Molecular identification of sei whales (*Balaenoptera borealis*) from the Netherlands

Guido O. Keijl, Dick S.J. Groenenberg, Aline M. Nieman, R. Becky Desjardins & Barbara Gravendeel

Naturalis Biodiversity Center, P.O. Box 9517, NL-2300 RA Leiden, The Netherlands,
e-mail: guido.keijl@naturalis.nl

Abstract: Sei whale (*Balaenoptera borealis*) is a very rare species in the North Sea. The remains of four whales identified as sei whales, originating from the Netherlands, are preserved in the zoological collection of Naturalis Biodiversity Center, Leiden, the Netherlands. A single bone in the Naturalis collection, found on the Dogger Bank just outside Dutch waters, was also identified as sei whale. Because there were doubts about identification of several specimens, three of them were genotyped, using partial *16S, COI, ND4L, CytB* and *D-loop* sequences, which are standard DNA barcoding markers. The holotype of *Sibbaldius schlegelii* (Flower, 1864) [= *B. b. schlegelii*] from Pekalongan, Indonesia, and a fin whale (*B. physalus*) from the Netherlands were included for comparison. Three of the six specimens turned out to be incorrectly identified. The results underscore the added value of molecular identification of skeletons, and of museum collections. On the basis of our results, we cannot distinguish between the southern hemisphere subspecies of sei whale, *B. b. schlegelii* and *B. b. borealis*.

Keywords: baleen whale, rorqual, genotype, museum collection, DNA barcode.

Introduction

The baleen whales included in the genus *Balaenoptera*, also called rorquals, all have a torpedo-shaped body, more or less pointed snout, sickle-shaped dorsal fin, and gular grooves from chin to belly. The smaller species are quite similar in colour and body dimensions and may therefore pose identification problems. For instance, despite centuries of whale hunting, an extant *Balaenoptera* was described only in the twenty-first century: Omura's whale (*B. omurai*) (Wada et al. 2003). As living whales only show little of themselves when surfacing, and an observation usually lasts some seconds at most, specific identification at sea is difficult or even impossible. When stranded, rorqual identification can be even more challenging: corpses are frequently decomposed, discoloured and/or incomplete, while their skulls, if still present, can only be identified by specialists. As a consequence, knowledge on distribution and population size of several species is incomplete (e.g. Jung et al. 2016).

Sei whale (*Balaenoptera borealis*) has a worldwide distribution, but is still poorly known. At present, this species seems to have smaller populations than many of its relatives (Christensen et al. 1992, Prieto et al. 2012, Hammond et al. 2013) and it is currently categorised as 'endangered' (IUCN 2018). In order to be able to protect it, it is important to know its distribution, potential division into separate populations, (sub)specific sta-

tus and identifying characters. In the North Sea, sei whale is extremely rare, probably because the feeding conditions are not suitable for this species. In the southern North Sea five rorqual species have been recorded, among which sei whale, but out of 88 *Balaenoptera* carcasses stranded in the Netherlands up to April 2019, only four have been identified as sei whale (e.g. Reid et al. 2003, Smeenk & Camphuijsen 2016, www.walvisstrandingen.nl). The remains of these are preserved in the scientific natural history collection of Naturalis Biodiversity Center in Leiden, the Netherlands. In this note, we report on three of these four specimens. Some were incomplete when they washed ashore, and identification was problematic. Remains of a whale identified as sei whale from just outside the Dutch territorial waters at the Dogger Bank consists of a single vertebra only. Therefore we performed a DNA-analysis for identification. (The sei whale that stranded on Maasvlakte, Zuid-Holland, on 9 November 1986 was not included in the sample, as this specimen was fresh and complete when it washed ashore and good photographs are still available; hence its identification is beyond any doubt (figuur 1).) The analyses presented us with a challenge, because the first sei whale for the Netherlands dated back to 1811. As the Naturalis collection is also in possession of the holotype of *Sibbaldius schlegelii* (Flower, 1864), currently considered a subspecies of *B. borealis* (Perrin et al. 2009) occurring in the Southern Hemisphere, we included this specimen in our sampling.

Material and methods

Skeletal fragments of six different whales were investigated (table 1): the type specimen of the southern sei whale, *Sibbaldius schlegelii* Flower, 1864, originating from Indonesia, and four whales identified as sei whales: three that stranded in the Netherlands between 1811-2006 and one that was collected at the Dogger Bank. A whale identified as fin whale (*B. physalus*) that stranded in the Netherlands was included as well. We presume that sei whale specimens from the North Sea represent the nominate subspecies.

Bone samples were taken by breaking off pieces of bone from the ear region using a pair of pliers; the fragments were collected in Falcon tubes. The pliers were cleaned with bleach and meticulously rinsed with demi-water before sampling the next specimen. DNA extractions were carried out in a dedicated ancient DNA facility of Leiden University and Naturalis Biodiversity Center, where no prior work on whales had been performed. To prevent contamination, all work on the type

Table 1. *Balaenoptera* specimen data, sequence results and updated (sub)specific identifications. Except for the type specimen of *Sibbaldius schlegelii*, which is from Java, Indonesia, all specimens are from the Netherlands or very close by. Cases in which the analysis conflicted with the original identification are indicated in bold.
Museum ID = initial (morphological) identification; RMNH = Naturalis collection number; Genetic ID = the results of the DNA analysis; *CB* = cytochrome B, *CR* = control region D-loop.

Museum ID	Locality	Year	RMNH	Reference	Sequence	Genetic ID
B.b. schlegelii	Pekalongan	1864	31166	Flower 1864	*16S, COI, ND4L*	***B. b. borealis***
B.b. borealis	Monnikendam	1811	31164	Weber 1922	*16S, COI, CR, ND4L, CB*	*B. b. borealis*
B.b. borealis	Dogger Bank	1953	11933	[unpublished]	*16S*	***B. acutorostrata***
B.b borealis	Rotterdam	1972	22579	van Bree & Husson 1974	*16S, COI, CR, ND4L, CB*	*B. b. borealis*
B.b. borealis	Texel	2005	41459	Camphuysen et al. 2008	*16S, COI, CR*	***B. acutorostrata***
B. physalus	Kwade Hoek	2006	41465	Camphuysen et al. 2008	*COI, CR B*	*B. physalus*

Table 2. Targeted region and primer sequences.

Gene	Primer name	Primer sequence (5'--> 3')	Length	Length amplicon	Without primers
16S	1081_16S_F	TACACCTAGAAGATTCCACAGTCC	24	121/122	70/71
	1201_16S_R	TCTCCTATACTTTAGATGTATGGTGAA	27		
COI	5875_COI_F	AATATAAAACCACCTGCCATGACC	24	124	77
	5998_COI_R	TAAGTAGCATGGTGATTCCGGCT	23		
ND4L	9998_ND4L_F	ACCTAATATCCGCACTACTCTGTC	24	103	55
	10100_ND4L_R	ATCATGTTAGCCAAGGTGAAGTGT	24		
CB	14437_CB_F	GATACCTACACGCAAACGGAGC	22	139	92
	14575_CB_R	GTGGCTATAACTGTGAATAGTAGGA	25		
D-loop	15649_D-loop_F	GCATTCAATTATTTTCACTACGAGCA	26	150	101
	15798_D-loop_R	TGGAGCGGCCATAAGAATCATTT	23		

specimen of *S. schlegelii* was carried out first, separate from the bone material of the other whales. Samples were ground using a mortar and pestle, while a Retsch Mill was used for larger fragments (collection numbers RMNH.MAM.41459 and RMNH.MAM.41465, table 1). Total genomic DNA was isolated using the silica extraction method from Rohland & Hofreiter (2007). Sequences obtained with this study have been deposited in GenBank (supplementary table S3: https://zoogdierwinkel.nl/content/lutra-62-1-2019).

Based on a MAFFT alignment (Katoh & Toh 2008) of full mitochondrial sequences of *Balaenoptera* from a number of studies (Arnason & Gullberg 1993, Arnason et al. 2004, Sasaki et al. 2005, 2006, Archer et al. 2013), primers targeting five small (103-150 bp) variable mitochondrial regions commonly used for DNA barcoding (*16S large ribosomal subunit*, *Cytochrome Oxidase subunit I*, *NADH dehydrogenase subunit 4L*, *Cytochrome B*, *control region D-loop*) were designed (table 2). PCRs were performed in 25 µl volumes using 1 µl template, 0.5 µl Phire® Hot Start II DNA Polymerase (Thermo Scientific), a final concentration of 1× Phire reaction buffer, 0.5 mM $MgCl_2$, 0.3 mg/ml BSA, 0.5 mM of each primer and 0.2 mM dNTPs. The used thermoprofile consisted of an initial denaturation for 3 minutes at 98˚C, followed by 40 cycles of – denaturation 5 seconds at 98˚C, annealing 10 seconds at 60˚C, extension 30 seconds at 72˚C – and a final extension of 5 minutes at 72˚C. PCR products were cloned with a TOPO® TA cloning kit (Life Technologies) following the manufacturer's protocol. Up to five colonies were picked to PCR the insert using primers M13-FP Forward and M13-pUC(-40) Reverse (Messing 1983). The colony-PCR amplicons were purified and Sanger sequenced in both directions on an ABI 3730xl sequencer (Applied Biosystems), using the last mentioned primers, at BaseClear (Leiden). The chromatograms were edited with Sequencher 4.10.1 (Gene Codes Corporation). For each marker the obtained sequences were aligned in Geneious v.10.2.3 (Kearse et al. 2012) with the corresponding regions from the full mitochondrial datamatrix, adding humpback whale (*Megaptera novaeangliae*) (Sasaki et al. 2005) as outgroup. These five alignments were subsequently concatenated.

Maximum likelihood phylogenies (100 bootstrap replicates) were generated with PhyML (Guindon et al. 2010) for both the alignment of the complete mitogenomes as well as the alignment of the selected regions. A comparison between these two should show whether the reduced dataset (together the selected regions representing ~2.4% of the complete mitogenome) would yield the same cladogram topol-

Figure 1. The sei whale that stranded on 9 November 1986 on Maasvlakte, Zuid-Holland. The picture is a bit blurry because it was taken during twilight, but the species is easily identified by the colours and pattern on flipper and throat and the length of the gular grooves. *Photo: A. Molenkamp (Naturalis).*

ogy. Uncorrected pairwise (P) genetic distances between species were calculated for each of the short targeted fragments, also in Geneious (same version). The number of variable and parsimony informative sites were calculated with PAUP 4.0a165 (Swofford 2003). Assignment of the obtained sequences to any of the known species of *Balaenoptera* was done based on NCBI nucleotide BLAST searches (https://blast.ncbi.nlm.nih.gov), assessing interspecific distances and the position of these sequences in the phylogeny reconstruction.

Results

Only three out of the six specimens appeared to be correctly identified. Two originally identified as sei whale turned out to belong to minke whale (*B. acutorostrata*) (table 1). The original identification as fin whale from the animal stranded at Kwade Hoek was confirmed by the analysis. The type of *S. schlegelii* could genetically not be discriminated from other sei whales.

There were strong fluctuations in amplification success between the different samples, which were not typically (collection) age related. In order of amplification success, the markers could be ranked (from highest to lowest success rate) as *16S,COI, CR, ND4L* and *CB*. Only for the sei whales of Monnikendam and Rotterdam all five markers were obtained. Least effective were the PCRs of the minke whale from the Dogger Bank. BLAST searches with each of the obtained sequences yielded unambiguous identifications (4e44 < E-value < 5e19; 99% < identity <100%; supplementary table S1: https://zoogdierwinkel.nl/content/lutra-62-1-2019). Also, there was no conflict in identification between the different markers. The selected regions showed some interspecific variation within *Balaenoptera*. Based on average uncorrected P-distances the markers could be ranked (from most to least informative) as *ND4, CR, 16S, CB* and *COI* (supplementary table S2: https://zoogdierwinkel.nl/content/lutra-62-1-2019). Within *Balaenoptera*, the average interspecific sequence divergence (all markers) was 11.7%. From a phylogenetic per-

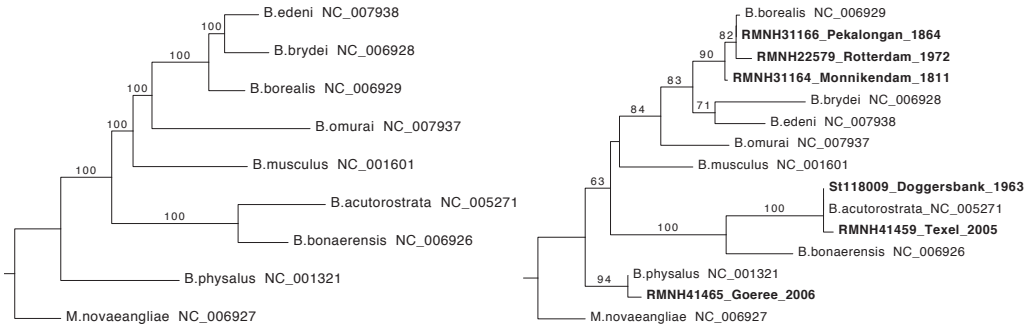

Figure 2. The left figure shows a fully resolved Bayesian phylogeny of *Balaenoptera* based on complete mitogenomic sequences (~16400 nt). The right figure shows the same phylogeny based on a subset of *16S, COI, CR, ND4L* and *CB* (sequences obtained in this study are indicated in bold).

spective the markers could be ranked (corrected for fragment length) from most to least parsimony informative: *ND4* (17,12), *CR* (37,21), *16S* (24,12), *CB* (25,12) and *COI* (16,10) (in parenthesis, the number of variable and parsimony informative characters, respectively). Fragment lengths without primers are given in table 2. The ML phylogeny inferred from full balaenopteran mitochondrial sequences was completely resolved (figure 2, left cladogram). The phylogeny based on the selected regions showed the same topology, although some nodes received lower bootstrap support (figure 2, right cladogram). Species clades, including taxa assessed in this study, were always highly supported.

Discussion

DNA analysis can aid accurate identification of historic cetacean specimens (e.g. Foote et al. 2012 and references therein), which (as in this case) may be up to 200 years old. The larger DNA-fragments in this study (*control region D-loop* and *cytochrome B*) amplified less successfully, yet no trend could be detected between amplification success and age of the specimens. Although the primers were generally designed to work for *Balaenoptera*, a bias in species specificity cannot be ruled out. For instance, PCRs with the *ND4L* and *CB* primers only resulted in amplicons for sei whale, while PCRs with *COI* and *CR* showed that these primers worked with one minke whale (RMNH. MAM.41459), but did not result in amplicons for the other one. Despite the successful amplifications, we could not separate the species pair minke whale – Antarctic minke whale (*B. bonaerensis*) with COI (table S2).

This study underpins the value of natural history collections. They offer the possibility to study material both morphologically and with DNA techniques: being stored in a suitable facility, specimens can be studied again and again if needed. Genetic analysis could result in successful identification when morphological characters are no longer available, for instance in case of incomplete or badly decomposed specimens. However, if conservation conditions are less than optimal, DNA can degrade quickly even in recently collected material and molecular analysis becomes impossible. Also, collecting cetacean bones from stranded animals is a costly affair, as is their storage. It is nevertheless recommended to store at least some parts of a stranded specimen, not only for *post hoc* identification, but also because new techniques could recover information that is presently impossible to get.

As the marine environment changes, so does the distribution of cetaceans (e.g. Santos et al. 2008, Nøttestad et al. 2015). Identification should therefore be done on basis of characters of specimens, not on known

(=present) distribution. Monitoring of cetaceans is generally considered important, e.g. for detecting population changes and conservation issues, and several techniques are in use, including acoustic surveys, aerial surveys and strandings analyses (Evans & Hammond 2004, MacLeod et al. 2005, André et al. 2011, Scheidat et al. 2011, Hammond et al. 2013). However, cetaceans are known to sometimes wander away from their area of regular occurrence (van Oort 1926, Anonymous 2005), while distribution of several species is still incomplete due to identification problems. Omura's whale, for instance, described from the Indo-Pacific Ocean and overlooked for centuries, was recently shown to occur in both the Indian and Atlantic Oceans as well (e.g. Cerchio et al. 2015, Cypriano-Souza et al. 2016, Jung et al. 2016). As identification of dead whales on the beach or in a collection may be as difficult as that of live individuals at sea, easier access to important morphological characters, like an identification key of cetacean remains, would be of great help.

The whale delivered to the Leiden museum by C.G.C. Reinwardt from Java was described by Flower (1864) as a species new to science: *Sibbaldius schlegelii*. This name was later synonymised with *B. borealis* by Tomilin (1946), although he proposed to retain it as a subspecies. As a result, two subspecies of sei whale are recognised until present (e.g. Rice 1998, Wilson & Reeder 2005, Perrin et al. 2009, IUCN 2018), despite the fact that Tomilin (1946) states that the two are only separable on basis of (maximum) body length and geographical distribution. Perrin et al. (2009) are right in pointing out that samples from northern and southern hemispheres should be compared more closely to find potential differences. On basis of our analysis, it is not possible to differentiate between sei whales from the Atlantic Ocean and the type of *S. schlegelii*, which originates from the Indo-Pacific. In cetaceans, body size alone cannot be used as a distinguishing character between (sub)species. We cannot rule out the possibility that population genetic markers, like microsatellites or RAD sequencing (e.g. Palsbøll et al. 1997, Attard et al. 2018), would show a difference between northern and southern populations, but it is unlikely that those data can be obtained from the type specimen of *Sibbaldius schlegelii*.

The geographical distribution of sei whale is still poorly known in the Atlantic (e.g. Prieto et al. 2012), but even more so in the Pacific. The northern population of the latter is considered to stay north of 40 °N in summer, but migrates south in winter, apparently almost into the tropics, similar to sei whales in the North Atlantic (IUCN 2018). Southern sei whale spend the (southern) summer between 40-60°S, while they occur as far north as 7°S in (southern) winter, at least in the Atlantic (da Rocha 1983). So, the distance between the southernmost records of northern sei whales and the northernmost records of southern sei whales may be as little as 2500 km. With the type specimen from *Sibbaldius schlegelii* from Java washing ashore at 7°S, one may wonder whether it really belonged to the southern sei whale population.

Sei whale populations from the Atlantic and Pacific are genetically distinct (Huijser et al. 2018), but until present the two are not (yet) separated as subspecies. Genetically distinct populations of any species could merit (sub)specific recognition, for instance when both prove to represent monophyletic clades. Genetic distance could lead to follow-up research, perhaps even revealing distinct and constant morphological characters (cf. Baker et al. 2002, Baker & Bradley 2006, Zink 2015).

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Samenvatting

Moleculaire determinatie van noordse vinvissen (*Balaenoptera borealis*) uit Nederland

Van de vijf soorten vinvis (*Balaenoptera*) die in de zuidelijke Noordzee zijn waargenomen, is alleen dwergvinvis (*Balaenoptera acutorostrata*) een regelmatige gast. Deze soort komt er het hele jaar voor; zijn verspreidingsgebied in de Noordzee reikt zuidelijk tot aan de zuidelijke rand van de Doggersbank. Gewone vinvis (*B. physalus*) en noordse vinvis (*B. borealis*) komen onder andere voor in de diepe noordelijke Noordzee, Bultrug (*Megaptera novaeangliae*) is in de hele Noordzee zeldzaam en blauwe vinvis (*B. musculus*) is er zeer zeldzaam. Toch zijn al deze soorten op de Nederlandse kust gevonden. Van noordse vinvis zijn er tot nog toe vier meldingen uit ons land: een uit de 19e eeuw (1811), de andere uit 1972, 1986 en 2005. Skeletresten van deze vier worden bewaard in de collectie van Naturalis Biodiversity Center in Leiden. Daarnaast is er in Naturalis een wervel afkomstig van de Doggersbank uit 1953, juist buiten het Nederlandse deel van het Continentale Plat, die eveneens aan noordse vinvis is toegeschreven. Omdat er twijfels waren over de juistheid van de determinaties, en vanwege de grote zeldzaamheid van noordse vinvis in de zuidelijke Noordzee, is DNA uit botten van drie van de veronderstelde noordse vinvissen onderzocht aan de hand van vijf markers: 16S, COI, ND4L, CytB en D-loop. Het exemplaar van 1986 is niet onderzocht, omdat determinatie van dit dier vanwege de zeer verse staat bij stranden, compleetheid van het kadaver en goede foto's, buiten kijf staat. Wel is het holotype van *Sibbaldius schlegelii* Flower, 1864 meegenomen in het onderzoek. Dit exemplaar is in het midden van de 19e eeuw gevonden op Java en naar Leiden gestuurd. De naam *B. borealis schlegelii* wordt gebruikt voor de ondersoort van de noordse vinvis die op het zuidelijk halfrond leeft en men is er altijd van uitgegaan dat dit exemplaar uit die populatie afkomstig was. Uit de resultaten van de DNA-analyse bleek dat twee als noordse vinvis gedetermineerde dieren dwergvinvissen bleken te zijn. Op grond van de gebruikte DNA-markers is er geen verschil aantoonbaar tussen het type van *schlegelii* en de nominaat uit de Noordzee. Dit onderzoek toont het belang aan van DNA-onderzoek, dat bovendien mogelijk blijkt te zijn aan de hand van oud botmateriaal, mits dat onder de juiste condities bewaard wordt. Ook het belang van collecties is hiermee opnieuw aangetoond. Daarnaast wordt gesteld dat het belangrijk is om altijd enig materiaal van gestrande walvissen te bewaren, zodat het voor eventueel toekomstig onderzoek gebruikt kan worden.

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