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Jeremy J. Austin, Julien Soubrier, Francisco J. Prevosti, Luciano Prates, Valentina Trejo, Francisco Mena & Alan Cooper

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# The origin of the enigmatic Falkland Islands wolf.

Jeremy J Austin[a,b,*], Julien Soubrier[a,*], Francisco J Prevosti[c], Luciano Prates[d], Valentina Trejo[e], Francisco Mena[f] and Alan Cooper[a,1]

[a] Australian Centre for Ancient DNA, School of Earth and Environmental Sciences, The University of Adelaide, North Terrace, Adelaide, South Australia, 5005. AUSTRALIA

[b] Sciences Department, Museum Victoria, Carlton Gardens, Melbourne, Victoria, 3001. AUSTRALIA

[c] División Mastozoología, Museo Argentino de Ciencias Naturales "Bernardino Rivadavia" - CONICET, Av. Angel Gallardo 470, C1405DJR, Buenos Aires, ARGENTINA

[d] División Arqueología, Museo de La Plata, La Plata, ARGENTINA

[e] Martín Alonso Pinzón 6511, Las Condes, Santiago. CHILE

[f] Centro de Investigación en Ecosistemas de la Patagonia (R10C1003), I. Serrano 509, Coyhaique CHILE

[*] These authors contributed equally to this work

[1]Corresponding author: alan.cooper@adelaide.edu.au

**Abstract**

The origin of the extinct Falkland Island wolf (FIW), *Dusicyon australis,* has remained a mystery since it was first recorded by Europeans in the 17th Century. It is the only terrestrial mammal on the Falkland Islands (also known as the Malvinas Islands) which lie ~460km from Argentina, leading to suggestions of either human-mediated transport or over-water dispersal. Previous studies used ancient DNA from museum specimens to suggest that the FIW diverged from its closest living relative, the South American maned wolf (*Chrysocyon brachyurus*) around 7 Ma, and colonized the islands ~330 ka by unknown means. In contrast, we retrieve ancient DNA from subfossils of an extinct mainland relative, *Dusicyon avus*, and reveal the FIW lineage became isolated only 16 ka (8-31 ka), during the last glacial phase. Submarine terraces, formed on the Argentine coastal shelf by low sea-stands during this period, suggest that the FIW colonized via a narrow, shallow marine strait, potentially while it was frozen over.

**Introduction**

During the Pleistocene (ca. 2.6 Ma–12ka) South America supported a diverse canid fauna including large hyper-carnivorous species (i.e. *Theriodictis* spp., *Protocyon* spp., [1-4]), as well as smaller species such as the Falkland Island wolf and a related fox found on the mainland, *D. avus* (Fig. 1). Most of these species became extinct during the Pleistocene, with *D. avus* extinct by the late Holocene, and the FIW extinct in the nineteenth century following human hunting [4-7]. The origin of the FIW has been a natural history mystery for over 320 years [8]. Following his encounter with the species in 1834, Darwin commented ‘As far as I am aware, there is no other instance in any part of the world, of so small a mass of broken land, distant from a continent, possessing so large a quadruped peculiar to itself’ [9]. The mystery is made even deeper by the absence of any other terrestrial mammals on the islands. While the flora and fauna of the islands show overwhelming Patagonian biogeographical affinities [10] the

physical isolation of the islands (~460 km from the South American mainland) has resulted in a number of theories to explain the origin of the FIW. These include semi-domestication and transport of a continental ancestor by humans sometime after their arrival in southern South America ~13-14.5 ka, [11,12] or natural dispersal via rafting on ice or over a land-bridge during Pleistocene glacial sea-level minima [1,13-15]. Recently, ancient DNA analysis revealed the FIW to be a unique South American endemic only distantly related to the living South American maned wolf (*C. brachyurus*) [7]. However, uncertainty about the canid phylogenetic tree and ambiguous and/or imprecise fossil calibrations limited the power of the analysis to an approximate divergence date for the FIW and maned wolf of 6.7 Ma (4.2-8.9 Ma). The molecular evolutionary rate estimated in this analysis was also used to calculate the time to the most recent common ancestor (TMRCA) of five museum specimens of the FIW as 330 ka (95% highest posterior density [HPD] 70-640 ka), and this date was used as a proxy for the colonization age of the Falkland Islands. However, even if this date estimate was approximately accurate, the colonization event could have been considerably older or younger than this depending on the demographic history, any subsequent lineage extinctions [16], and the relationship between the FIW and unsampled mainland relatives [17]. Furthermore, the use of external fossil calibrations to date recent events is known to be problematic due to the temporal dependency of molecular rates [18,19]. Either way, while the estimated 330 ka date and wide error margins (70-640 ka) clearly predates human arrival, it provided little evidence as to how the FIW might have colonized the islands.

Critically, the genetic analysis of the FIW did not include the putative mainland close-relative *D. avus*, whose phylogenetic relationship has not been tested. *D. avus* had a clear archaeological association and temporal overlap with modern humans until extinction ~3 ka (6), raising the possibility of human transport to the islands. To investigate this issue and characterize the genetic diversity and phylogenetic relationships of this extinct canid, we

extracted and sequenced 1069 bp of ancient mitochondrial DNA from six specimens of *D. avus* collected across Argentina and Chile, ranging in age from 7800 – 3000 y BP. We also compared the genetic signals with morphological data from nearly all the extinct and living species of South American canids, a wide sample of other Caninae, and fossils of the extinct subfamilies Hesperocyonidae and Borophaginae [3].

## Results

### *Sequences*

A combined 1069 bp of mtDNA COII and cytochrome *b* sequence was obtained from six out of seven *D. avus* specimens (Supplementary Table S3). No samples produced PCR products for the four nuclear gene targets. All PCR and sequencing results were replicated at least once and produced identical results, suggesting a minimal contribution from damage-related artefacts.

### *Topology*

Both ML and Bayesian molecular analyses confirm that *D. avus* is the closest relative of the FIW with strong statistical support (Fig. 2), and only six fixed transition differences separate the FIW and *D. avus* (Supplementary Fig. S1, uncorrected sequence divergence = 0.56%). Ten additional variable sites defined three haplotypes within the FIW (from five individuals) and four haplotypes in *D. avus* (from six individuals) (Supplementary Fig. S1). Strong support is also recovered for the monophyly of the *Dusicyon* clade, its sister taxon relationship to the maned wolf, with the bush dog as the outgroup. While *D. avus* was weakly recovered as paraphyletic in the topological analysis, *D. avus* and the FIW were strongly supported as reciprocally monophyletic in analyses using a molecular clock and either external or internal calibrations (Fig. 3 and Supplementary Fig. S5).

*Dating*

Date randomization tests confirmed that sufficient signal existed in the dated concatenated sequences from the six *D. avus* individuals to provide appropriate internal temporal calibration information at the tip of the tree to facilitate molecular dating ([19], Supplementary Information). Bayesian analyses estimate that the FIW diverged from *D. avus* recently, around 16.3 ka (95% HPD 7.9-31.1 ka). The TMRCA for the *D. avus*, and for the FIW, specimens were estimated at 15.0 ka (95% HPD 8.5-25.2 ka) and 4.5 ka (95% HPD 0.6-10.6 ka), respectively (Fig. 3). In contrast, analyses using several deep, and poorly constrained canid fossil calibration points originally used in Slater *et al.* [7] produced much older age estimates, close to those previously reported (Supplementary Information, Supplementary Table S4). Interestingly, while the low intra-specific mtDNA diversity within the FIW ($\pi$ =0.0021) was consistent with that of other insular species, the diversity within *D. avus* was similar ($\pi$ =0.0026), despite the much larger geographic and ecological range of the samples. Furthermore, no phylogeographic patterns were evident within the *D. avus* sequences (Supplementary Fig. S1) despite the wide sampling area.

## Discussion

The estimated latest Pleistocene date of 16 ka (8-31 ka) for the origin of the FIW is consistent with events associated with the Last Glacial Maximum (LGM) ca. 26-19 ka [20], and is considerably younger than the previously estimated date of 330 ka [7]. The accuracy of molecular divergence date estimates is directly related to the quality of calibration data, and their proximity to the date of interest. In this regard, the use of deep fossil calibration points has been shown to be inappropriate for intraspecific and recent interspecific divergences [18,19] such as those within *Dusicyon*. The radiocarbon dates associated with the ancient sequences of *D. avus* provide much closer calibration points to the Late Pleistocene events under consideration than the early canid fossils used in Slater *et al.* [7], and importantly do not suffer

from the same uncertainty over taxonomic identification and phylogenetic position. Although additional internal calibration points would no doubt help refine the inferred dates, the randomization analyses confirm that the heterochronous Holocene *D. avus* sequences contain sufficient temporal information to calibrate the recent evolutionary history of *Dusicyon* (Supplementary Information and Supplementary Fig. S4).

The data do not support a recent origin for the FIW via human transport from a source population of *D. avus* on the South American mainland due to the estimated 16 ka divergence date and the reciprocal monophyly of the two species in calibrated analyses. While the confidence interval (8-31 ka) for the divergence event overlaps with the earliest human presence in Patagonia (13-14.5 ka, [11,12]) and human agency cannot therefore be ruled out, the genetic isolation of the FIW (presumably via transfer to the Falkland Islands) would have had to occur only once during the earliest phases of human occupation in Patagonia (*i.e.* >8 ka), which seems improbable. Furthermore, the estimated 16 ka divergence date is unlikely to be an over-estimate due to the existence of undetected and phylogenetically closer source populations of *D. avus*, because the geographically widespread *D. avus* samples have low nucleotide diversity and lack phylogeographic structure. Indeed, the young TMRCA of ~15 ka for *D. avus* and the lack of geographic structure and low genetic diversity suggest that the range of this species has recently expanded across the Patagonian and Pampean regions, potentially from a LGM refugium.

A pre-human dispersal of the FIW requires either a land-bridge during low sea-level stands or a marine crossing, but the absence of other terrestrial mammals on the Falkland Islands strongly argues against a continuous land-bridge connection to the mainland. The particularly shallow slope of the Argentine continental shelf means that lowered sea levels dramatically reduce the size of the marine strait separating the Falkland Islands, which currently has a minimum depth of ca. 160 m [21]. During the height of the LGM (26-19 ka) global sea levels

were around 130 m lower, which exposed an enormous coastal plain off the Argentine coast, while the Falkland islands landmass was about four times larger than the present [21-23]. Four pronounced submarine terraces detected on the coastal shelf (Fig. 4) are thought to record low sea-stands at various points during the LGM [24]. The two shallowest have been dated ca. 11 ka (-35/-40 m isobath) and 15 ka (-80/-90 m) [21], with the timing of the latter being consistent with Meltwater Pulse 1A, associated with the onset of the retreat of the West Antarctic Ice Shelf [20]. Two deeper terraces situated at the -110/-120 m and the -130/150m isobaths remain undated, but are thought to represent low sea-stands during earlier phases of the LGM. The deepest terrace (-130/150m) is presumably the oldest and corresponds to the lowest isostatic conditions, as it has not been degraded through subsequent near-shore activity. The depth of the terrace matches the estimated minimal sea level during the LGM (ca. -130m), but could also relate to earlier glacial events [24]. However, the difference between the -110/120m and -130/150m terraces is consistent with a rapid 10m rise from LGM lowstand sea levels associated with the 19-20 ka Meltwater Pulse caused by the widespread retreat of northern hemisphere ice sheets [20]. Either way, the molecular date estimate for the divergence of the FIW of 16 ka (range 8-31 ka) is consistent with colonization during the LGM, when the submarine terraces indicate the marine strait was both narrow and shallow.

A low sea-stand between the -130/-150 m isobaths would drastically reduce the size of the marine strait separating the Falkland Islands, potentially to just 20-30 km (Fig. 4), with an estimated minimum depth of 10-30 m. It is possible that the ancestors of the FIW were transported across this strait by ice-rafting during this period, as has been suggested, but it seems more likely that such a shallow marine strait would be periodically frozen over by continuous sea ice and/or glacial outflow to produce an ephemeral connection that could act as a filter, rather than a corridor. Individuals or packs of the ancestor of FIW pursuing marine food sources (e.g. seals, penguins, seabirds) on the margins of the ice would have an increased likelihood of colonization compared to other South American endemic mammals that would

not cross large ice-fields due to either habitat or behaviour. An interesting question is why previous glacial maxima did not lead to other mammal colonisations of the Falkland Islands, as the marine strait is thought to have been shallower during earlier phases of the Pleistocene prior to the erosion of soft sediments [21]. It is possible that if there were such previous residents they may have gone extinct without leaving a fossil record.

The greatly improved resolution provided by genetic data from the closest mainland relative allows us to conclude that the ancestor of the FIW likely colonized the Falkland Islands via mobile or static ice, crossing a narrow strait during the Last Glacial Maximum in Patagonia.

## Materials and Methods

### *Samples*

Seven *D. avus* teeth representing different individuals (Supplementary Table S1) were obtained from four sites in Patagonia: La Marcelina, Rio Negro (one tooth), Loma de los Muertos, Rio Negro (one tooth) and Perro 1 site, Tierra del Fuego (two teeth) in Argentina; and Baño Nuevo-1 Cave (three teeth) in Chile. One premolar from Baño Nuevo-1 cave has been dated to 7860 ± 78 cal. yr BP (UCIAMS-19490, Supplementary Table S1), while specimens from Perro 1, Loma de los Muertos and La Marcelina 1 were dated to 3085 ± 133, 3072 ± 151, and 3814 ± 117 cal. yr BP (AA75297, AA83516, AA90951; 6, 23 Supplementary Table S1) respectively. A second specimen from Baño Nuevo-1 cave was undated but assigned a prior mean age of 7500 cal. yr BP (6000-9000 cal. yr BP 95% range, Supplementary Table S1) based on an archaeological association with dated remains from the same site. Teeth were obtained from recently described specimens that have diagnostic morphological features of *D. avus* (e.g., large lower carnassial, a lower fourth premolar with a second distal accessory cusp and a narrow distal cingulum; see [4,6]). *D. australis* exhibits differences in dental morphology from *D. avus* including a more reduced protocone in the P4, smaller metaconid in the m1, and taller and more acute principal cusps of the premolars (see

[6]). These characters are generally associated with a more carnivorous diet [25,26] and potentially reflect the limited dietary breadth of *D. australis* [27,28].

*Molecular Analyses*

To avoid the potential for contamination of *D. avus* samples with contemporary canid DNA or previously amplified FIW PCR products [7], all pre-PCR work was performed in a dedicated ancient DNA laboratory geographically separated (by ~1.5km) from post-PCR and other molecular biology laboratories at the Australian Centre for Ancient DNA, University of Adelaide, South Australia. No contemporary canid DNA had ever been present in the pre-PCR laboratory. The ancient DNA facility includes HEPA-filtered positive air pressure with one-way air flow, overhead UV lights, individual work-rooms, the use of dead-air glove boxes with internal UV lights for DNA extractions and PCR set-up, regular decontamination of all work areas and equipment with sodium hypochlorite, PPE including full body suit, face mask, face shield, boots and triple-gloving and strict one-way movement of personnel (shower > freshly laundered clothes > ancient DNA laboratory > post-PCR laboratory).

A negative extraction control was included with every set of DNA extractions and all extractions were carried out in small sets and generally included samples from phylogenetically divergent non-canid species. DNA was extracted using a modified silica-based method [29] designed to maximize recovery of PCR-amplifiable DNA from ancient bone and tooth specimens while minimizing co-extraction of PCR inhibitors.

Short fragments of mitochondrial (166-240 bp) and nuclear (108-173 bp) DNA were amplified by PCR, to assemble a 652 bp fragment of the mtDNA COII gene, a 394 bp fragment of the mtDNA cytochrome *b* gene, and four nuclear loci (CH21, VANGL, VTN(SNP), VTN(indel), see [7]) (Supplementary Table S2). One microlitre of extract, in

parallel with extraction controls and negative PCR controls, were amplified in a 25 ul PCR containing: 1x Platinum *Taq* High Fidelity Buffer (Invitrogen), 2 mM $MgSO_4$, 0.4 mM each primer, 0.25 mM each dNTP, 0.5 U Platinum *Taq* DNA Polymerase High Fidelity, 1mg/ml RSA (Sigma-Aldrich) and sterile $H_20$. PCRs were run on a Palmcycler (Corbett Research) under the following conditions: initial denaturation at 94°C for 1 min; 50 cycles of denaturation at 94°C for 15 sec; primer annealing at 55°C for 15 sec; elongation at 68°C for 30 sec; a final elongation step at 68°C for 10 min. PCR products were visualized under UV light on a 3.5% agarose gel stained with ethidium bromide. Successful amplifications were purified using Ampure (Agencourt) according to manufacturer's instructions and sequenced directly using Big Dye chemistry and an ABI 3130XL Genetic Analyzer (Applied Biosystems). All positive PCR and sequencing results were repeated to ensure reproducibility.

*Sequence Alignment*

COII and cytochrome *b* sequences were generated from six *D. avus* specimens and aligned with the available sequences from five FIW specimens, and the eight South American canids used by Slater *et al.* [7] (maned wolf - *Chrysocyon brachyurus*, bush dog - *Speothos venaticus*, crab-eating fox -*Cerdocyon thous*, small-eared dog - *Atelocynus microtis*, sechuran fox - *Lycalopex sechurae*, culpeo fox - *Lycalopex culpaeus*, pampas fox – *Lycalopex gymnocercus*, hoary fox - *Lycalopex vetulus)*. The South American canids have previously been shown to be monophyletic [7].

*Phylogenetic Analyses*

To study the phylogenetic placement of *D. avus*, we performed both Bayesian (MrBayes [30]) and maximum likelihood (PhyML [31]) analyses on the entire data set (*D. avus*, FIW and eight South American canids, as described above). The best substitution model was selected through comparison of Bayesian information criteria scores using ModelGenerator v0.85 [32].

In addition, a haplotype network showing genealogical relationships between all *D. avus* and FIW sequences was generated using statistical parsimony implemented in TCS v1.17 [33]. Nucleotide diversity within *D. avus* and FIW was calculated using DnaSP v5.10.01 [34].

We also performed a total evidence analysis combining the new mitochondrial sequences with previously reported morphological, behavioural and genetic data for South American canids [35]. The individual mitochondrial sequences of *D. avus* and FIW were each combined into a single consensus sequence using the program Bioedit 7.0.5.3 [36], and the variable sites were scored as polymorphic using the IUPAC code. The mitochondrial sequences were added to a previously published "total evidence" matrix [3] containing dental and skeletal characters, behavioral and life history traits [37], and 22 nuclear and three mitochondrial genes from a wide sampling of living canids and several fossil representatives (for whom only dental and skeletal data was available). Sequence gaps were coded as a 5$^{th}$ state. The combined matrix was analyzed under Maximum parsimony with the software TNT version 1.1 [38] under equal weighting (SI). Trees were obtained from heuristic searches with 1000 random-addition sequence replicates and TBR branch swapping, supplemented by a TBR round on the resulting shortest-length trees. Additional searches were conducted using the sectorial-searches, tree-drifting and tree-fusing algorithms [39], but they found the same trees. Branch support was quantified with symmetrical-resampling jackknifing frequencies and frequency differences (5000 resamples; [40]). The trees were rooted with the basal canid *Hesperocyon gregarius*. Branches were collapsed following the rule number 1, where any branch with at least one reconstruction with 0 changes is collapsed (minimal branch length = 0; see [41]).

*Calibration using Fossils versus Internal Radiocarbon Dates*

To examine the problems caused by the temporal dependency of molecular dates, we performed molecular dating analyses using either internal (radiocarbon), or external (fossil), dates. The advantages of using internal calibrations to study recent evolutionary events are

well established [18,19], and given a divergence event thought to be late Pleistocene (natural dispersal) or Holocene (human dispersal), the internal radiocarbon dates for *D. avus* appear far more appropriate than external fossil dates of 4-32 Ma (with largely unknown error margins) and uncertain taxonomic/phylogenetic position.

Radiocarbon ages were converted to calibrated ages using the CALIB 6.0.1 software available at http://intcal.qub.ac.uk/calib/ [42,43], using the Southern Hemisphere SHCal04 curve [44] and two sigma ranges. A Bayesian phylogenetic analysis was performed using the FIW and *D. avus* sequences to estimate the TMRCA of each species, and their immediate ancestor, using the *D. avus* radiocarbon dates as the sole calibration points. The best substitution model was selected through comparison of Bayesian information criteria scores using ModelGenerator v0.85 [32]. Phylogenetic analyses were performed with BEAST 1.6.2 [45] using a strict molecular clock (analyses using an uncorrelated lognormal relaxed clock could not reject the strict clock assumption), and the Bayesian skyride demographic model [46] to account for demographic changes through time. The results were processed with Tracer v1.5 [47] to check that each sampled parameter had an effective sample size over 200.

To test whether the signal from the radiocarbon dates associated with the ancient sequences is sufficient to calibrate the *Dusicyon* phylogeny, a 'date randomization test' [48] was conducted. This test consists of randomizing all dates associated with the sequences (including modern ones), and then replicating the phylogenetic analysis. Ten replicates of the BEAST phylogeny described in the main text were performed using different iterations of randomized dates.

To examine the impact of using external canid fossils as calibration points, we repeated the initial molecular analysis of the FIW by Slater *et al.* [7], but with the addition of six new sequences from *D. avus* (Supplementary Information).

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**Contributions**

A.C. and J.J.A conceived the study, F.J.P., L.P., V.T., and F.M. provided samples, J.J.A., J.S. and F.J.P. performed the experiments, and A.C., J.S. and J.J.A wrote the manuscript with input from all authors.

**Competing financial interests**

The authors declare no competing financial interests.

**Figures**

**Figure 1. Specimen distribution**

Distribution map of the specimens analysed. The geographical locations of *Dusicyon avus* specimens known from late Pleistocene to late Holocene sites are shown as orange dots, with the localities of sampled specimens as orange stars. The latter are 1: La Marcelina 1, Rio Negro, Argentina; 2: Loma de los Muertos, Rio Negro, Argentina; 3: Baño Nuevo-1 Cave, Chile; 4: Perro 1 site, Tierra del Fuego, Argentina. Five specimens of Falkland Islands Wolf (FIW) (red) from museum collections were previously analysed, 5: Falkland or Malvinas Islands.

**Figure 2. Phylogenetic position of *D. avus* and FIW**

Phylogenetic tree showing the topological placement of *D. avus*, FIW and South American canids. The same topology was recovered using Bayesian (MrBayes, black support values) and maximum likelihood analyses (PhyML, grey support values). The FIW forms a

monophyletic group separate from the mainland *D. avus*, which is weakly supported as being paraphyletic, with the maned wolf as the closest living relative to the extinct *Dusicyon* clade.

**Figure 3. Dated phylogeny of *D. avus* and FIW (*D. australis*)**

Unconstrained phylogeny of *D. avus* and FIW using a molecular clock, with the radiocarbon dated ancient samples as temporally-proximate calibration points. The two *Dusicyon* species are strongly supported as reciprocally monophyletic clades in this analysis, and when external canid fossil calibration points are used (Supplementary Fig S5). The two species are estimated to have diverged during the last glacial phase (7.9-31.1 ka), with the upper bound overlapping with the first human presence in Patagonia [11,12]. The topology is a maximum clade credibility tree from BEAST with 95% HPD (highest probability density) of the calculated node ages represented as grey bars. The use of ancient samples to provide tip-date calibrations led to considerably younger TMRCA estimates for the *Dusicyon* clades than previous estimates (~330 ka for FIW [7]), which relied on deep canid fossil calibrations of imprecise phylogenetic and temporal position.

**Figure 4. Submarine terraces between the mainland and the Falkland Islands**

The four submarine terraces (Levels I to IV) along the continental shelf (light blue) between the South American continent and the Falkland Islands: Level I = -25/-30 m isobath; Level II = -85/-95 m; Level III = -110/-120 m and Level IV = -130/-150 m [21]. Terrace I has been dated ca. 11 ka, and Terrace II ca. 15 ka [21], with the latter being consistent with the global Meltwater Pulse 1A, associated with the West Antarctic Ice Sheet [20]. Terraces III and IV are undated but are thought to reflect earlier phases of the LGM, with the depth differential matching the rapid 10m rise from the LGM lowstand caused by the global Meltwater Pulse at 19-20 ka [20]. The projected sea level position at -140m is drawn in grey, representing the peak low seastand during the LGM (data from Ponce *et al.* [21]). At this point the Falkland Islands would have been separated from the mainland by a shallow marine strait estimated to be as

little as 20 km wide and only 10-30m deep. It is suggested that this is likely to periodically have been covered by ice, or facilitated ice rafting, during peak glacial conditions.

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