PEM	840	Baleen	21/06/82	Swarkops River mouth		
22	Wild		Skin biopsy	28/09/05		32 41.08S	17 59.74E
23	ISAM		Soft tissue	15/05/06	Gouritzmond		
24	ISAM		Soft tissue	18/03/07	Stillbaai		
25	ISAM	ZM 41283	Baleen				
26	ISAM	ZM 41244(92/12)	Baleen	10/08/92	Kleinbaai, Bloubergstrand		
27	ISAM	ZM 39830	Bone-skull	15/08/63	Milnerton beach-lighthouse		
28	DEA	MCM 2008/11	Skin	04/08/08	Olifantsbos, Cape Peninsula		
29	DEA	MCM 99/13	Skin	01/11/99	Glencairn beach, False Bay		
30	DEA	MCM2002/4	Skin	09/05/02	Mudge Point, Hermanus		
31	DEA	MCM 2003/8	Skin	01/08/02	Table Bay docks		
32	DEA	MCM 2003/8	Skin	17/06/03	Jakkalsfontein		
33	DEA	MCM2003/113	Skin	26/04/03	Dana Bay, MB		
34	DEA	MCM 2008	Skin	11/08	Muizenberg		
35	RVWS		Skin biopsy	12/2010	N Atlantic		
36	RVWS		Skin biopsy	01/2011	S Madagascar	28º 4S	48º.2E
37	ISAM		Foetus	?	MV Sierra		
38	ISAM	ZM 39958	Baleen	11121983	Table Bay Harbour		
39	ISAM		Skin, baleen	April 2012	Buffalo Bay		
40	ISAM		Skin	May 2012	Kleinbaai		
41	PEM	PEM4636	Skin	29/03/2012	Maitland River mouth		
42	PEM	PEM4653	Skin	11/05/2012	Blue Horizon Bay		
43	NDP		Skin	Jan 2012	Walvis Bay		

44	NDP		Skin	June 2012	Walvis Bay	34.03628	23.41618
45	Wild	BW1	Skin biopsy	2042012	Plettenberg Bay		
46	Wild	BW2	Skin biopsy	2042012	Plettenberg Bay	34.08675	23.42158
47	Wild	BW3	Skin biopsy	2042012	Plettenberg Bay	34.16913	23.41558
48	Wild	BW4	Skin biopsy	3042012	Plettenberg Bay	34.03775	23.39542
49	Wild	BW5	Skin biopsy	3042012	Plettenberg Bay	34.0113	23.47683
50	Wild	BW6	Skin biopsy	3042012	Plettenberg Bay	34.12683	23.43276
51	Wild	BW7	Skin biopsy	4042012	Plettenberg Bay	33.99736	23.5543
52	Wild	BW8	Skin biopsy	4042012	Plettenberg Bay	33.99728	23.5613
53	Wild	BW9	Skin biopsy	5042012	Plettenberg Bay	34.08545	23.41895
54	Wild	BW10	Skin biopsy	5042012	Plettenberg Bay	34.06076	23.4177
55	Wild	BW11	Skin biopsy	5042012	Plettenberg Bay	34.05965	23.42317
56	Wild	BW12	Skin biopsy	11042012	Plettenberg Bay	34.01260	23.48300
57	Wild	BW13	Skin biopsy	13042012	Plettenberg Bay	34.0593	23.4274
58	Wild	BW14	Skin biopsy	13042012	Plettenberg Bay	34.07403	23.39891
59	Wild	BW15	Skin biopsy	18042012	Plettenberg Bay	34.12097	23.4134
60	Wild	BW16	Skin biopsy	22062012	East London	32.8944	28.15505
61	Wild	BW17	Skin biopsy	17082012	False Bay	34.17487	18.55727
62	Wild	BW18	Skin biopsy	18082012	False Bay	34.17101	18.58525
63	Wild	BW19	Skin biopsy	18082012	False Bay	34.25738	18.619
64	Wild	BW20	Skin biopsy	18082012	False Bay	34.24762	18.60332
65	Wild	BW21	Skin biopsy	18082012	False Bay	34.19846	18.52594
66	Wild	BW22	Skin biopsy	23082012	False Bay	34.11949	18.5147

67	Wild	BW23	Skin biopsy	23082012	False Bay	34.19128	18.63601
68	Wild	BW24	Skin biopsy	23082012	False Bay	34.20761	18.65402
69	Wild	BW25	Skin biopsy	23082012	False Bay	34.1863	18.60914
70	Wild	BW26	Skin biopsy	24082012	False Bay	34.16134	18.65459
71	Wild	BW27	Skin biopsy	22032013	Plettenberg Bay	34.14729	23.41065
72	Wild	BW28	Skin biopsy	24032013	Plettenberg Bay	34.07913	23.39539
73	Wild	BW29	Skin biopsy	25032013	Plettenberg Bay	34.02551	23.52082
74	Wild	BW30	Skin biopsy	28032013	Plettenberg Bay	34.16635	23.36704
75	Wild	BW31	Skin biopsy	28032013	Plettenberg Bay	34.16996	23.36767
76	Wild	BW32	Skin biopsy	5042013	Plettenberg Bay	34.16441	23.46141
77	Wild	BW33	Skin biopsy	6042013	Plettenberg Bay	34.07588	23.45184
78	Wild	BW34	Skin biopsy	11042013	Plettenberg Bay	34.06365	23.4732
79	Wild	BW35	Skin biopsy	12042013	Plettenberg Bay	34.11415	23.59485
80	Wild	BW36	Skin biopsy	12042013	Plettenberg Bay	34.02042	23.54149
81	Wild	BW37	Skin biopsy	13042013	Plettenberg Bay	34.12695	23.4211
82	Wild	BW38	Skin biopsy	13042013	Plettenberg Bay	34.09232	23.48634
83	Wild	BW39	Skin biopsy	7052013	False Bay	34.18938	18.73845
84	Wild	BW40	Skin biopsy	7052013	False Bay	34.12019	18.5773
85	Wild	BW41	Skin biopsy	8052013	False Bay	34.10033	18.57143
86	Wild	BW42	Skin biopsy	11052013	False Bay	34.13228	18.49152
87	Wild	BW43	Skin biopsy	12052013	False Bay	34.11345	18.5888
88	Wild	BW44	Skin biopsy	12052013	False Bay	34.1222	18.64432
89	Wild	BW45	Skin biopsy	12052013	False Bay	34.0927	18.6595
90	Wild	BW46	Skin biopsy	12052013	False Bay	34.14714	18.6962

91	Wild	BW47	Skin biopsy	12052013	False Bay	34.14811	18.69244
92	Wild	BW48	Skin biopsy	12052013	False Bay	34.14174	18.66666
93	Wild	BW49	Skin biopsy	10082013	False Bay	34.25159	18.73582
94	Wild	BW50	Skin biopsy	10082013	False Bay	34.12012	18.70015
95	Wild	BW51	Skin biopsy	19082013	False Bay	34.2568	18.62858
96	Wild	BW52	Skin biopsy	23082013	False Bay	34.19211	18.5323
97	Wild	BW53	Skin biopsy	23082013	False Bay	34.18452	18.53082
98	Wild	BW54	Skin biopsy	2082013	Plettenberg Bay	34.13736	23.44129
99	Wild	BW55	Skin biopsy	2092013	Plettenberg Bay	34.16222	23.41961
100	Wild	BW56	Skin biopsy	2092013	Plettenberg Bay	34.18313	23.29155
101	Wild	BW57	Skin biopsy	2092013	Plettenberg Bay	34.18967	23.28212
102	Wild	BW58	Skin biopsy	5092013	Plettenberg Bay	34.05288	23.39849
103	Wild	BW59	Skin biopsy	2092013	Plettenberg Bay	34.18928	23.32329
104	Wild	BW60	Skin biopsy	12092013	Plettenberg Bay	34.0657	23.53479
105	Wild	BW61	Skin biopsy	17092013	Plettenberg Bay	34.08241	23.40592
106	Wild	BW62	Skin biopsy	17092013	Plettenberg Bay	34.10933	23.48392
107	Wild	BW63	Skin biopsy	22092013	Plettenberg Bay	34.17833	23.383055
108	Wild	BW64	Skin biopsy	22092013	Plettenberg Bay	34.17868	23.343276
109	Wild	BW65	Skin biopsy	22092013	Plettenberg Bay	34.17542	23.347943
110	Wild	BW66	Skin biopsy	22092013	Plettenberg Bay	34.14563	23.35694
111	DEA	SFRI10/19	Skin (male, 12.63m)	30082010	Sopiesklip	34.75381	19.5556

Appendix 2. The history of samples 37 and 38, recounted by PBB.

A male Bryde's whale foetus (#37) ca 35 cm long was presented to ISAM as having belonged to T. Haraldsen, ex-captain of the "pirate" whaling catcher-factory ship MV *Sierra*. As this vessel's operations were largely concentrated on the offshore population of Bryde's whales on the west coast of southern Africa (Best, 1996), and for security reasons excluded inshore waters on the South African coast, it is highly likely that this specimen originated from the offshore population, and it was treated such in analysis.

On 11 December 1983, a 14.7m male Bryde's whale was found floating dead but fresh in Ben Schoeman dock, Table Bay harbour. Its skin was intact and bore a large number of healed oval scars on the peduncle and flanks. There was also a large vertical abrasion about mid-length on the left side, suggestive of a ship strike. It was towed out to sea on the same day, but washed up on 15 December at Koeberg Power station, 40 km to the north. It was measured on 16 December, a testis collected and measured (41.5 x 12.5 x 6 cm) with cestode *Phyllobothrium* cysts recorded in the blubber, and a section of baleen plates collected before the carcase was buried on the beach. The baleen was presented to the museum in February 1984 and accessioned as ZM 39958 (#38).

The size, scarring and timing all indicate that this was most likely to be a representative of the offshore population that was struck by a ship at sea and carried inadvertently on its bow into the docks. Unfortunately, the baleen was either never labelled or subsequently lost its accession tag, but during a search of the ISAM collection in 2011 a section of unlabelled baleen was found that in description closely matched that of ZM 39958, and this was sampled on that assumption.