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The enigma of the landlocked Baikal and Caspian seals addressed through phylogeny of phocine mitochondrial sequences

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The endemic seals of Lake Baikal (*Phoca sibirica*) and of the Caspian Sea (*Phoca caspica*) inhabit ancient continental basins that have remained isolated from primary marine seal habitats for millions of years. The species have been united with the Arctic ringed seal, *Phoca hispida*, into (sub)genus *Pusa*, but the age and route of invasions to/from the continental basins remain controversial. A phylogenetic analysis of nine northern phocines based on three mitochondrial genes (Cyt*b*, COI, COII, total 3369 bp) provided no support for the monophyly of the *Pusa* group. The three species are involved in an apparent polytomy with the boreal harbour seal, *Phoca vitulina*, and grey seal, *Halichoerus grypus*. From the average estimated interspecies divergence (4.1%), the radiation of this group plausibly took place in the Late Pliocene 2–3 Mya. This dating does not fit the prevailing hypotheses on the origin of the landlocked taxa in association with Middle Pleistocene glacial events, or of the Caspian seal as a direct descendant of Miocene fossil phocines of the continental Paratethyan basin. The current phocine diversity more likely results from marine radiations, and the continental seals invaded their basins through Plio-Pleistocene (marine) connections from the north. The palaeohydrography that would have enabled the invasions at that time still remains an enigma.

ADDITIONAL KEYWORDS: glacial relicts – Paratethys – Phocidae – phylogeography – Pleistocene glaciations – *Pusa*.

INTRODUCTION

The origins and phylogenetic relationships of the endemic landlocked seals in Lake Baikal and in the Caspian Sea present a long-lasting biogeographical enigma (Nordqvist, 1899; Chapskii, 1955b; Davies, 1958; McLaren, 1960; Kozhova & Izmesteva, 1998; Koretsky, 2001). The Baikal seal, *Phoca sibirica* Gmelin, and the Caspian seal, *Phoca caspica* Gmelin, belong to the subfamily Phocinae, northern true seals, in the true seal family Phocidae (Table 1). The two landlocked species inhabit two old Eurasian continental basins that have been isolated from primary marine seal habitats for millions of years. Lake Baikal currently lies 456 m above sea level and is connected to the Arctic Ocean through 3800 km of the Angara–Enisei rivers (Fig. 1). It is the oldest lake on Earth (*c*. 28 Myr old), and the Baikalian region is unlikely to have been directly reached by sea since the Jurassic (Kozhova & Izmesteva, 1998). The Caspian basin has been mostly isolated from the surrounding seas since the Late Miocene, although transient connections through the Black Sea basin to the Mediterranean have existed still in the Pleistocene (Dumont, 1998; Zubakov, 2001).

Morphologically and ecologically, the Baikal seal and the Caspian seal are considered closest to the circumarctic ringed seal, *Phoca hispida* Schreber, a similarly small seal species that also breeds on ice and even comprises landlocked populations in boreal postglacial lakes (Nordqvist, 1899; Chapskii, 1955b; McLaren, 1960; Bininda-Emonds & Russell, 1996). Together, these three species are sometimes distinguished as a separate genus *Pusa* (Scheffer, 1958; De Muizon, 1982; Koretsky, 2001; Deméré, Berta &

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Table 1. A classification of modern phocine seal species (primarily after Chapskii, 1955a; Koretsky, 2001; also cf. Carr & Perry, 1997; the subfamilial and tribal assignment of *Cystophora cristata* varies widely in different classifications)

Classification	Common name
PHOCIDAE	
[Monachinae (subfamily)]	Monk seals and southern true seals, nine species
Phocinae	Northern true seals (phocines)
Erignathini (tribe)	
*Erignathus barbatus**	Bearded seal
Cystophorini	
*Cystophora cristata**	Hooded seal
Phocini	
Histriophocina (subtribe)	
Pagophilus groenlandicus	Harp seal
*Histriophoca fasciata**	Ribbon seal
Phocina	
*Halichoerus grypus**	Grey seal
*Phoca vitulina**	Harbour seal
*Phoca largha**	Larga seal
Phoca (*Pusa*) *hispida**	Ringed seal
Phoca (*Pusa*) *sibirica**	Baikal seal
Phoca (*Pusa*) *caspica**	Caspian seal

Asterisks indicate taxa included in this study.

Adam, 2003) but, more generally, *Pusa* is treated as a subgenus within *Phoca* (Chapskii, 1955a; McLaren, 1960; Burns & Fay, 1970; Bonner, 1994). A variety of views on the relationships within this group have emerged from comparative morphological analyses. These have clustered either *P. sibirica* and *P. caspica* (Nordqvist, 1899; Pastukhov, 1969; Taimisto, 1990); *P. hispida* and *P. caspica* (Timoshenko, 1975); *P. hispida* and *P. sibirica* (Bininda-Emonds & Russell, 1996; Koretsky, 2001); or proposed *P. sibirica* as intermediate between *P. caspica* and *P. hispida* (Koyama *et al*., 1997). Although the phylogeny of the eight marine phocine taxa has been previously clarified by cytochrome *b* sequencing (Árnason *et al*., 1995; Mouchaty, Cook & Shields, 1995; Perry *et al*., 1995), and recently by sequences of the nearly complete mitochondrial genome (Davis *et al*., 2004), the two continental 'relict' species were never included in these broader assessments. However, from a restriction fragment length polynorphism (RFLP) analysis of whole mtDNAs within the *Pusa* group, Sasaki, Numachi & Grachev (2003) suggested a contentiously close relationship between *P. sibirica* and *P. hispida*.

From a biogeographical point of view, a number of hypotheses have previously been presented to explain the origin of the Caspian and Baikal seals. Most of these hypotheses assume monophyly of the three *Pusa* group seals, but differ in views about the timescale of the continental invasion (Quaternary vs. Tertiary) and the distribution of the common ancestor (oceanic vs. Paratethyan, see below).

According to the Quaternary hypotheses, the colonization of the continental basins by Arctic *P. hispida*

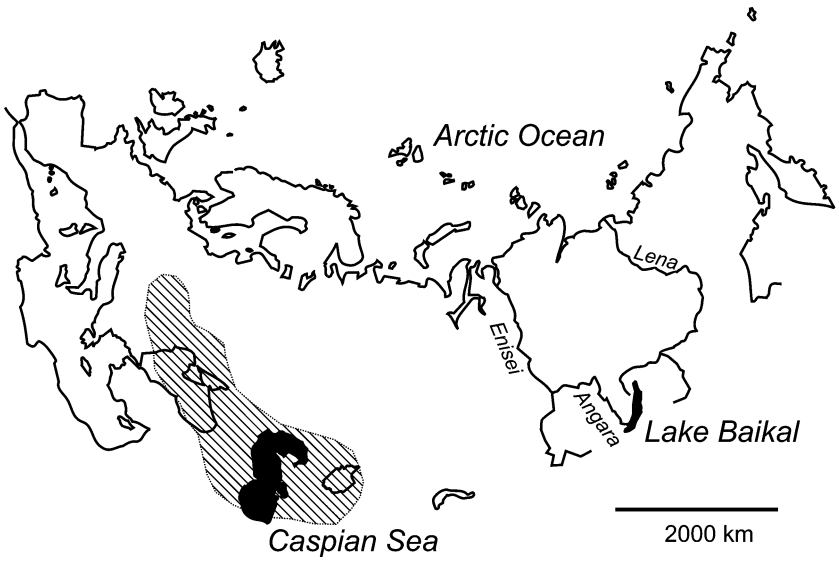

Figure 1. A schematic map of the northern Palearctic shows the isolated distributions of the Baikal and Caspian seals. The hatching illustrates the area covered by the epicontinental Paratethys Sea at times, *c*. 15–5.5 Mya.

has been facilitated by marine inundations of Arctic coasts, and particularly by creation of large ice-dammed lakes in central Siberia during the major continental glaciations in the Middle Pleistocene (≤ 0.9 Mya) (Pirozhnikov, 1937; Chapskii, 1955b; Davies, 1958). The inundations would have helped access to the Baikal basin via the Enisei–Angara river system (Repenning, Ray & Gricorescu, 1979). The ice-dammed lakes also had temporary downstream connections towards the Caspian Sea (Arkhipov *et al*., 1995), which could have accounted for the presence of the 'arctic' *P. caspica* along with a number of typically arctic crustacean genera in the deep cold waters of the Caspian (Pirozhnikov, 1937; Segerstråle, 1957). Arctic adaptive features in the landlocked seals, in particular their breeding on ice and white natal hair, would seem to support this hypothesis. The period of the maximum continental glaciations *c*. 200–300 kya has been perceived as the most plausible time for such invasions (Davies, 1958; Repenning *et al*., 1979).

Arguments for the Tertiary hypothesis for the *Pusa* affinities relate to the rich fossil phocine fauna in the Late Miocene strata of the Paratethys, an inland basin that covered areas of south-east Europe and the current Ponto–Caspian region in the Miocene times (Fig. 1). It was suggested that P. caspica is a direct resident descendant of these Paratethyan seals (Grigorescu, 1976). The current affinities have been connected with this idea in two different ways. McLaren (1960) suggested that the *Pusa* group dates back to pre-Pliocene times and that the ancestors of *P. sibirica* and *P. caspica* invaded their continental basins by Late Miocene marine connections (> 5 Mya). Alternatively, all three *Pusa* species have been regarded as descendants of a Miocene Paratethyan progenitor (Ray, 1976; Árnason *et al*., 1995; Koretsky, 2001).

To assess various hypotheses on the zoogeographical and phylogenetic history of the Caspian and Baikal seals, we analysed 3369 bp of mitochondrial DNA sequence representing three protein-coding genes (the cytochrome *b*, cytochrome oxidase I, and cytochrome oxidase II: Cyt*b*, COI, and COII) from nine phocine taxa. From these data, we address the phylogenetic affinities of *P. sibirica* and *P. caspica* and, more generally, the unity of the suggested *Pusa* group, and discuss the power of mtDNA sequence data in resolving these questions. Molecular divergence provides an insight to the timescale of continental invasions and allows a critical evaluation and rejection of some of the previous biogeographical hypotheses.

MATERIAL AND METHODS

The sequences of the entire COI (1545 bp), COII (684 bp), and Cyt*b* (1140 bp) genes were obtained from single individuals of each of *P. hispida*, *P. sibirica*, *P. caspica*, *Phoca largha* Pallas (larga seal, or spotted seal; University of Alaska Museum collection UAM Mamm 18613), *Histriophoca fasciata* (Zimmermann) (ribbon seal; UAM Mamm 19029), *Cystophora cristata* (Erxleben) (hooded seal; UAM Mamm 36480), and *Erignathus barbatus* (Erxleben) (bearded seal; UAM Mamm 36477). Sequences were deposited in GenBank under accession numbers AY140962–AY140982. In addition, we used published mtDNA-sequence data from *Halichoerus grypus* (Fabricius) (grey seal X74002; Árnason *et al.*, 1993) and *Phoca vitulina* L. (harbour seal X63726; Árnason & Johnsson, 1992).

DNA from seal muscle tissue was extracted using a standard SDS-proteinase K digestion procedure followed by ethanol precipitation (Bruford *et al*., 1992). Segments containing the Cyt*b* and the COI-COII genes were amplified separately. Polymerase chain reaction (PCR) primers for these two regions and additional sequencing primers (Table 2) were designed to match conserved regions in aligned sequence of

Table 2. Primers used for amplification and sequencing

Primer name	Sequence	Target
PCR primers		
L6223	5′-GAGCCCCCATAGTTAGATTTAC	COI-COII
H872	5′-AACTGTGGCATTTCATTAAAGG	
L15015	5′-CATCATTATTCCCACATGGA	Cyt*b*
H16325	5′-GGGGTTGTTACCTCTTCCT	
Internal sequencing primers		
L6673	5′-TAGCCCATGCCGGGAGCATC	COI
L7083	5′-TATTAGGAATAGTTTGAGCA	COI
H7868	5′-ATTGAGAAAGACATAAGGGTT	COI
L7820	5′-GAAAGGAAGGAGTCGAACC	COII
L15507	5′-ATCATTTTGAGGAGCAACAG	Cyt*b*

Primer names refer to site numbering the complete harbour seal mitochondrial genome X63726.

the grey seal, harbour seal, dog (*Canis familiaris*, NC002008; Kim *et al.*, 1998) and domestic cat (*Felis catus*, NC001700; Lopez, Cevario & O'Brien, 1996). PCR reactions were performed in a total volume of 20 L, with approximately 20 ng of template DNA, 5 pmol each primer, 0.25 U AmpliTaq DNA polymerase (PE Biosystems), 1 × PCR buffer II (PE Biosystems), 2.5 mM $MgCl_2$ and 200 M of each nucleotide (Amersham Pharmacia Biotech). The thermal cycling profiles were: 95 C for 3 min followed by 30 cycles of 95 C for 45 s, annealing (COI–COII: 58 C, Cyt*b* 52 C) for 45 s and 72 C for 45 s. Unincorporated nucleotides and primers were removed either enzymatically using shrimp alkaline phosphatase and exonuclease I (Amersham Pharmacia Biotech) or using the GFX DNA purification kit (Amersham Pharmacia Biotech). Sequencing reactions were made using the fluorescent BigDye-terminator sequencing kit (PE Biosystems). Unincorporated dye terminators were removed with Centri-Sep gel filtering columns (Princeton Separations, Inc.). Sequences were resolved on an ABI Prism 377 sequencer.

Statistics of molecular diversity were calculated and phylogenetic analyses performed using PAUP*4.0b8 (Swofford, 1998) unless indicated otherwise. Trees were constructed from the total sequence data set using the criteria of maximum parsimony (MP; equal weights, exhaustive search, branches with zero minimum length collapsed during the run, ACCTRAN optimization) and maximum likelihood (ML; exhaustive search), and from distance estimates using the neighbour-joining (NJ) algorithm. The choice of the mutation model for the ML analysis and distance estimation was based on a hierarchical log likelihood test procedure implemented in MODELTEST 3.04 (Posada & Crandall, 1998), using a 1% significance cut-off level, and gamma distribution among-site rate heterogeneity approximated with four discrete rate categories. The reliability of the phylogenetic trees was assessed by bootstrapping (1000 replicates) and by calculating decay indices for the MP tree branches (Bremer, 1994) using the program TREEROT (Sorenson, 1999).

In order to evaluate different *a priori* phylogenetic hypotheses, particularly the monophyly of *Pusa* and sister-taxon relationships of its component members, likelihood scores and MP tree lengths under alternative constrained tree topologies (Fig. 2) were evaluated using the tests of Shimodaira & Hasegawa (1999) and Kishino & Hasegawa (1989) (1000 bootstrap replicates using full optimization, one-tailed tests).

The molecular clock hypothesis was tested by comparing the log likelihood scores (using the Kishino–Hasegawa likelihood ratio test) of trees found with and without enforcing molecular clock in the ML search. The pinniped divergence times were estimated using an external reference point for calibration (i.e. as proportion of the cat-dog divergence) using the nonparametric rate smoothing (NPRS) method (Sanderson, 1997). For NPRS analysis, cat, dog, finback whale (*Balaenoptera physalus*, NC 001321; Valverde, Marco & Palo, 1994) and hippopotamus (*Hippopotamus amphibius*, NC 000889; Ursing & Árnason, 1998) sequences were first added to the seal data set. The mutation model was re-estimated for this data with the MODELTEST procedure and a maximum likelihood phylogram was constructed (heuristic search, ten repetitions with the taxon input order randomized). The whale and hippopotamus were only used as an outgroup to root the ML-tree, and were pruned prior to the actual NPRS analysis, performed with the TREEFINDER software (Jobb, 2003).

Although the current analysis is based on single sequences from each taxon, it may be noted that the reciprocal monophyly of mtDNAs in the three *Pusa* group taxa focal to the study is well corroborated in assessments of larger data sets (e.g. RFLP data of Sasaki *et al*., 2003; our unpublished control region data).

RESULTS

The data set comprised 3369 nucleotide sites, of which 719 were variable among the nine phocine taxa (Table 3). The alignment contained no gaps and all the sequences consisted of full length open reading frames. Most of the substitutions observed were synonymous; there was marked variation in the proportions of variable synonymous and nonsynonymous sites among the genes (COI 143:1–Cyt*b* 19:1).

In the analyses, the COI, COII and Cyt*b* sequences were treated as a single data set. The substitution model chosen based on the hierarchical test was the HKY85 model (Hasegawa, Kishino & Yano, 1985) with a transition to transversion ratio of 18.84, nucleotide frequencies A: 0.311, C: 0.276, G: 0.149, and T: 0.264, and a correction for rate heterogeneity between sites (Γ-distribution with shape parameter $\alpha = 0.154$). The

Table 3. Descriptive statistics for three mtDNA genes in the data set of nine seal taxa

	COI	COII	Cyt*b*	Total
Number of sites	1545	684	1140	3369
Number of variable sites	347	140	232	719
Parsimony informative sites	148	55	98	301
Base frequency (%)				
A	27	34	31	29.9
C	26	26	31	27.5
G	18	15	13	15.7
T	29	25	25	26.9

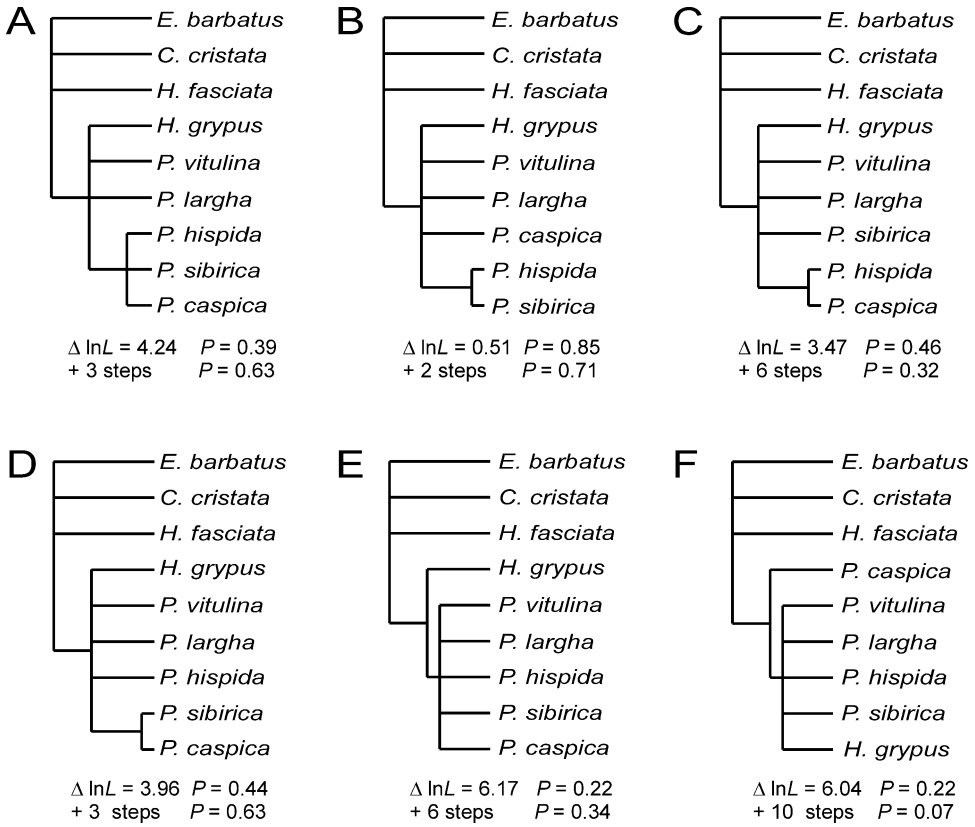

Figure 2. Evaluated alternative tree topologies representing different phylogenetic and biogeographical hypotheses. A, monophyly of the *Pusa* group. B–D, different sister-taxon relationships within *Pusa*. E, monophyly of the genus *Phoca* (including the *Pusa* group). F, Caspian ancestry of Phocina. Below each tree are shown the differences in log likelihood and in tree length between the best trees compatible with the constrained tree topology vs. those of the unconstrained ML tree (Fig. 3C; $-\ln L = 9017.27$) and MP tree (Fig. 3A; 983 steps), respectively. *P*-values are from the corresponding Shimodaira–Hasegawa and Kishino–Hasegawa tests (see Material and methods).

Table 4. Estimates of interspecific sequence divergence at three mitochondrial protein-coding genes assuming the HKY85+Γ substitution model with shape parameter $\alpha = 0.154$

	1	2	3	4	5	6	7	8
1. *Phoca hispida*	–							
2. *Phoca sibirica*	0.031	–						
3. *Phoca caspica*	0.035	0.027	–					
4. *Halichoerus grypus*	0.041	0.034	0.033	–				
5. *Phoca vitulina*	0.054	0.047	0.048	0.056	–			
6. *Phoca largha*	0.043	0.035	0.038	0.048	0.021	–		
7. *Histriophoca fasciata*	0.141	0.152	0.150	0.145	0.157	0.154	–	
8. *Cystophora cristata*	0.183	0.169	0.196	0.218	0.191	0.191	0.217	–
9. *Erignathus barbatus*	0.321	0.364	0.364	0.351	0.381	0.369	0.337	0.367

pairwise HKY85+Γ distance estimates are shown in Table 4.

Unweighted parsimony tree search revealed one most parsimonious tree of 983 steps, which involved an unresolved trichotomy among the clades of *P. sibirica* (*P. caspica* + *H. grypus*) and (*P. vitulina* + *P. largha*) (Fig. 3A). Along with *P. hispida*, these five taxa constitute the subtribe Phocina *sensu* Chapskii (1955a) (see Table 1). The data clearly delimited this Phocina group from the more basal *H. fasciata*, *C. cristata*, and

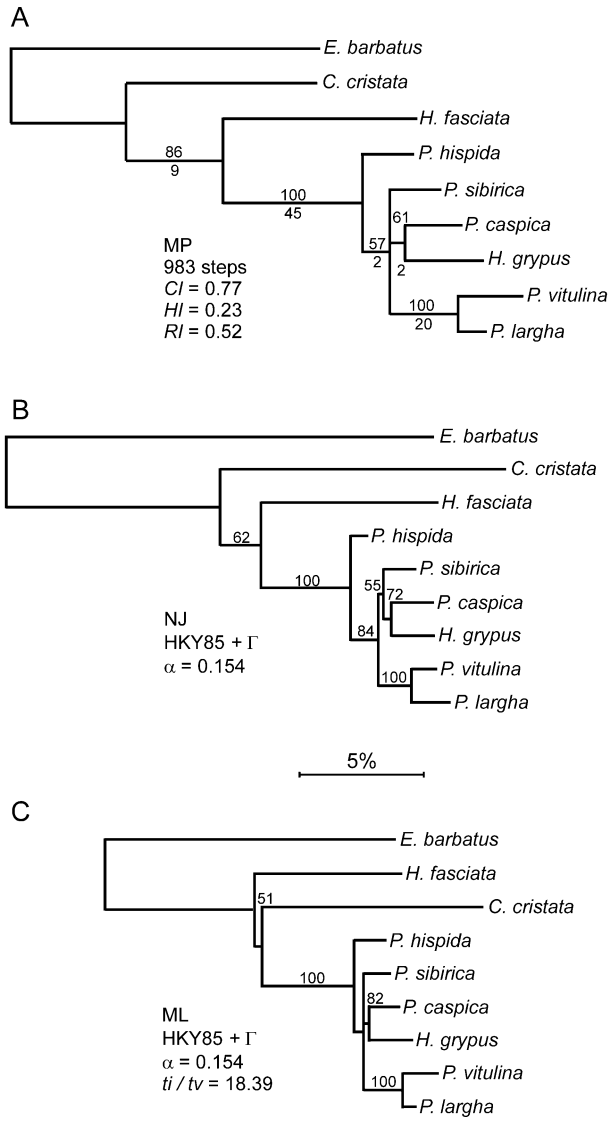

Figure 3. Reconstructions of phocine mtDNA relationships. A, unweighted maximum parsimony tree. The numbers below branches are decay indices, numbers above branches indicate bootstrap support (%, from 1000 replicates). Also indicated are the tree length and consistency (CI), retention (RI), and homoplasy (HI) indices (all characters included). B, neighbour-joining tree based on HKY85+Γ corrected distances. C, maximum likelihood tree under the HKY85+Γ substitution model.

E. barbatus. The topologies of the NJ and ML trees were similar to the MP tree; the main difference was in the relative positions of *C. cristata* and *H. fasciata* (Fig. 3B, C).

In the tree reconstructions, the five Phocina lineages appeared as a tight cluster and their branching order was poorly resolved (Fig. 3). There was no indication of a monophyly of the *Pusa* trio (*P. hispida*, *P. sibirica*, *P. caspica*) or of a sister group relationship between any two of these in the MP tree. There was weak support for a (*P. caspica* + *H. grypus*) clade, and for a basal position for *P. hispida* within the Phocina. However, none of the six alternative phylogenetic hypotheses presented by the constrained trees in Figure 2 could be strictly ruled out by the data.

The likelihood ratio test indicated marked heterogeneity of substitution rates among lineages ($\Delta\ln L = 10.21$, d.f. = 7, $P = 0.005$). We applied the nonparametric rate smoothing method (Sanderson, 1997) to obtain approximate estimates for relative ages of the major phocine branching events in a framework of a larger carnivore phylogeny. With a ML tree constructed under a GTR+Γ+I substitution model (Rodriguez *et al*., 1990), chosen for the broader data set (Γ shape parameter $\alpha = 1.227$, proportion $I = 0.585$ of invariant sites; tree not shown), the method suggested that the age of the first Phocinae branching event (*E. barbatus* split) was 26.5% of the cat–dog divergence, and the basal Phocina branching 4.5% of it (or, Phocina radiation 17% of the age of *E. barbatus* split). When NPRS was applied to the seal data only, using the HKY85+Γ trees estimated above (Fig. 3B, C, position of root determined by inclusion of dog sequence), the relative ages of the seal clades were similar or slightly older (age of Phocina root 18–21% of *E. barbatus* split).

DISCUSSION

Phylogenetic resolution

Despite the amount of sequence data, the relationships among the Phocina mtDNA lineages were not conclusively resolved. Five lineages, including the focal *P. sibirica* and *P. caspica*, make a virtually unresolved cluster in the trees. The grey seal *H. grypus*, currently assigned to a monotypic genus, remains inseparable from *Phoca s.s.* (including the *Pusa* group). The problem of a paraphyly of *Phoca s.l.* (which also includes *Pagophilus groenlandicus* and *H. fasciata*; Burns & Fay, 1970) with respect to *Halichoerus* was already noted in previous mtDNA studies (Árnason *et al*., 1995; Mouchaty *et al*., 1995; Perry *et al*., 1995; Davis *et al*., 2004).

Although the monophyly of the *Pusa* group cannot be confidently ruled out with the current data, the phylogenetic reconstructions in Figure 3 show no particular affinities between the circum-arctic *P. hispida* and the two landlocked species, contrary to the conclusion from (or assumption in) most previous examinations. The preferred MP phylogeny actually shows *P. hispida* as a basal Phocina lineage, and a trichotomy of *P. sibirica*, (*P. caspica* + *H. grypus*) and (*P. vitulina* + *P. largha*). Nevertheless, apart from the

most recent *P. vitulina–P. largha* split, *P. sibirica* and *P. caspica* actually are the closest taxa in terms of pure sequence similarity (Table 4), and the three *Pusa* taxa would cluster together in a simple UPGMA phenogram. The evaluation of alternative topologies also shows virtually no difference between the optimal trees (Fig. 3) and those constrained to reflect *Pusa* monophyly or a *P. hispida–P. sibirica* sister relationship (Fig. 2A–D). The basal position of *P. hispida* in the optimal trees may reflect a slower substitution rate in that branch.

The power of mtDNA data in resolving close cladogenetic events (such as those within the Phocina) depends on several factors, including the limited resolution of the molecular genealogy due to finite sequence length and the Poisson variance of the substitution process, problems arising from unequal rates among branches, and interspecific mtDNA captures (Avise, 2000; Slowinski, 2001). The latter should not pose a problem for resolution of continental colonization events, which by definition involve allopatric isolation. In practice, the single most important confounding effect for the phylogenetic resolution is the potential discordance of true gene and species trees caused by retention of ancestral polymorphisms and lineage sorting (Edwards & Beerli, 2000; Nichols, 2001). This effect is governed by the long-term effective (female) population size $N_{e(f)}$ before the initial radiation. If the N_e (and thus the variability) of the ancestral population has been large, the problem cannot be circumvented by mtDNA data only, whatever the sequence length (Edwards & Beerli, 2000).

For an insight to the importance of this effect, we use current population sizes or levels of polymorphism as a guide to evaluate the ancestral situation in Phocina. The level of microsatellite variability of the probably largest and most stable contemporary phocine population, the Arctic *P. hispida*, suggests a long-term N_e of the order 3×10^4 to 3×10^5 individuals (Palo *et al.*, 2001). Theoretically, an equilibrium $N_{e(f)} = 5 \times 10^4$ would imply an expected variation ($2 \times$ SD) in coalescence time of $2 \times N_{e(f)} = 10^5$ generations, or 10^6 years for a seal species with a 10-year generation interval (equation 1 in Edwards & Beerli, 2000). The intraspecific mtDNA diversity within *P. hispida* similarly suggests a continuously large population size, with several deep lineages surviving since an ancient population expansion. The average divergence of three deep intraspecific *P. hispida* Cyt*b* lineages corresponds to 34% of the average interspecies Phocina divergence (J.U. Palo, H. Hyvärinen and R. Väinölä, unpubl. data). A similar ratio of intra vs. interspecific diversities is seen in a large mtDNA RFLP data set (Sasaki *et al.*, 2003). Such high levels of polymorphism in the Phocina ancestor would easily have masked a true population branching order different from those in Figure 3, or a potential true species polytomy, while allowing the heterogeneity of divergences now seen among the lineages (Table 4). Although the abundant *P. hispida*, supported by the extensive Pleistocene Arctic ice habitat, might not be an appropriate analogue to represent the variability of the ancestral pre-Pleistocene phocine, even the current lineages within the landlocked Caspian seal species appear of similar age (Sasaki *et al.*, 2003).

Divergence times

Even though mtDNA is widely used for addressing vertebrate divergence times, the appropriate rate calibrations remain poorly established. Applying the 'standard' mammalian mtDNA calibration of *c.* 2% Myr^{-1} (Brown, George & Wilson, 1979; Avise, 2000), the major Phocina radiation (average $D = 4.1\%$) would appear to be 2 Mya. However, generalizing from distant calibration points is sensitive to the substitution model employed, and also to the apparent rate heterogeneity between the lineages examined (Arbogast *et al.*, 2002). We applied the nonparametric rate smoothing method to partly cope with these issues; seal divergences were related to the cat–dog divergence dated from multigene data with reference to palaeontological evidence from a number of other taxa (Springer *et al.*, 2003). With a cat–dog split at 55 (50–60) Mya, the basal Phocinae divergence would be put at $26.5\% \times 55 = 14.6$ (13.3–15.9) Mya. This is in line with the previously suggested age for phocine seals; monachines and phocines were well separated by 15 Mya (Ray, 1976; Berta & Wyss, 1994). The radiation of the five main Phocina mtDNA lineages, at 17–21% of the basal seal divergence using various trees, would then be estimated starting at 2.5–3.1 (2.3–3.3) Mya. Corresponding estimates for the closest interspecies mtDNA relationships in the cluster (Fig. 3, Table 4) would be in the range 1.7–2.6 (1.6–2.8) Mya. The average divergence rate of the three mtDNA genes studied here within the Phocina would have been approximately 1.5% Myr^{-1}.

Again, due to ancestral polymorphism in the splitting populations, the divergence of molecular lineages is expected to predate the actual species divergence by an average of $N_{e(f)}$ generations, under assumption of equilibrium ancestral population (Edwards & Beerli, 2000). For example, for ancestral $N_{e(f)}$ of 10 000–100 000 (see above), and an average 10-year generation interval (Palo *et al.*, 2001), the average overestimation from molecular data would be 0.1–1.0 Myr, which would bring the hypothesized biogeographical events correspondingly closer. Under a scenario of a concurrent radiation of five main lineages, a reasonable approximation of the species divergence date could also be given by the youngest of the estimated

interspecies coalescences (1.7–2.6 Myr, above). From these considerations, also the continental invasions by *P. sibirica* and *P. caspica* would appear to have been relatively concurrent events that most probably took place in the Late Pliocene. The time scale thus estimated for the Phocina radiation is close to that suggested by Árnason *et al*. (1996b), who argued that the advance of Arctic ice *c*. 2.7 Mya would make an appropriate palaeoclimatical reference point to fix the *P. vitulina–H. grypus* (= Phocina) divergence time, even to be used for calibration of general mammalian mtDNA rate.

Yet the time scale suggested above is clearly different from two other recent estimates based on mitochondrial data. First, Árnason *et al*. (1996a) later implied a notably older 7 Mya divergence for the same two Phocina lineages; this was based on applying the amino acid divergence rate of Cetartiodactyla to other mammals. Second, from RFLP of whole mtDNA genomes, Sasaki *et al*. (2003) suggested a mere 0.4 Myr age for the *P. sibirica–P. hispida* split, and a hierarchically higher 0.7 Myr divergence of these taxa from *P. caspica*. The difference between those estimates and ours seems unexpected and needs comment. First, the basal Phocina (and *Pusa*) divergences in the Sasaki *et al*. (2003) data are actually similar to ours (3.5–4% total mtDNA divergence, vs. our 4.1% corrected average coding gene divergence). Second, their calibration was mistakenly based on a general 2% Myr^{-1} mammalian substitution rate (= 4% Myr^{-1} divergence rate), instead of the more conventional 2% divergence rate, or the *c*. 1.5% divergence rate implied by our treatment. Third, their dating was based on net interspecies distance, which accounts for (i.e. subtracts) the average intraspecific variation, as with our ancestral polymorphism considerations above. These calibration-related considerations can reconcile their data with our interpretation on the basal Phocina radiation 2–3 Mya. However, the apparent close sister-relationship of the Baikal and ringed seals suggested by the Sasaki *et al*. (2003) results does not fit our data. The effective sequence length assessed in their RFLP scan was substantially shorter than that in the present study.

Biogeographical hypotheses

Our data and calibration suggest a concurrent Late Pliocene radiation for the Phocina group of seals. Although the phylogenetic relationships of *P. sibirica* and *P. caspica* could not be assertively resolved, several previously presented hypotheses on the origin of the landlocked taxa and the timescale of the phocine radiations (see Introduction) can be rejected.

Most of the presented Tertiary hypotheses relate to the diverse Paratethyan fossil seal fauna of Late Miocene age, *c*. 13–8 Mya (for a review, see Koretsky, 2001). The current *P. caspica* has often been proposed as a direct descendant of one of these Paratethyan phocines, retained in the continental basin since the Miocene (Grigorescu, 1976; Árnason *et al*., 1995). According to Ray (1976) and Koretsky (2001), the *Pusa* group would stem from Paratethyan seals while the predecessors of other phocines would have continuously inhabited the North Atlantic since their divergence from the monachines (15 Mya). The ancestors of Arctic *P. hispida* and Baikalian *P. sibirica* would later have escaped the Caspian along hypothetical continental routes (Repenning *et al*., 1979; Koretsky, 2001). Under this hypothesis, *Pusa* should make a phylogenetically distinct cluster, diverged from the remaining phocines (including *P. vitulina* and *H. grypus*) several million years ago. Moreover, at least Repenning *et al*. (1979) implied that *P. caspica* should be clearly basal to the terminal (*P. hispida*, *P. sibirica*) clade of Middle Pleistocene age. The relatively concurrent radiation of the five main Phocina lineages (Fig. 3) in itself appears to refute this hypothesis.

Árnason *et al*. (1995) in turn suggested that the entire tribe Phocini (or *Phoca s.l.*, including all extant phocines except *E. barbatus*, *C. cristata*; Table 1) would stem from Paratethyan ancestors and that it even originally radiated in the Paratethys in the late Miocene *c*. 6 Mya. This group is united by a chromosome number $2n = 32$ (Árnason, 1974) and a white natal hair. A number of the Paratethyan lineages, excluding the ancestors of *P. caspica*, would then have emigrated to the Arctic, where the rest of the modern Phocina group radiated 2–3 Mya (particularly *P. hispida*, *P. vitulina* and *H. grypus*). Although this view also denies the unity of *Pusa*, it implies that *P. caspica* should be the (distinctly) basal lineage within the entire Phocina, which is not supported by our data.

Instead of the previous hypotheses of a Paratethyan ancestry, the inferred Post-Miocene radiation of several Phocina species appear to better accord with a common origin in northern seas. This hypothesis also more plausibly accounts for the ice-breeding habit and the white natal hair shared by all the Phocina taxa. Given that these features are synapomorphic, it is unlikely that they would have arisen in the subtropical waters of the Miocene Paratethys (cf. Perry *et al*., 1995; Koretsky, 2001; Deméré *et al*., 2003). Current fossil taxonomy also no more implies particular affinities between the modern Phocina and the Miocene Paratethyan seals (cf. Ray, 1976; Koretsky, 2001). The most likely link between the Paratethyan and the current northern seals may be in an 11–12 Myr old Paratethyan fossil attributed to the extant genus *Histriophoca* (Koretsky, 2001), a relationship that only marginally could fit the concepts of molecular divergence above (Fig. 3).

A northern marine origin of the landlocked seal taxa has been supported by another line of biogeographical thought, which attributes the current continental seal occurrences to direct geological effects of the Pleistocene glaciations. Following the earlier hypotheses of Högbom (1917) and Berg (1928) of the origin of a number of 'Arctic marine' crustaceans and fishes in the deep cold waters of the Caspian Sea, Pirozhnikov (1937) and Davies (1958) suggested that both the Caspian Sea and Lake Baikal were colonized by *P. hispida* from the Arctic basin in the Middle Pleistocene, maybe as late as 200 kya. The hypothesis assumed that the Arctic marine ancestors were trapped in lakes that formed in front of expanding ice-sheets and ultimately discharged south along rivers draining to the Caspian basin (Arkhipov *et al*., 1995).

Repenning *et al*. (1979) in turn implied a Middle Pleistocene northern colonization only for the Baikal seal. Similarly, a relatively recent *P. hispida–P. sibirica* relationship (contrasted to a Pliocene divergence of *P. caspica*) was suggested from biogeography of seal parasites (Kozhova & Izmesteva, 1998). During the Plio-Pleistocene, Lake Baikal has been connected to the north variously through the Angara–Enisei and Lena rivers (Kozhova & Izmesteva, 1998). Although *P. hispida* may penetrate into rivers far inland, the potential means of ascent to the current Baikal altitude remain unexplained. At any rate, these hypotheses of a Middle Pleistocene penetration of the landlocked seals in connection with the major glaciations since *c*. 900 kya (Arkhipov *et al*., 1995) would imply notably closer affinities between the landlocked and marine taxa than indicated by our data, and are therefore rejected.

From our data, it thus seems most likely that both the Caspian and Baikal seals represent Late Pliocene immigrants from the northern seas. The radiation of the Phocina seals may have been triggered by the climatic cooling and the subsequent appearance of the polar ice-cap at that time, and the associated glacial eustatic cyclicity favouring recurrent allopatric isolation (cf. Hoberg & Adams, 1992; Árnason *et al*., 1995). Recent evidence indicates considerable intensification of continental glaciations already in Late Pliocene 2.7 Mya (Kleiven *et al*., 2002). Yet, no tenable palaeohydrographical scenario to account for the continental immigration at that relatively early time can so far be presented. Ray (1976), Repenning *et al*. (1979) and Koretsky (2001) speculated on Pliocene or Early Pleistocene continental waterways to explain the Pusa affinities and distribution, but they assumed a Caspian origin of the group and of the dispersal. Koretsky (2001) even favoured a Caspian-to-Baikal-to-Arctic way of invasion. These authors referred to Pliocene transgressions that would have brought the Ponto-Caspian and Arctic waters to relatively close proximity *c*. 3 Mya (see also Steininger & Rögl, 1984), but no actual palaeogeographical data for such a direct connection exist. By contrast, Zubakov (2001) suggested that the Caspian seal would have immigrated through a Mediterranean connection at this relatively cool transgressive phase, *c*. 3.4 Mya.

Although our data are only marginally compatible with the timescale of the earlier suggested Pliocene links, invasion through a direct Pliocene Arctic–Caspian waterway is supported by another biogeographical argument, related to the notion of *P. caspica* as a member of the broader 'Arctic marine' zoogeographical community in the Caspian basin (Pirozhnikov, 1937). As with the seal, molecular data suggest that the invasions of the crustaceans *Mysis* and *Gammaracanthus* most likely predated the major Middle Pleistocene glaciations (Väinölä, 1995; Väinölä, Vainio & Palo, 2001); in contrast to the hitherto prevailing glacial invasion hypothesis. The dispersal and environmental requirements of these taxa are more constrained than of phocids; evidently, their immigration has required a proper direct coldwater connection that also would have enabled the access for the seal.

The unusually rich, predominantly endemic fauna of Lake Baikal does not have prominent Arctic-marine affinities of comparable age. The closest parallel to the Baikal seal to suggest recent colonization from the north has been seen in the endemic Baikal omul *Coregonus migratorius*, until lately considered conspecific with the Arctic cisco *C. autumnalis* (Kozhova & Izmesteva, 1998). Molecular data, however, refute any close relationship between these fishes, and rather suggest the Baikal basin as the origin of dispersal (Politov, Bickham & Patton, 2004; Sukhanova *et al*., 2004). The proposed Late Pliocene age of the Baikal seal coincides with the origin of the current type of environment and climate in the basin (i.e. a cool deep-water lake; Kozhova & Izmesteva, 1998). It also conforms to molecular time frame estimates of other zoogeographical events in the basin. These include the origin and radiation of the endemic cottoid fish species flock of Lake Baikal (Kontula, Kirlichik & Väinölä, 2003), and diversification of a number of (sometimes cryptic) endemic invertebrate lineages, whereas much of the diversity also appears to be older (Sherbakov, 1999; Väinölä & Kamaltynov, 1999). The survival of this diversity indicates a relative stability of the Baikalian animal communities across the Pleistocene glacial dynamics, to support the peculiar lacustrine top predator through these times.

Conclusion

Our mtDNA data provide no clear support for the conventionally assumed close sister-lineage relationships among the *Pusa* group seals in the Arctic seas and in

the continental Caspian and Baikal basins. Although the monophyly cannot be refuted, the radiation of these seals appears to have coincided with their divergence from other, morphologically more dissimilar marine taxa (Phocina). The time scale suggested by the molecular data poorly fits previous hypotheses of the continental invasions of the endemic seals. Relationships appear to be too distant to comply with dispersal through Middle Pleistocene glacial lakes, and too close to conform with a Miocene Paratethyan relict ancestry of the Caspian seal. More likely, the radiation of the Phocina started in the northern seas of Late Pliocene times 2–3 Mya, and was accompanied by invasion of the continental basins. An Arctic ancestry also plausibly accounts for the emergence of the white natal hair shared by the landlocked taxa and perpetuation of the ice breeding habit in the Phocini. Nevertheless, the actual geographical conditions that would have facilitated the continental invasions in those times still remain undocumented and enigmatic.

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