The Laptev Sea walrus *Odobenus rosmarus laptevi*: an enigma revisited

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The walrus (*Odobenus rosmarus*) is in some current systematic schemes divided into three subspecies: *O. r. rosmarus* in the North Atlantic, *O. r. divergens* in the North Pacific and *O. r. laptevi* in the Laptev Sea. These three subspecies have been described as differing in body size, but the taxonomic status of *O. r. laptevi* is disputed. The current study applies molecular and morphometric methods to assess the taxonomic status of *O. r. laptevi* and to analyse the systematic and phylogeographic relationships between the three purported walrus subspecies. Tusk length and tusk circumference were measured from the few skulls available of *O. r. laptevi*, and the obtained values were within the ranges reported for Pacific walruses. Thus, morphologically, subspecies status for *O. r. laptevi* is not supported according to the Amadon–Mayr '75% rule'. Phylogenetic analyses and haplotype networks based on mitochondrial nucleotide sequence data of NADH dehydrogenase 1, 16S rRNA, cytochrome oxidase I and the D-loop of the control region of the historic *O. r. laptevi* bone material and contemporary *O. r. rosmarus* and *O. r. divergens* showed that the Laptev Sea walrus groups with individuals from the North Pacific. Thus, the mitochondrial sequence data do not support the recognition of three walrus subspecies as reciprocally monophyletic evolutionary units with independent evolutionary histories. Only *O. r. rosmarus* and *O. r. divergens* meet this criterion with the present sampling. Accordingly, we recommend that *Odobenus r. laptevi* be abandoned and the Laptev walrus instead be recognized as the westernmost population of the Pacific walrus, *Odobenus r. divergens*. However, further research is recommended to assess whether the Laptev walrus could be considered as a significant unit in terms of conservation and management, since it is unique in several ecological parameters.

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Introduction

The walrus (*Odobenus rosmarus*) has a circumpolar distribution in the Arctic. Three subspecies are currently recognized. *Odobenus rosmarus rosmarus* is distributed in the North Atlantic and *O. r. divergens* is distributed in the North Pacific (Fay 1985). The third subspecies, *O. r. laptevi* from the Laptev

Sea, was recognized by Chapskii (1940) but its taxonomic status is disputed (Fay 1981, 1985). While Heptner *et al.* (1996), Rice (1998) and Wozencraft (2005) retained the subspecies status of *O. r. laptevi*, Fay (1985) considered its taxonomic status uncertain because of the very limited number of specimens available for comparative assessment of the phenotypic variation of the Laptev specimens. After having re-examined the available specimens Fay (1985) stated that 'the Laptev walruses, though very small, are most similar in form to *O. r. divergens*'. However, the morphological data revealed by Fay's re-examination have never been published.

Although the three tentative walrus subspecies have been described as differing in body size, there are no published morphometric comparisons that include the Laptev walrus. It is generally assumed that the Pacific walrus is the largest subspecies while Atlantic walruses from Eastern Canada tend to be the smallest (Fay 1981, 1985). The size of the Laptev walrus is intermediate according to the information provided by Chapskii (1940). However, Knutsen & Born (1994) found no statistical difference in the asymptotic standard length of male walruses from Greenland and the Pacific, although they found the Pacific form to be heavier than those from Greenland. McLaren (1993) suggested that Atlantic walruses were of the same size as Pacific walruses, and Wiig & Gjertz (1996) also concluded that the bodies of male Atlantic walruses at Svalbard can be as large as those of male Pacific walruses. Knutsen & Born (1994) and Garlich-Miller & Stewart (1998) suggested that body size in Atlantic walruses may differ significantly among subpopulations.

Only a few genetic studies have so far been conducted on walruses. Cronin *et al.* (1994) assessed genetic differentiation between the Atlantic and the Pacific subspecies based on mtDNA markers. Both subspecies had distinct monophyletic mtDNA haplotypes that supported their distinct taxonomic status. The genetic structure within Atlantic walrus populations has been fairly well-studied (Andersen *et al.* 1998; Andersen & Born 2000; Born *et al.* 2001). Based on RFLP patterns of the mitochondrial NADH dehydrogenase 1 (ND1), ND2 and ND3/ND4 genes and variation at 11 microsatellite loci, Andersen *et al.* (1998) concluded that gene flow is very restricted between North-West Greenland and more eastern Atlantic populations. However, there is only limited data available on the population structure of the Pacific walrus (Scribner *et al.* 1997; Jay *et al.* 2004), and walruses from the Laptev Sea region have never been included in any molecular analysis.

The Laptev walrus has been classified as data deficient in the IUCN Red List (Seal Specialist Group 1996), and listed in the category Species of Concern by Reijnders *et al.* (1997). In the Red Book of Russia the Laptev Sea walrus is considered an endemic subspecies that is potentially vulnerable due to low population size, limited range, and increased exposure to anthropogenic stress (Vishnevskaya & Bychov 1990). A proper taxonomic assessment of the Laptev walrus is therefore important in the context of future management of the population. Thus, the aim of the present study was to test the taxonomic status of *O. r. laptevi* by analysing the systematic and phylogeographic relationships among the three purported walrus subspecies using molecular and morphometric methods. For testing that Laptev walrus represents a distinct subspecies we follow the criteria that the three walrus subspecies should be reciprocally monophyletic evolutionary units with independent evolutionary histories (Moritz 2002; Zink 2004). We also follow the '75% rule' (Amadon 1949; Mayr 1969), which states that 75% of a population must effectively lie outside 99% of the range of other populations for given defining morphological characters. We analysed contemporary biopsies from *O. r. rosmarus* and *O. r. divergens*, and all currently available material from the Laptev walrus, that is, 12 skulls from the mammal collection of the Zoological Institute, Russian Academy of Sciences, St Petersburg, Russia.

Materials and methods

Samples

Bone material from 12 walruses, collected during 1886–1936 at three different localities in the Laptev Sea (Table 1, Fig. 1), was analysed. This material has been archived in the collection of the Zoological Institute of the Russian Academy of Sciences, St Petersburg. Unfortunately, Chapskii (1940) did not provide a careful description of the material he used for the subspecies description of *O. r. laptevi*. According to age of the samples and a notion by Chapskii (1940) pointing to the Zoological Institute of the Russian Academy of Sciences, St Petersburg, this material may have in part been used by him. F.H. Fay (unpublished data, available from the corresponding author) stated that most skulls were regarded as being from adults. DNA was also extracted from 28 skin biopsies, either frozen or stored in EDTA buffer, from contemporary walrus populations collected from North-West and East Greenland, Svalbard, Franz Josef Land, and Alaska. Fifteen sequences of the mtDNA control region (CR) from individuals collected throughout the distribution area (except the Laptev Sea) previously determined in the Arnason laboratory were also included in this study. In sum, the three subspecies *O. rosmarus laptevi*, *O. r. divergens* and *O. r. rosmarus*, were represented by 12, 16 and 27 samples, respectively (Table 1).

Morphometry

The circumference of the tusk and the thickness of the lower jaw were identified by Chapskii (1940) as important characters in the original description of the Laptev walrus. Thus, these morphological characters were chosen for comparison of the Laptev walrus skulls to material from Atlantic and Pacific

Table 1 Details for the walrus samples used in this study.

Sample	Sequence ID	Locality	Collecting date	Sex (age)	Latitude degree N	Longitude degree E/W	GenBank accession — Amplicon I	II	III
O. r. laptevi: Skulls from the Zoological Institute Russian Academy of Sciences, St Petersburg, Russia ($N = 12$)									
11129*	LAP01	Zhokhov Island	20 July 1913	Female†	75.60	152.50 E	EU728456	EU728488	EU728523
27654*	LAP02	Pronchishcheva Bay	September 1934	Male	76.00	113.50 E	n.d.	EU728489	EU728524
8696*	LAP03	New Sibir Island	1902	Female†	75.30	149.00 E	n.d.	n.d.	n.d.
27653*	LAP04	Pronchishcheva Bay	August 1934	Female†	76.00	113.50 E	n.d.	n.d.	n.d.
11116*	LAP05	Malyi Taimyr Island	27 August 1913	Male†	77.50	107.00 E	n.d.	n.d.	EU728525
27658*	LAP06	Pronchishcheva Bay	September 1935	n/a	76.00	113.50 E	n.d.	EU728490	EU728526
27652*	LAP07	Pronchishcheva Bay	6 September 1934	n/a	76.00	113.50 E	n.d.	EU728491	EU728527
4838*	LAP08	Kotelnyi Island (Novosibirskye Islands)	1886	n/a	74.90	140.00 E	n.d.	n.d.	n.d.
27651*	LAP09	Pronchishcheva Bay	6 September 1934	Female	76.00	113.50 E	EU728457	EU728492	EU728528
11114*	LAP10	Begichev Island (mouth of Khatanga River)	1912	Male†	74.30	112.00 E	n.d.	EU728493	EU728529
11123*	LAP11	Begichev Island (mouth of Khatanga River)	1912	n/a	76.20	112.00 E	EU728458	n.d.	n.d.
27656*	LAP12	Pronchishcheva Bay	8 October 1936	n/a	76.00	113.50 E	EU728459	EU728494	EU728530
O. r. divergens: Skin biopsies samples ($N = 13$)									
PAC531	PAC01	Savoonga/Diomede, Alaska	7 June 1994	Male (18)	64.75‡	167.75 W‡	EU728460	EU728496	EU728531
PAC536	PAC02	Savoonga/Diomede, Alaska	7 June 1994	Male (25)	64.75‡	167.75 W‡	EU728461	EU728497	EU728532
PAC537	PAC03	Savoonga/Diomede, Alaska	7 June 1994	Male (16)	64.75‡	167.75 W‡	EU728462	EU728498	EU728533
PAC539	PAC04	Savoonga/Diomede, Alaska	25 June 1994	Male (16)	64.75‡	167.75 W‡	EU728463	EU728499	EU728534
PAC541	PAC05	Savoonga/Diomede, Alaska	29 May 1994	Male (27)	64.75‡	167.75 W‡	EU728464	EU728500	EU728535
PAC543	PAC06	Savoonga/Diomede, Alaska	29 May 1994	Male (24)	64.75‡	167.75 W‡	EU728465	EU728501	EU728536
PAC544	PAC07	Savoonga/Diomede, Alaska	6 October 1994	Female (17)	64.75‡	167.75 W‡	EU728466	EU728502	EU728537
PAC545	PAC08	Savoonga/Diomede, Alaska	29 May 1994	Male (14)	64.75‡	167.75 W‡	EU728467	EU728503	EU728538
PAC546	PAC09	Savoonga/Diomede, Alaska	29 May 1994	Male (24)	64.75‡	167.75 W‡	EU728468	EU728504	EU728539
PAC547	PAC10	Savoonga/Diomede, Alaska	3 June 1994	Female (14)	64.75‡	167.75 W‡	EU728469	EU728495	EU728540
PAC548	PAC11	Savonga/Diomede, Alaska	3 June 1994	n/a	64.75‡	167.75 W‡	EU728470	EU728505	EU728541
PAC550	PAC12	Savoonga/Diomede, Alaska	1994	n/a	64.75‡	167.75 W‡	EU728471	EU728506	EU728542
PAC551	PAC13	Savoonga/Diomede, Alaska	1994	n/a	64.75‡	167.75 W‡	EU728472	EU728507	EU728543

Table 1 *Continued.*

Sample	Sequence ID	Locality	Collecting date	Sex (age)	Latitude degree N	Longitude degree E/W	GenBank accession — Amplicon		
							I	II	III
O. r. rosmarus: Skin biopsies samples (*N* = 15)									
STu25	ATL01	Tusenøyane, Svalbard	30 August 1993	n/a	77.00	22.00 E	EU728473	EU728508	EU728544
STu26	ATL02	Tusenøyane, Svalbard	30 August 1993	n/a	77.00	22.00 E	EU728474	EU728509	EU728545
STu27	ATL03	Tusenøyane, Svalbard	30 August 1993	n/a	77.00	22.00 E	EU728475	EU728510	EU728546
SL28	ATL04	Lågøya, Svalbard	20 August 2004	n/a	80.30	18.20 E	EU728476	EU728511	EU728547
SL29	ATL05	Lågøya, Svalbard	20 August 2004	n/a	80.30	18.20 E	EU728477	EU728512	EU728548
69TH	ATL06	Thule, NW Greenland	21 March 1990	Female	77.50	72.00 W	EU728478	EU728513	EU728549
456TH	ATL07	Thule, NW Greenland	16 March 1990	Female	77.50	72.00 W	EU728479	EU728514	EU728550
457TH	ATL08	Thule, NW Greenland	21 March 1989	Male	77.50	72.00 W	EU728480	EU728515	EU728551
493TH	ATL09	Thule, NW Greenland	29 March 1990	Male	77.50	72.00 W	EU728481	EU728516	EU728552
781TH	ATL10	Thule, NW Greenland	25 April 1990	Female	77.50	72.00 W	EU728482	EU728517	EU728553
J2SCO	ATL11	Scoresby Sound, E Greenland	23 August 1989	Male	70.50	21.40 W	EU728483	EU728521	EU728554
J7SCO	ATL12	Scoresby Sound, E Greenland	1990	Male	70.50	21.40 W	EU728484	EU728522	EU728555
J13SCO	ATL13	Scoresby Sound, E Greenland	03 July 1992	Male	70.50	21.40 W	EU728485	EU728520	EU728556
10SCO	ATL14	Scoresby Sound, E Greenland	06 October 1993	Male	70.50	21.40 W	EU728486	EU728518	EU728557
12SCO	ATL15	Scoresby Sound, E Greenland	21 June 1993	Male	70.50	21.40 W	EU728487	EU728519	EU728558
O. r. divergens: Sequenced earlier in the U. Arnason laboratory (*N* = 3)									
OROSALASKA	PAC14	Alaska	n/a	n/a	n/a	n/a	n.d.	n.d.	EU728562
1829ALASKA1	PAC15	Alaska	n/a	n/a	n/a	n/a	n.d.	n.d.	EU728563
OROSALASKA	PAC16	Alaska	n/a	n/a	n/a	n/a	n.d.	n.d.	EU728564
O. r. rosmarus: Sequenced earlier in the U. Arnason laboratory (*N* = 12)									
OROS19TH	ATL16	Thule, NW Greenland	25 May 1989	n/a	77.50	72.00 W	n.d.	n.d.	EU728559
OROS522TH	ATL17	Thule, NW Greenland	28 May 1989	n/a	77.50	72.00 W	n.d.	n.d.	EU728560
OROS2TH	ATL18	Thule, NW Greenland	13 May 1989	n/a	77.50	72.00 W	n.d.	n.d.	EU728561
OROS10FrJo	ATL19	Franz Josef Land	2 September 1992	n/a	79.90	50.00 E	n.d.	n.d.	EU728565
OROS11FrJo	ATL20	Franz Josef Land	2 September 1992	n/a	79.90	50.00 E	n.d.	n.d.	EU728566
OROS1FrJo	ATL21	Franz Josef Land	2 September 1992	n/a	79.90	50.00 E	n.d.	n.d.	EU728567
OROS7FrJo	ATL22	Franz Josef Land	2 September 1992	n/a	79.90	50.00 E	n.d.	n.d.	EU728568
OROS9FrJo	ATL23	Franz Josef Land	2 September 1992	n/a	79.90	50.00 E	n.d.	n.d.	EU728569
OROS2Svalb	ATL24	Tusenøyane, Svalbard	21 July 1992	n/a	77.00	22.00 E	n.d.	n.d.	EU728570
OROS3Svalb	ATL25	Tusenøyane, Svalbard	21 July 1992	n/a	77.00	22.00 E	n.d.	n.d.	EU728571
OROS4Svalb	ATL26	Tusenøyane, Svalbard	22 July 1992	n/a	77.00	22.00 E	n.d.	n.d.	EU728572
OROS5Svalb	ATL27	Tusenøyane, Svalbard	22 July 1992	n/a	77.00	22.00 E	n.d.	n.d.	EU728573

*Collection number of the Zoological Institute, Russian Academy of Sciences, St Petersburg, Russia; †according to F. Fay (unpublished data); ‡exact location of sampling site not known; n.d., sequence not determined; n/a, data not available.

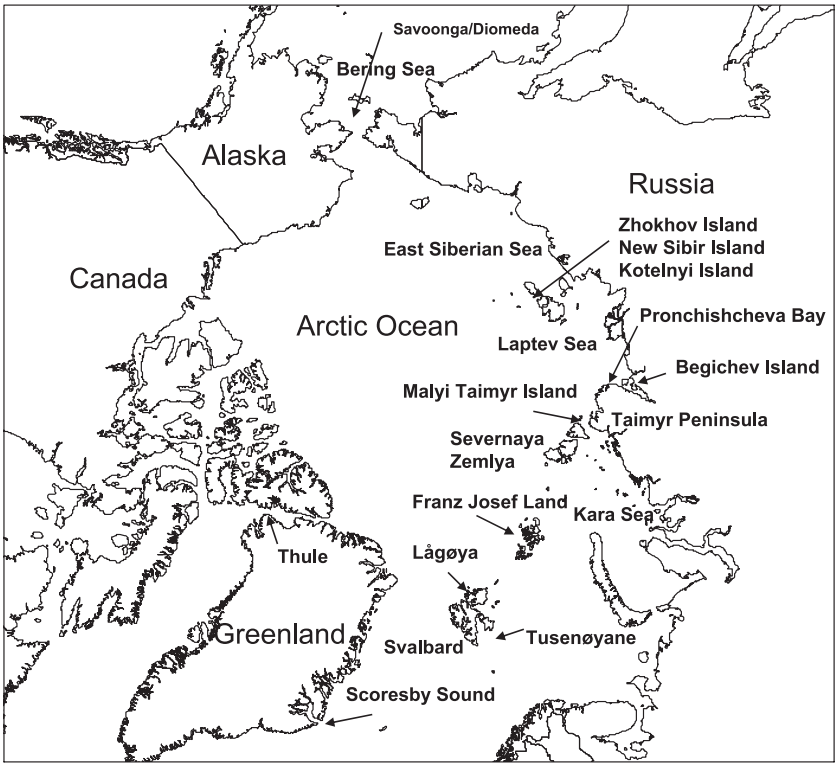

Fig. 1 Sampling sites of walruses used in this study (indicated with arrows).

walrus populations. Circumference of the tusk was measured at the gum, and the thickness of the lower jaw was measured at its narrowest part, that is, mandible thickness behind the postcanines. Five additional measurements were made on the 12 Laptev walrus skulls in the collection of the Zoological Institute, Russian Academy of Sciences, St Petersburg (as described by Wiig & Gjertz (1996) and Wiig *et al.* (2007)), including: (i) the length of the tusk, measured along the front of the tusk; (ii) mandible length; (iii) mandible height; (iv) mandible depth behind the postcanines; and (v) mandible thickness.

Ancient DNA analyses

When working with historical samples it is of utmost importance to follow stringent procedures because the minute amount, and the degraded nature, of historic (or ancient) DNA make the procedures particularly sensitive to contamination (Cooper & Poinar 2000; Gilbert *et al.* 2005; Rohland & Hofreiter 2007). The introduction of foreign DNA can happen at any stage in the analysis of ancient DNA, rendering precautions mandatory. Therefore, chopping and grinding of bone material was conducted in a different building — physically isolated from the DNA facility at the Natural History Museum in Oslo. Furthermore, at the DNA laboratory where analyses of old bone material were performed there has been no previous handling of any modern material from walruses, and all analyses (i.e., from DNA extraction through sequence determinations) of modern walrus material for this study were done at a different DNA facility (CEES laboratory facility at the University of Oslo, located on a different campus). All DNA extractions and PCR reactions of old material were set-up in PCR workstations equipped with UV-light in a dedicated DNA facility separated from facilities where subsequent purifications and sequencing of the products were performed.

Ancient DNA extraction

Small samples of bone material were chopped off specimens with a chisel and hammer and ground into a fine powder using a mortar under liquid nitrogen. Approximately 0.1 g of bone powder was subsequently transferred into a 2.0-mL screw-capped centrifugation tube and DNA was extracted according to Borge *et al.* (2007).

Modern DNA extraction

For DNA extraction of modern samples, the EZNA Tissue DNA kit (Omega Bio-tek, Doraville, GA) was used according to the manufacturer's instructions, except for the final elution step where the DNA was eluted in 2×100 μL elution buffer to recover DNA in higher concentration.

Table 2 Primers used in this study for the amplification of walrus mtDNA target regions. ND1 (except from ND1-1F) and COI primers were designed based on sequences from the complete *Odobenus rosmarus* mitochondrial genome (GenBank accession AJ428576; Arnason *et al*. 2002).

Amplicon	Primer name	Forward primer sequence (5′–3′)	Primer name	**Reverse primer sequence (5′–3′)**
I-1	ND1-1F	ACCCCGCCTGTTTACCAAAAACAT*	ND1-3R	TGTCCTGATCCAACATCGAG
I-2	ND1-4F	GGGATAACAGCGCAATCCTA	ND1-6R	TGGTCGTAAAGGTTCCTTGG
I-3	ND1-7F	GGACCATACGGACTTCTCCA	ND1-9R	CCAGGAAGAATAGGGCGAAT
I-4	ND1-10F	CAGAATTAGTATCAGGCTTCAACG	ND1-12R	GGTATGGGCCCGATAGCTTA
II-1	COI-1F	ACAAGGACATCGGCACTCTC	COI-1R	GCTCCGATTATTAGGGGAACT
II-2	COI-2F	GCCCATGCATTCGTAATAATC	COI-2R	GATGGTCCTGCGTGAGCTA
II-3	COI-3F	GAACCGGATGAACCGTCTAC	COI-3R	CCGCTGTAATTAATACGGACCA
II-4	COI-4F	CTCCCGCAATATCCCAATAC	COI-4R	TTCCGAATCCTGGTAGAATGA
III-1	DL-1F	GCCTATTGCCGGTATAATCG	DL-1R	TGTGATGGTACAGTAGGGGTGA
III-2	DL-2F	CTGACGCCCTACCATTCATA	DL-2R	AAGGGTTGCTGGTTTCTCG
III-3	DL-3F	AATTCACTTGGTCCGTCAAGC	DL-3R	TTATGTGTGATCATGGGCTGA
III-4	DL-4F	TGGGACATCTCGATGGACTT	DL-4R	CGTGTATGTCCTGTGACCATT

*From Cronin *et al.* (1994).

PCR amplifications and sequencing

Three regions of the mitochondrial DNA (mtDNA), were targeted (nucleotide positions refer to the complete *O. rosmarus* mitochondrial genome, GenBank accession AJ428576; Arnason *et al*. 2002): (i) a *c.* 1930 base pairs (bp) amplicon I covering *c.* 820 bp of the 16S rRNA, the tRNA-Leu of the ND1 and the tRNA-Ile genes (nucleotide positions 1936–3866); (ii) a 685-bp amplicon II covering parts of the cytochrome oxidase subunit I (COI) gene (positions 5398–6083); and (iii) a *c.* 700 bp amplicon III covering the tRNA-Thr and the tRNA-Pro genes as well as *c.* 560 bp of the D-loop of the non-coding CR (positions 15 313–16 015). Walrus-specific internal primers were designed using software Primer3 (Rozen & Skaletsky 2000) to amplify four overlapping fragments of *c.* 500 bp each of the ND1 region (Table 2). The COI and CR segments were amplified as one fragment in the modern samples but for the old samples internal primers were designed in order to amplify overlapping fragments of *c.* 200–240 bp each. ND1 and COI primers were designed based on sequences from the complete *O. rosmarus* mitochondrial genome (see above), while CR primers were designed in conserved areas, identified on the basis of an alignment of sequences determined in the Arnason laboratory.

PCR amplifications were performed in 25 μL reaction volumes using either PCR beads (GE Healthcare, Little Chalfont, UK) or *PfuTurbo* DNA polymerase (Stratagene, La Jolla, CA), 1× Reaction Buffer, 0.2 mM of each dNTP, 0.04% bovine serum albumin (BSA), 1 μM of each primer, and 50–100 ng contemporary DNA or 2 μL of ancient DNA extract (DNA concentration not quantified). Thermal cycling conditions were the same for all three amplified mtDNA regions: 94 °C for 2 min; 32 cycles of 94 °C for 50 s, 48 °C for 50 s and 72 °C for 1 min; and a final extension at 72 °C for 10 min. PCR products were purified using a third volume of 10 times diluted exoSAP-IT (USB Corporation, Cleveland, OH) per reaction. Cycle sequencing, using the same primers as in the PCR reaction, was performed in 10 μL reactions using 2 μL BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA), 1× Sequencing Buffer, 10 pmol primer, and 250–500 ng cleaned PCR product. Sequencing products were purified with ethanol precipitation and analysed using an ABI 3100 Genetic Analyser (Applied Biosystems). Modern samples were sequenced at the CEES ABI-laboratory, University of Oslo, using an ABI 3730 Genetic Analyser. Forward and reverse sequences were assembled and edited using Sequencher ver. 4.1.4 (GeneCodes, Ann Arbor, MI). The mitochondrial nucleotide sequences have been deposited in GenBank (accession numbers EU728456–EU728573).

Data analyses

All sequences were unambiguously aligned manually using the software BioEdit (Hall 1999). The following mtDNA data matrices were subjected to parsimony analyses using the program TNT (Goloboff *et al*. 2003): (i) amplicon I separately; (ii) amplicon III separately; and (iii) all three regions combined excluding samples which were not represented in all data sets (orphans). In order to investigate monophyly of the subspecies, parsimony analysis of the ND1 data set was performed including sequences from mtDNA genome data of three otariid outgroup taxa obtained from GenBank (*Callorhinus ursinus* AM181016, *Eumetopias jubatus* AJ428578, *Zalophus californianus* AM181017). All analyses were performed using a traditional search approach (equal weights, 100 random replications saving 10 trees per replicate). The trees obtained were further swapped using TBR and a strict consensus tree was estimated. To estimate support for internal branches, parsimony jackknifing (Farris *et al*. 1996) was performed in TNT. One thousand replicates were conducted, each performing TBR branch swapping with 10 random entry orders saving 1 tree per replicate. Partition homogeneity test were performed using PAUP* 4 beta 10 win (Swofford 2003).

Genealogical relationships among individual walrus haplotypes were estimated using the software TCS (Clement *et al.* 2000), which is based on the method of Templeton *et al.* (1992) to infer population level genealogical networks. The statistical parsimony procedure in this analysis was developed specifically to reconstruct intraspecific gene trees when differences among haplotypes are low. Furthermore, the algorithm identifies potential recombinant haplotypes and provides a quantitative assessment of deviations from parsimony, that is, how much ambiguity is likely to exist in bifurcating tree reconstructions. Statistical parsimony has been demonstrated to outperform traditional phylogenetic approaches when the level of divergence among sequences is low (e.g. Crandall 1994, 1996). Using a 95% statistical confidence ('connection limit'), the analysis was performed on the CR data matrix, which represents most individuals and appeared to be the fastest-evolving marker of the three mtDNA regions included in this study (see also Pesole *et al.* 1999). Since the high number of missing characters for some of the samples from the Laptev Sea could bias downstream analyses, this latter data set was modified by (i) deleting one of the Laptev samples (11129), since it contributed only with 252 bp, (ii) deleting a polyT region and three single nucleotide positions that introduced several gaps into the alignment, and (iii) replacing the missing information in sequences of the Laptev samples 27651 and 27654 with the consensus of the other Laptev Sea sequences according to the criterion that more than 75% of the sequences shared the consensus.

Similarly, based on the CR data, genetic structure among the walrus populations included in this study was investigated. Five populations were defined based on the geographical locations of the sample collections: (i) Alaska; (ii) Laptev Sea; (iii) Svalbard plus Franz Josef Land; (iv) E Greenland; and (v) NW Greenland. The latter three subpopulations have previously been identified (Andersen *et al.* 1998; Andersen & Born 2000; Born *et al.* 2001). First, an analysis of molecular variance (AMOVA; Excoffier *et al.* 1992) was conducted with the program ARLEQUIN (Schneider *et al.* 2000) testing differentiation among groups of populations according to subspecific affinity, that is, whether the data would group into two or three subspecies. Next, alternative groupings of the populations were tested using a spatial analysis of molecular variance (SAMOVA; Dupanloup *et al.* 2002). This method is based on a simulated annealing procedure that aims to maximize the proportion of total genetic variance due to differences between groups of populations (F_{CT}). Using the software SAMOVA 1.0 <http://web.unife.it/progetti/genetica/Isabelle/samova.html>, analyses were performed based on 100 simulated annealing steps with $K = 2$ and 3 (K = the number of geographically defined groups). Standard diversity indices (e.g. Nei 1987; Tajima 1983, 1993) were computed with Arlequin (Schneider *et al.* 2000).

Results

Morphometric data

The morphometric data obtained from the 12 skulls from Laptev Sea walruses are listed in Table 3. Only data for mandible length, height, depth and thickness and tusk length for females and tusk circumference for both sexes of the *O. r. laptevi* samples were available for comparative use. The mean mandible length, height, depth and thickness for females were 21.1, 4.7, 7.0 and 1.6 cm, respectively, ($N = 3$). The mean tusk length was 29.3 cm for females ($N = 3$), but for males there was only one complete specimen (40.8 cm). The mean tusk circumference was 12.9 cm for females ($N = 4$) and 18.7 cm for males ($N = 4$).

Molecular data

Because of the degraded and fragmentary nature of DNA from historic samples, only short segments can be targeted using carefully designed PCR primers. Nine (75%) of the historic bone samples from the Laptev Sea walruses rendered useful DNA for genetic analysis, though not all of these samples yielded successful amplicons with all primer combinations. Amplicon I was successfully amplified for only four historic samples. For the COI region, *c.* 185 bp were amplified for seven samples. The most extensive data set was obtained for amplicon III where five historic samples yielded concatenated full-length sequences (707–708 bp) and three yielded concatenated partial sequences (252–403 bp). Full nucleotide

Table 3 Morphometric measurements of the Laptev walrus samples from the collection of the Zoological Institute, Russian Academy of Sciences, St Petersburg, Russia. Many measurements could not be determined due to fragmentation of the samples.

Sample	11 114	11 116	27 652	27 654	8696	11 129	27 651	27 653	11 123	27 658
Sex	Male	Male	Male	Male	Female	Female	Female	Female	unknown	unknown
Mandible length (cm)	24.7	24.0	—	—	20.7	—	20.8	21.8	—	—
Mandible height (cm)	6.4	5.8	—	—	4.3	—	4.7	5.0	—	—
Mandible depth (cm)	8.9	8.4	—	—	6.6	—	6.8	7.6	—	—
Mandible thickness (cm)	2.7	2.7	—	—	1.3	—	1.7	1.8	—	—
Tusk circumference (cm)	22.3	15.4	18.0	19.0	11.8	11.6	13.0	15.2	11.8	16.4
Tusk length (cm)	—	40.8	—	—	32.0	25.0	31.0	—	17.5	32.4

sequences were determined for the three targeted mtDNA regions for the contemporary samples, except from one partial amplicon I sequence from a sample PAC548 from Alaska.

The amplicon I and amplicon II data sets both included a total of 35 sequences, and the complete alignments were 1939 and 686 characters, respectively. The amplicon III data set included 51 sequences and 709 characters, of which 54 were variable. Ten of these nucleotide positions included insertions/deletions (indels) and 32 were potentially phylogenetically informative. Since the high number of missing characters for some of the samples from the Laptev Sea could bias downstream analyses, this latter data set was modified. The resulting data set, which included 50 amplicon III sequences and 698 characters, was used for determining haplotypes and performing the network and AMOVA analyses (see below). The resulting alignment that was used for all further analyses can be obtained upon request.

Parsimony analyses

The amplicon I data matrix yielded six most-parsimonious trees of 426 steps (consistency index CI = 0.87, retention index RI = 0.92). The three outgroup taxa used in this analysis rendered all walrus samples a strongly supported monophyletic clade in the strict consensus tree (Fig. 2). Within the walrus clade, the Atlantic individuals grouped together in one clade that was a sister clade to that containing all Laptev Sea walruses and nearly all Pacific walrus individuals. One individual from Alaska (PAC531) was resolved as a sister to this larger clade, although there was no strong support for this in the jackknife resampling analysis.

The analysis of amplicon III yielded more than 1000 most-parsimonious trees of 77 steps (consistency index CI = 0.61, retention index RI = 0.91). The unrooted strict consensus tree (data not shown) provides a better visual resolution of the clades than the amplicon I consensus tree, particularly with respect to the walruses from the Pacific and the Laptev Sea. Again, the Laptev Sea samples were nested within the Pacific walrus samples. Two groups, the Atlantic and the Pacific/Laptev Sea walrus, respectively, formed well-supported monophyletic clades. Within the Pacific/Laptev Sea clade, a well-supported group consisting of four Alaskan individuals was present, including the PAC531 individual that remained unresolved in the amplicon I analysis. Within the Atlantic clade, the samples from NW Greenland formed a well-supported group in both analyses.

Partition homogeneity tests performed on (i) the three amplicons, and (ii) 16S rRNA, ND1, COI and CR revealed no significant differences ($1 \geq P \geq 0.694$). Subsequent analyses using the combined data set yielded similar results to those reported above (Fig. 3), although the Pacific/Laptev clade was less well-resolved than with the amplicon III data alone where 188 most-parsimonious trees of 139 steps were produced

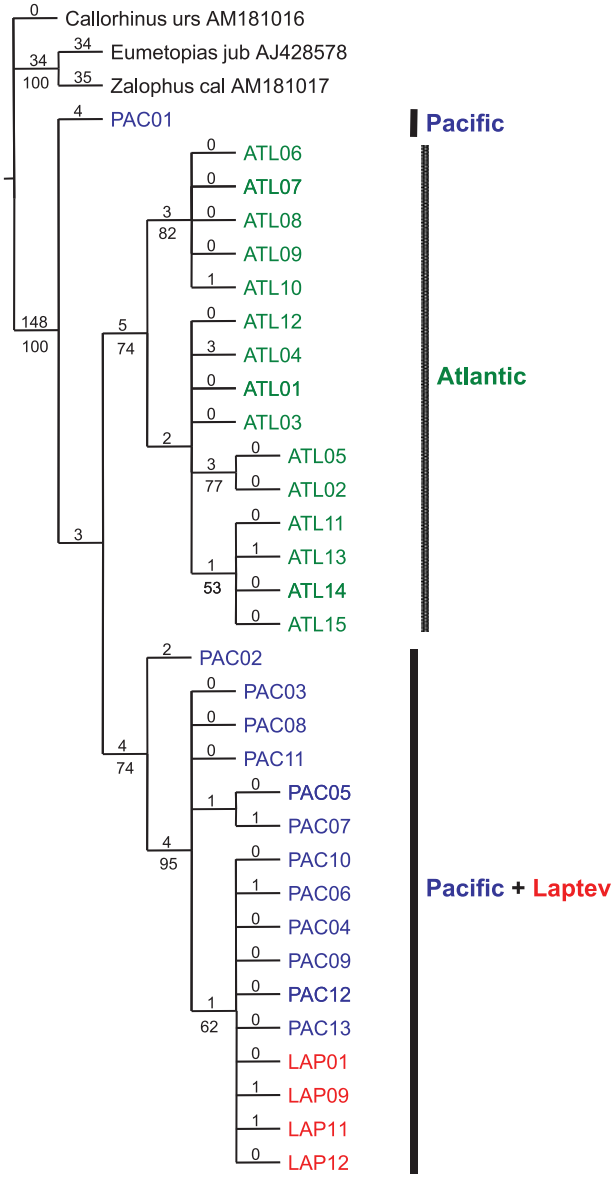

Fig. 2 Strict consensus tree of six trees based on walrus and outgroup 16S rRNA, tRNA-Leu of the NADH dehydrogenase 1 (ND1) and tRNA-Ile (amplicon I) sequences. Branch lengths (number of unambiguous nucleotide substitutions as optimized by Winclada (Nixon 2002)) are shown above branches and jackknife values > 50 are shown below the branches. Three otariid outgroup taxa were retrieved from GenBank: Northern fur seal (*Callorhinus ursinus*, AM181016), Steller sea lion (*Eumetopias jubatus*, AJ428578), and California sea lion (*Zalophus californianus*, AM181017). Walrus samples from the Laptev Sea are shown in red, Pacific samples in blue and Atlantic samples in green.

(consistency index CI = 0.76, retention index RI = 0.96). A strongly supported (100% jackknife support) NW Greenland clade was resolved as the sister group to a well-supported clade

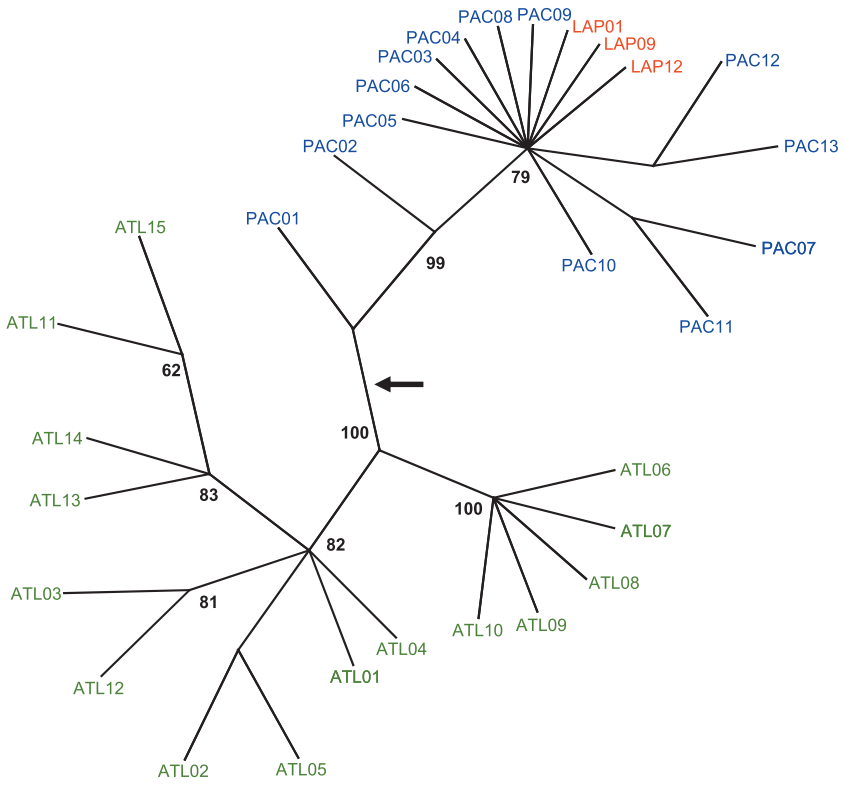

Fig. 3 Unrooted strict consensus of 139 trees based on a combined matrix of partial 16S rRNA, ND1, control region, and COI (amplicons I, II and II) sequences. Jackknife values > 50 are shown at the nodes. Walrus samples from the Laptev Sea are shown in red, Pacific samples in blue and Atlantic samples in green. The black arrow indicates the split between the Atlantic and Pacific + Laptev Sea samples.

consisting of the remaining Atlantic samples. The PAC531 individual was strongly supported as being sister to the Atlantic walrus clade in this analysis.

Network construction

A statistical parsimony network construction based on the amplicon III sequences resulted in a single network (Fig. 4), which unambiguously separated the Atlantic and Pacific haplotypes. Nonetheless, some loops (i.e., more than one most-parsimonious connection of haplotypes) were found between these two groups. Two Pacific haplotypes were separated from both the Atlantic haplotypes and the remaining Pacific and Laptev haplotypes by several mutational changes (internodes plus dots in the network). Consequently, the Laptev haplotypes were more closely connected to the remaining Pacific haplotypes than to either of the two other Pacific groups or the Atlantic haplotypes. More mutational changes were detected among the Pacific samples, suggesting greater haplotype differentiation in this region.

Genetic substructure

AMOVA was used to partition haplotype diversity for amplicon III into variation: (i) among groups (subspecies), (ii) among populations as defined according to sampling region (Table 4), and (iii) within populations (Excoffier *et al.* 1992). Analyses were performed twice, testing different *a priori* definitions of groups. In the first test, two groups were defined, that is, *O. r. rosmarus* (group I), and *O. r. laptevi* and *O. r. divergens* pooled (group II). In the second test, the three subspecies were taken as separate groups. In both tests most molecular variation was attributed to differentiation among groups (subspecies), at least when compared to differentiation among or within populations (Table 5). However, there was no significant support for genetic differentiation into three vs. two subspecies. Also, when analysed with SAMOVA there was no significant support for alternative subgroupings (results not shown). However, the *P*-value decreased slightly ($P = 0.07$) when samples were separated into: (i) 'NW Greenland'; (ii) 'Pacific' plus 'Laptev'; and (iii) 'E Greenland' plus 'Svalbard and Franz Josef Land'.

Nucleotide diversity was calculated as an average over all loci, and ranged from 0.0004 (NW Greenland) to 0.0128 (Alaska) (Table 4). Although the number of haplotypes from Alaska and Svalbard (including Franz Josef Land) was the same, the number of polymorphic sites was considerably larger in the Alaskan population.

Discussion

Walruses have a discontinuous circumpolar distribution (Fig. 1) and are found in areas with drifting arctic pack ice over continental shelf areas (Rice 1998). The Atlantic walrus (*O. r. rosmarus*) is distributed from the Eastern Canadian Arctic to the Kara Sea, including western and eastern Greenland,

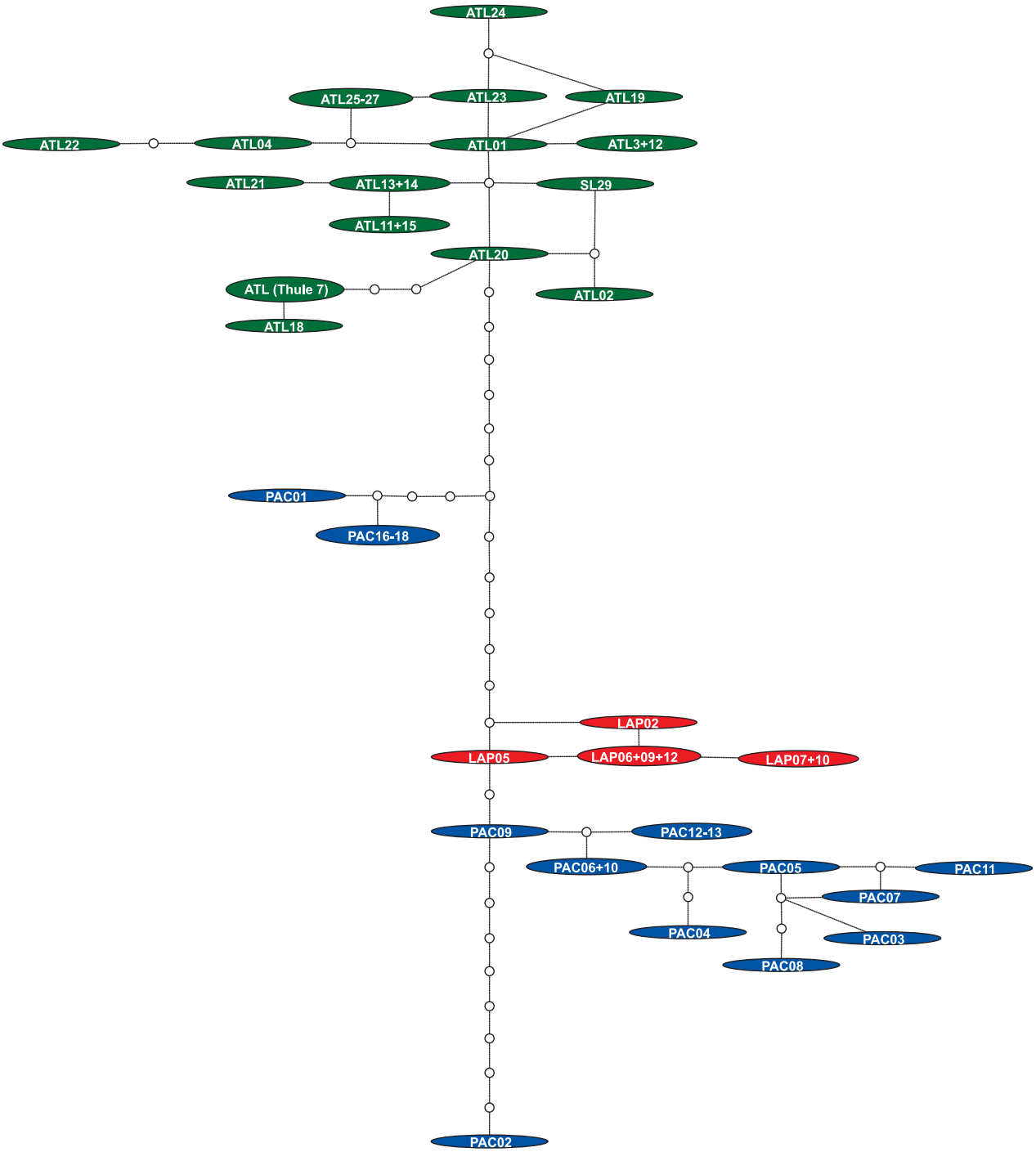

Fig. 4 Statistical parsimony haplotype network based on walrus partial D-loop of the non-coding control region (amplicon III) sequences. Walrus samples from the Laptev Sea are shown in red, Pacific samples in blue and Atlantic samples in green.

Svalbard, Franz Josef Land and Novaya Zemlya (Fay 1982; Born *et al.* 1995). The Pacific subspecies (*O. r. divergens*) is distributed in the Pacific Arctic from Cape Shelagskyi in Siberia to Point Barrow in Alaska and in the Bering Sea from Karaginskyi Bay in Kamchatka to Bristol Bay in Alaska. The Laptev walrus (*O. r. laptevi*) is distributed between the eastern part of the Kara Sea, the Laptev Sea and the western part of the East Siberian Sea (Rice 1998).

Table 4 Molecular diversity indices based on amplicon III sequences (parts of the D-loop of the control region) measured for walrus grouped according to the sampling localities of the individuals, i.e. Alaska, Laptev Sea, NW Greenland, E Greenland, and Svalbard (includes samples from Franz Josef Land).

	Alaska	Laptev	NW Greenland	E Greenland	Svalbard
Number of samples	16	7	8	5	14
Number of haplotypes	12	4	2	3	12
Number of sites	698	698	698	698	698
Number of polymorphic sites	30	3	1	4	14
Nucleotide diversity (s.d.)*	0.0128 (0.0070)	0.0015 (0.0013)	0.0004 (0.0005)	0.0026 (0.0021)	0.0050 (0.0030)
Pi (s.d.)†	8.9417 (4.3496)	1.0476 (0.7848)	0.2500 (0.3113)	1.8000 (1.2360)	3.4835 (1.8889)

*Nucleotide diversity calculated as an average over all sites.

†Mean number of differences between all pairs of haplotypes in the sample.

Table 5 Molecular variance (AMOVA) of mtDNA using haplotype frequencies based on amplicon III sequences covering parts of the D-loop of the control region. The two analyses consider two different groupings of walrus populations (two vs. three subspecies). The populations were defined according to the sampling localities of the individuals, i.e. Alaska, Laptev Sea, NW Greenland, E Greenland, and Svalbard plus Franz Josef Land.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*F*-statistics	*P*-value
Two subspecies hypothesis: *O. r. rosmarus* vs. *O. r. divergens*						
Among *two* groups/subspecies	1	128.916	4.50472	56.99	F_{CT} = 0.56994	0.09842
Among populations within groups	3	38.860	1.23635	15.64	F_{SC} = 0.36373	< 0.00001
Within population	45	97.323	2.16274	27.36	F_{ST} = 0.72637	< 0.00001
Three subspecies hypothesis: *O. r. rosmarus* vs. *O.r. laptevi* vs. *O. r. divergens*						
Among three groups/subspecies	2	142.450	3.76333	52.25	F_{CT} = 0.52246	0.10149
Among populations within groups	2	25.327	1.27709	17.73	F_{SC} = 0.37127	< 0.00001
Within population	45	97.323	2.16274	30.02	F_{ST} = 0.69975	< 0.00001

Chapskii (1940) considered the Laptev walruses different from Atlantic and Pacific walruses based on their specific geographical location and their intermediate size. He was obviously aware of the weakness of the evidence, as he himself stated that the data '... [were] not completely sufficient for any categorical statement ...' (Chapskii 1940). Nevertheless, he formally described *O. r. laptevi* as a separate subspecies but did not provide more than a half-page description for the new taxon.

According to Chapskii (1940), adult males have tusks with a mean circumference at the base of 16.8 cm (N = 5). Later, Popov (1960a) studied 300 walrus specimens and concluded that maximum tusk length in adult walruses from the Laptev Sea was 65 cm in males and 58 cm in females. He found an average tusk length for mature males (body length 300+ cm) of 45–47 cm and 34–36 cm for females. The tusk circumference in females was 9–14 cm with a peak at 12 cm, and 14–21 cm with a peak at 18 cm in males. For Laptev walruses considered 3+ years old (estimated according to body size), Tavrovskii (1971) reported a mean tusk length of 35.3 cm (range 21–47 cm; cv 20.2) for males and 25.4 cm (range 10–43; cv 29.0) for females. The number of animals included in this study was not explicitly stated but was likely N = 24 males and N = 169 females (based on table 1 in Tavrovskii (1971)). According to Fay (1982 fig. 79, p. 115), very few tusks of Pacific walrus have a circumference larger than 23 cm. The mean circumference of tusks longer than 30.0 cm is < 20.0 cm (interpreted from Fay 1982). On Svalbard Wiig & Gjertz (1996) found tusk circumferences between 13 and 24 cm, with a mean of 18.7 cm, for 38 anaesthetized males selected for their large size. Consequently, based on literature and the data presented here (Table 3) there is no clear support for the hypothesis that male tusk circumference of walruses from the Laptev Sea is different from Pacific (*O. r. divergens*) or Atlantic (*O. r. rosmarus*) walruses.

Chapskii (1940) suggested that the thickness of the lower jaw was distinctive among Laptev walruses. He listed mean values of 4.04 cm for males (N = 4) and 1.98 cm for females (N = 2) for walruses from the Chukchi and Bering Seas. In our study, measurements for Laptev males were 2.7 cm (N = 2) and Laptev females had an average of 1.6 cm (N = 3). These measurements are well within the range for this character in Atlantic walruses from Arctic Canada studied by Wiig *et al.* (2007). Therefore, Atlantic and Laptev walruses cannot be separated based on this character. Unfortunately, comparable data do not exist for Pacific walruses. However,

even if more comprehensive information did exist for contemporary walruses, the morphological information on the Laptev walrus is so fragmentary that comparative statistical analyses would be impossible. To summarize, based on the available morphological data *O. r. laptevi* cannot be considered a subspecies in its own, at least not when applying the '75% rule' (Amadon 1949; Mayr 1969), which states that 75% of a population must effectively lie outside 99% of the range of other populations for a given defining character or set of characters.

Based on mitochondrial sequence data, there is also no support for the Laptev walrus being considered a discrete taxonomic entity since the individuals included in this study were nested within the Pacific walrus clade, rendering the subspecies *O. r. divergens* paraphyletic. Our reasoning is in line with concepts of subspecies being defined as reciprocally monophyletic evolutionary units with independent evolutionary histories (Moritz 2002; Zink 2004). Consequently, the walruses in the Laptev Sea region can at best be considered the westernmost population of the Pacific walrus *O. r. divergens*, at least based on our limited sampling.

It is possible, nonetheless, that Laptev Sea walruses could become an isolated group over time. Walruses are thought to occur in the region on a year-round basis and heavy winter ice conditions in the northern Laptev Sea might restrict their winter movements. Probably Laptev walruses concentrate their winter (Fay 1982; Fay *et al*. 1984; Solovieva 2001) breeding aggregations in the Great Siberian Polynya (Sjare & Stirling 1996), which could result in them becoming genetically isolated from other groups. However, given the prognosis of marked reductions in sea ice extent and thickness throughout much of the Arctic (e.g. ACIA 2006), it is just as likely that there will be increased mobility of walruses among populations in the future.

Observation of walruses in the Kara Sea along the northwestern coast of Taimyr and west of Severnaya Zemlya are rare (Belikov *et al*. 1998a,b). However, walruses have been observed in leads and channels in the northern Kara Sea and in the area east of Severnaya Zemlya. These animals are presumably from the Atlantic subspecies. The Laptev walrus is most numerous in the western part of the Laptev Sea near the coast of south-eastern Taimyr (Chapskii 1940). There are very few walruses along the eastern coasts of Severnaya Zemlya (Chapskii 1940; Shereshevskii 1960; Popov 1960b; Belikov *et al*. 1998b) and it is unlikely that walruses move into the Kara Sea from the east (Chapskii 1940). One can only speculate as to the reason for the few walruses that occur in the eastern part of the Severnaya Zemlya, but their scarcity in this area might be related to food availability. The shelf area is relatively narrow and deep water is encountered rapidly to the north and east of the Severnaya Zemlya. In more southern and central areas of the Laptev Sea, the shelf is very wide and the sea is shallow creating a more ideal habitat for the benthic feeding walrus. Large areas of the Laptev shelf are shallower than 50 m and the mean depth of the southern part is only around 20 m. The Taimyr Peninsula (*c.* 95°E) might represent the border between the distribution of Atlantic and Pacific walruses.

Although migration of marine mammals between the Atlantic and Pacific oceans is considered rare, significant genetic exchange might occur among some populations. For example, Borge *et al*. (2007) have recently shown that bowhead whales (*Balaena mysticetus*) show no significant genetic differentiation between historical samples from the Spitsbergen stock in the North Atlantic and the current Bering-Chukchi-Beaufort (BCB) stock in the North Pacific. These data suggest that migration of bowhead whales might have occurred over Northern Siberia within the last 10 000 years. The occurrence of bowheads along the entire Siberian coast has also been suggested by Belikov & Boltunov (2002). No migration events by walruses have been reported between their distribution areas in the Atlantic and the Pacific. However, Andersen *et al*. (1998) did report a walrus from East Greenland with a 'Pacific haplotype'. Further studies based on more comprehensive sampling are required to address this issue in more detail.

Today, there is a distributional break of 500–600 km between *O. r. divergens* and walruses in the Laptev Sea (Fay 1982; Belikov *et al*. 1996; Belikov & Boltunov 2005). However, the genetic similarity between Laptev Sea and Pacific walruses found in our study may indicate that the walrus populations in these two regions were once much larger and perhaps formed a continuum. Heavy exploitation from 1930 to 1950 dramatically reduced the number of walruses in the Laptev Sea region (Belikov & Boltunov 2005). During the same period Pacific walruses were also heavily depleted due to over-hunting, primarily in the former Soviet Union (Fay *et al*. 1989). These depletions may be the cause for the distributional gap recorded today. However, other reasons such as bathymetric restrictions may also cause such a distributional gap.

In the present molecular data set, the geographical populations of Atlantic walrus, that is, those from West Greenland and those from the North-East Atlantic, appear less variable than Pacific walruses from Alaska. This may indicate bottlenecks in these populations during the displacement of the Atlantic walrus during the last Ice Age (Andersen *et al*. 1998). Of course, such bottlenecks may also be correlated with the species' dispersal. However, given the limited current sampling, it is not possible to choose among these potential alternative explanations.

The molecular conclusions of the current study yield no support for considering the Laptev walrus (*O. r. laptevi*) a discrete taxonomic entity, at least not following the concept of subspecies being reciprocally monophyletic evolutionary units with independent evolutionary histories. Additionally,

the fragmentary morphological measurements of walruses from the Laptev Sea fall within the range of the Pacific subspecies. The nucleotide sequence data of the mitochondrial 16S rRNA, ND1 and COI genes, as well as the non-coding CR, identify the Laptev walrus as a population of the Pacific walrus *O. r. divergens*. Accordingly, the data support the abandonment of the subspecies name *O. r. laptevi* and the recognition of the Laptev walrus within *O. r. divergens*. However, recognizing the Laptev walrus as a subpopulation of *O. r. divergens* does not exclude consideration of these animals as a significant unit in terms of conservation and management (Ryder 1986; Waples 1991). The Laptev walrus is unique in several ecological parameters and further research is certainly warranted in order to secure its future persistence. Sampling of biopsies of extant Laptev Sea walruses combined with more extensive ecological studies may be an important emphasis for future research on this enigmatic population. We consider such data essential in the context of a broad phylogeographical survey for understanding the population dynamics, genetic divergence, and migratory patterns in this distinct pinniped lineage.

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