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Ancient DNA Clarifies the Evolutionary History of American Late Pleistocene Equids

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Abstract Hippidions are past members of the equid lineage which appeared in the South American fossil record around 2.5 Ma but then became extinct during the great late Pleistocene megafaunal extinction. According to fossil records and numerous dental, cranial, and postcranial characters, *Hippidion* and *Equus* lineages were expected to cluster in two distinct phylogenetic groups that diverged at least 10 MY, long before the emergence of the first *Equus*. However, the first DNA sequence information retrieved from *Hippidion* fossils supported a striking different phylogeny, with hippidions nesting inside a paraphyletic group of *Equus*. This result indicated either that the currently accepted phylogenetic tree of equids was incorrect regarding the timing of the evolutionary split between *Hippidion* and *Equus* or that the taxonomic identification of the hippidion fossils used for DNA analysis needed to be reexamined (and attributed to another extinct South American member of the equid lineage). The most likely candidate for the latter explanation is *Equus (Amerhippus) neogeus*. Here, we show by retrieving new ancient mtDNA sequences that hippidions and *Equus (Amerhippus) neogeus* were members of two distinct lineages. Furthermore, using a rigorous phylogenetic approach, we demonstrate that while formerly the largest equid from Southern America, *Equus (Amerhippus)* was just a member of the species Equus caballus. This new data increases the known phenotypic plasticity of horses and consequently casts doubt on the taxonomic validity of the subgenus *Equus (Amerhippus)*.

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Two groups of equids were present in Late Pleistocene South America, *Hippidion* and *Equus*. *Hippidion* appeared in the South American fossil record around 2.5 Ma, shortly after the Great American Interchange, and is clearly different from *Equus*, which appeared only around 1.0 million years ago (MA). Cladistic analysis of dental, cranial, and postcranial characters separate *Hippidion* and *Equus* into two different clades, which share the North American late-Miocene *Pliohippus* as a common ancestor around 10 MA (Prado and Alberdi 1996). The first clade includes *Equus*, *Astrohippus*, and *Dinohippus*, while the second consists of

Hippidion. Alternatively, MacFadden (1997) has suggested that *Equus* is derived from *Dinohippus*, and *Hippidion* from *Pliohippus* sensu lato (including *Astrohippus*), implying that the divergence between *Dinohippus* and *Pliohippus* occurred prior to 10 MA.

A molecular phylogeny proposed by Orlando et al. (2003) raised a completely different picture, with *Hippidion* nesting within a paraphyletic *Equus* group. These authors analyzed two mitochondrial DNA sequences of the hypervariable region I (HVR-I) from two Chilean fossils attributed to *Hippidion saldiasi*. The results raised questions about the generic classification (*Hippidion versus Equus*) of several fossil equids. This misidentification was potentially due to the pronounced shortening of the distal limbs in both hippidiform and *Equus* (*Amerhippus*) horses, a likely convergent adaptation to life in sloped terrains.

Alberdi et al. (2005) raised concerns about this interpretation and called for further genetic analysis. Central to their comment was an extensive morphological survey of almost all available collections of fossils from South America, which confirmed that the teeth samples used for the ancient DNA analyses undoubtedly belonged to *Hippidion*. They concluded that the phylogenetic DNA clustering obtained by Orlando et al. (2003) required deep taxonomic revision and should be checked using molecular data from remains that indisputably belong to *Equus* (*Amerhippus*) species.

Seven additional *Hippidion* fossils were independently analyzed (Weinstock et al. 2005) and resulted in virtually identical HVR-I sequences to Orlando et al. (2003). Additional sequence data from the second hypervariable region (HVR-II) confirmed that *Hippidion* formed part of a paraphyletic *Equus*, and postdated the emergence of the genus *Equus* (Weinstock et al. 2005). Most of these seven new specimens consisted of phalanges and showed morphological characteristics typical of *Hippidion* (e.g., two short tuberosities in the first phalanges, in contrast to the single, long, and V-shaped forms found in *Equus*).

Consequently, it seems unlikely that the nine *Hippidion* samples that have been genetically analyzed so far could all be misattributed, supporting the new phylogenetic arrangement where *Hippidion* is not a separate lineage dating back more than 10 Ma. However, to fully clarify this issue, it is necessary to obtain sequences from an *Equus (Amerhippus)* specimen. In this study, eight samples unambiguously related to *Equus (Amerhippus)* (Table 1) were subjected to ancient DNA extraction. A new *Hippidion saldiasi* specimen from Patagonia was also analyzed to further check the homogeneity of the mtDNA gene pool of the species (Table 1). DNA was extracted as previously described (Orlando et al. 2003, 2006; Weinstock et al. 2005) using appropriate ancient DNA methods and controls. A 183-bp fragment of the cytochrome b gene was targeted by PCR using the previously described cytb2L/cytb2H primers (Orlando et al. 2003). Furthermore, we designed a new set of primers to recover six short overlapping DNA fragments encompassing 546 bp of the horse mtDNA HVR-I (Table 2). All PCR reactions were conducted in a total volume of 25 µl using either Taq Gold (2.5 units; Perkin-Elmer), 1 × buffer, 2 mM $MgCl_2$, 1 mg/ml BSA, 250 µM of each dNTP, and 0.5–1 µM of the different primers (France), or Taq Hifi (1.5 units; Invitrogen), 1 × buffer, 2 mM $MgSO_4$, 2 mg/ml BSA, 250 µM of each , and 1 µM of each primer (ACAD). PCR conditions were the following: for Taq Gold, activation (92°C, 10 min), 50 cycles of DNA denaturation (92°C, 40 s), primer annealing (50°C, 40 s), and extension (72°C, 40 s), with a final elongation step of 10 min; and for Taq HiFi, activation (94°C, 1 min), 50 cycles of DNA denaturation (94°C, 20 s), primer annealing (51°C, 20 s), and extension (68°C, 40 s), with a final 10-min elongation step.

We successfully recovered DNA sequences for two *Equus* (*Amerhippus*) specimens (CH423 and CH425) and for the additional *Hippidion saldiasi* sample (ACAD1652) (Table 2). Notably, the two samples that gave maximal sequence length information (ACAD1652 and CH423) both originated from arid cave deposits (Table 1). For each DNA fragment, the final sequence was determined from the consensus of clones from at least two independent PCR products to minimize the impact of artifactual substitutions induced by DNA damage (Hofreiter et al. 2001). A total of 48 PCR products and 381 clones were analyzed in Lyon (Table 2). In addition, the mtDNA HVR-I sequences of fragments 15492F-15625R, 15668F-15847R, and 15950F-16083R of specimen CH423 were independently replicated with complete sequence identity at the Australian Centre for Ancient DNA (Adelaide). The mtDNA HVR-I sequence of the new *Hippidion* specimen from Patagonia was highly similar to previously reported *Hippidion* sequences (Orlando et al. 2003; Weinstock et al. 2005) (see branch lengths of the phylogenetic tree reported in Fig. 1). The sequences were deposited in GenBank under accession numbers EU030679–EU030682.

Importantly, the *Equus (Amerhippus)* sequences are highly divergent from both the existing and the new *Hippidion* sequences (Fig. 1). This convincingly demonstrates that the genetically analyzed *Hippidion* samples were not misattributed *Equus* (*Amerhippus*) specimens, in agreement with Alberdi et al. (2005) and Weinstock et al. (2005). On the contrary, the *Equus* (*Amerhippus*) sequence reveals similarities with several caballine horse haplotypes including members of Thoroughbred, Quarter, and Shire breeds. Over 546 bp, the CH423 haplotype shows complete identity to previously reported horse mtDNA HVR-I sequences (accession nos. AF072976, AF072980, AF072991, AY246192, AY246193, AY246216,

Table 1 List of the samples analyzed in this study

Species	Reference	Collection no.	Sample	Geographic origin
Equus (*Amerhippus*) *neogeus*				
Stored at the Museo de La Platà (MLP)	CH422	MLP 42 VI 24 16	m1 right	Tres Arroyos, BA province
	CH425	MLP 44 XII 29 23	p3	Arroyo Tapalqué (Olavarría), BA province
	CH426	MLP 63 VI 10 61	p2	Quequén Salado-Indio Rico, BA province
	CH427	MLP 91 VI 5 1	m2	Magdalena Cantera de Vialidad, BA province
	CH428	MLP 94 II 1 57	P2	Quequén Salado, BA province
	CH429	MLP 71 X 17 2	m3 left	Paso Otero, BA province
Stored at INCUAPA (Universidad Nacional del Centro de la provincial de BA, Olavarria)	CH423	None	M1-2	Inti Huasi, SL province
	CH424	None	M1-2	Centinela del Mar, BA province
Hippidion saldiasi	ACAD1652	IP 45575	Limb bone	Cueva Lago Sofia, Patagonia

Note. BA, Buenos Aires; SL, San Luis; IP, Institute de Patagonia. All the *Equus (Amerhippus)* specimens are related to the Lujanian Land Mammal Age. The morphology of the teeth is characteristic of *Equus* (*Amerhippus*) as the presence of triangular protocones, the distal part of the protocones longer than the mesial sections, with enamel wrinkles in several cases, pre- and postfossettes in the upper cheek teeth with developed folds in the upper teeth; and the double-knot with rounded metaconid-angular metastylid, shallow linguaflexid in general and more angular in p3–p4 and more open in m1–m2; the ectoflexid varies from deep to shallow and never connects to the linguaflexid in premolars in the lower teeth (for a detailed definition of tooth characters, see Figs. 1 and 2 in Alberdi et al. 2005). Besides, the comparisons of the measurements of these two teeth with the same teeth of the different species of horses from South America indicate a big size, similar to that of *Equus* (*Amerhippus*) *neogeus* (Prado and Alberdi 1994; Alberdi et al. 2003). On the contrary, the dentition of *Hippidion* genus is primitive, similar to the *Pliohippus* type, with an oval-elongate protocone that is more or less rounded, and with an anterior and posterior hypoconal groove that varied with the different stages of wear in the upper teeth; the metaconid-metastylid double knot is reduced and rounded, the buccal borders of the protoconid and hypoconid are rounded, the linguaflexid depth is moderate, and the ectoflexid is deeper in the molars than in the premolars in the lower teeth. The dental pattern varies with to the degree of wear (age) as in *Pliohippus*

Table 2 PCR results

Forward primer (5′-)	Reverse primer (5′-)	CH423	CH425	ACAD1652
15492F ATTCACCCTCATGTGCYATGTCAGTA	15625R ACATGCTTATTATTCATGG	3/20*	3/28	3/24
15562F TAYCCTATTAACGSCCCTATGTAC	15708R TGTTGRCTGGAAATGATTTG	2/14	–	3/23
15668F TCGTGCATACCCCATYCAA	15847R AGATGCCAGGTATAGTTTCA	7/56*	–	4/35
15788F TCCTCGCTCCGGGCCCAT	15945R TGTGAGCATGGGCTGATTAGTC	4/22	–	2/15
15889F CTTTCCCCTTAAATAAGACA	16018R CTTTGACGGCCATAGCTGAGT	4/34	–	3/23
15950F ACTGTGGTTTCATGCATTTG	16083R TTGCTGATGCGGAGTAATAA	4/32*	–	3/31
CYTB2L AACTGCCTTCTCATCCGTCA	CYTB2H AAAAGTAGGATGATTCCAAT	3/24	–	–
Total		27/202	3/28	18/151
Length (bp)		689	89	542

Note. Position numbers are given according to the location of the first nucleotide of the primer over the complete mtDNA sequence of *Equus caballus* (accession no. NC_001640). Above slash: number of independent PCR products analyzed. Below slash: number of total number of clones sequenced to deduce the final consensus sequence

* Independently reproduced by direct sequencing at ACAD

AY246217, AY246230, DQ297636, DQ327891, DQ327917). The same holds true for the CH425 specimen, though in this case, the sequence information retrieved is rather short (i.e., 89 bp), and consequently the list of putatively identical horse haplotypes is longer.

To examine the phylogenetic position of the *Equus (Amerhippus)* sequences in relation to horses and hippidions, we performed analyses using a mtDNA data set of HVR-I sequences for a range of equids available in GenBank. Sequences shorter than 500 bp, or exhibiting large stretches of undetermined nucleotide positions (e.g., *Hippidion* under accession no. DQ007561), were discarded, leaving a final data set of 348 sequences. Redundant or very similar haplotypes (pairwise Kimura2 distances <0.005) as well as two highly divergent caballine horse haplotypes (accession nos. AY049718–AY049719) were eliminated. In total, the final data comprised 105 sequences of extant and extinct equids (available upon request; Fig. 1). The sequence information was used to construct maximum-likelihood (PHYML online; Guindon and

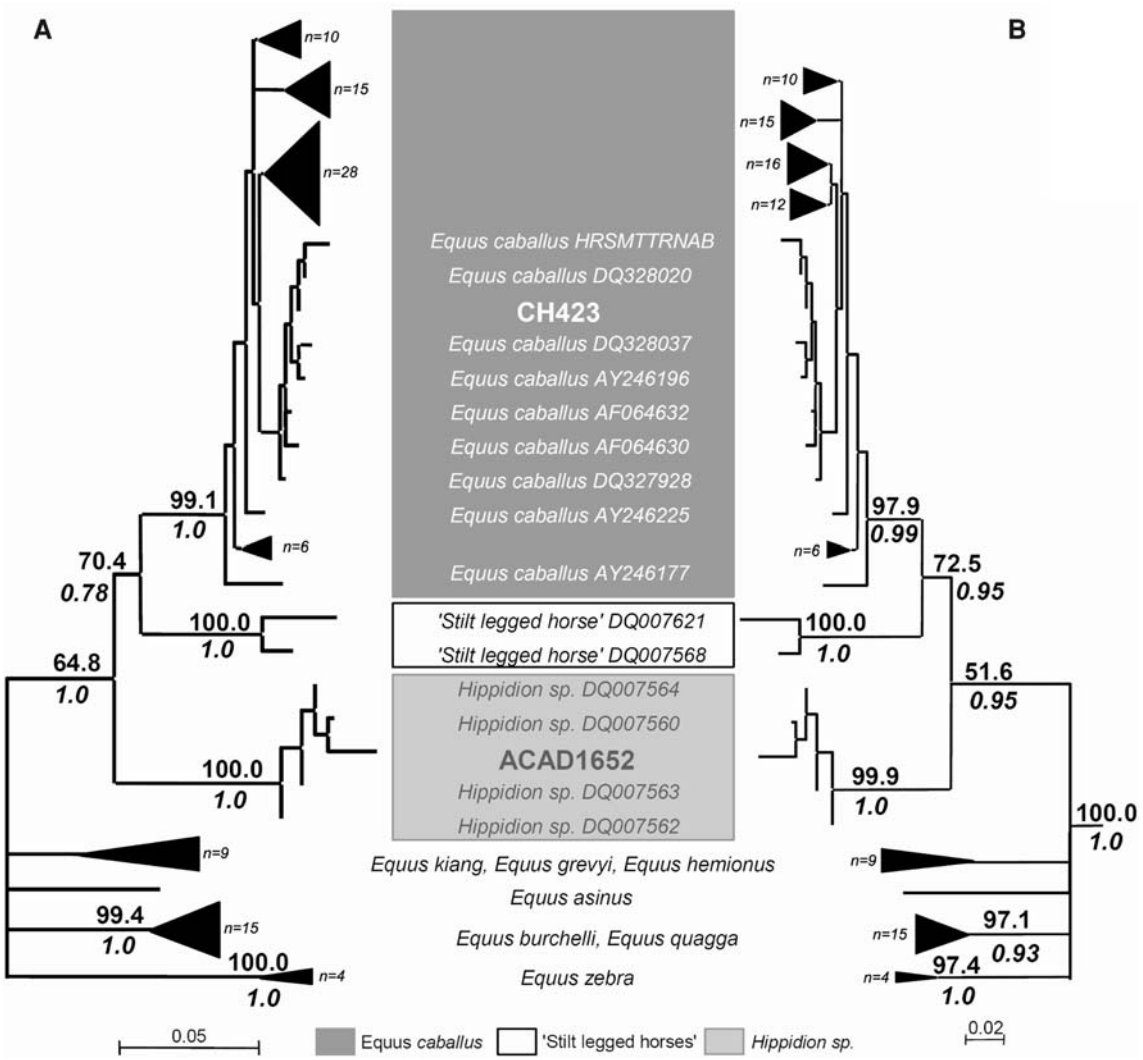

Fig. 1 Phylogenetic relationships as shown by the mtDNA HVR-I sequence data. (**A**) Midpoint-rooted phylogenetic tree. GTR+G4 + I, 488 sites, 105 sequences; ML $\alpha = 0.417$, I = 0.432, log*lk* = –3350.39608; Bayes $\alpha = 0.237$, I = 0.504. (**B**) Two rhinos (*Ceratotherium simum* and *Rhinoceros unicornis*; accession nos. NC001808-NC001779) were used as outgroups as by Weinstock et al. (2005): GTR+G4+I, 475 sites, 107 sequences; ML $\alpha = 0.292$, I = 0.163, log*lk* = –4246.20573; Bayes $\alpha = 0.217$, I = 0.237. Bootstrap values (%) and posterior probabilities are indicated above and below the principal nodes of the tree, respectively. As the data sets include an extensive number of taxa, some parts of the tree have been compressed (black triangles) using the MEGA software. The number of sequences included in each of these groups is reported. Accession numbers are as follows: *Equus asinus*—NC_001788; *Equus burchelli*—AF220916–AF220924; *Equus caballus*—AF354425, AF354426, AF354427, AF354428, AF354429, AF354431, AF354432, AF354433, AF354434, AF354436, AF354437, AF354438, AF354439, AF354440, AF354441, AF169009, AF169010, AF014406, AF014407, AF014408, AF014409, AF014411, AF014412, AF014413, AF014414, AF014415, AF014416, AF064627, AF064628, AF064629, AF064630, AF064631, AF064632, AY049718, AY049719, AY049720, AY246174, AY246175, AY246176, AY246177, AY246178, AY246179, AY246180, AY246181, AY246184, AY246186, AY246187, AY246190, AY246192, AY246195, AY246196, AY246197, AY246198, AY246211, AY246212, AY246214, AY246219, AY246220, AY246221, AY246222, AY246225, AY246226, AY246229, AY246231, Y246234 , AY246235, AY246236, AY246240, AY246241, AY246242, AY246243, AY246248, AY246253, AY246254, AY246256, AY246257, AY246259, AY246261, AY246266, AY246267, AY246271, DQ297634, DQ297635, DQ297637, DQ297638, DQ327893, DQ327897, DQ327900, DQ327903, DQ327904, DQ327905, DQ327908, DQ327915, DQ327916, DQ327918, DQ327919, DQ327920, Q327921 , DQ327923, DQ327924, DQ327925, DQ327926, DQ327927, DQ327928, DQ327929, DQ327934, DQ327935, DQ327936, DQ327937, DQ327942, DQ327944, DQ327945, DQ327946, DQ327948, DQ327950, DQ327951, DQ327956, DQ327957, DQ327958, DQ327959, DQ327960, DQ327968, DQ327969, DQ327970, DQ327971, DQ327973, DQ327974, DQ327976, DQ327981, DQ327982, DQ327983, DQ327984, DQ327986, DQ327989, DQ327990, DQ328002, DQ328005, DQ328007, DQ328012, DQ328015, DQ328018, DQ328020, DQ328021, DQ328023, DQ328025, DQ328034, DQ328035, DQ328037, DQ328039, DQ328040, DQ328042, DQ328043, DQ328044, DQ328045, DQ328050, DQ328052, DQ328053, DQ328054, DQ328056, HRSMTTRNAA, HRSMTTRNAB, HRSMTTRNAC; *Equus grevyi*—AF220928–AF220930; *Equus hemionus*—AF220934–AF220937; *Equus kiang*—AF220933, AY569539; *Equus quagga*—AY914318–AY914323; *Equus zebra*—AF22025–AF22027, AF220931; *Hippidion saldiasi*—DQ007560, DQ007562–DQ007564; "stilt-legged" horses—DQ007568, DQ007621

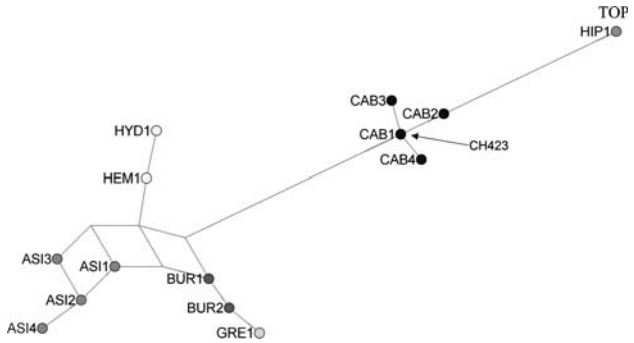

Fig. 2 Median spanning network showing the proximity of the 137-bp cytochrome *b* in horses and *Equus (Amerhippus)*. All the available cytochrome *b* sequences of equids were retrieved from GenBank and redundant haplotypes were eliminated using the DNAsp software (Rozas et al. 2003). Additionally, three highly divergent horse sequences were discarded (accession nos. DQ236094, AY819736–AY819737). The arrow indicates the location of the cytochrome *b* sequence from the CH423 specimen. Accession numbers are as follows: *Equus asinus* (ASI1- to -4)—AF380135, AF380133, AF380132, AF380130; *Equus burchelli* (BUR1, -2)—AY534349, DQ470804; *Equus caballus* (CAB1- to 4)—DQ223537, DQ223533, DQ297658, DQ297640; *Equus grevyi* (GRE1)—X56282; *Equus hemionus* (HEM1)—DQ464015; *Equus hydruntinus* (HYD1)—DQ464013; *Hippidion saldiasi* (HIP1)—AY152859

Gascuel 2005) and Bayesian (MrBayes 3.1.2; Huelsenbeck and Ronquist 2001) trees using the best model of molecular evolution according to the AIC criterion of Modeltest (Posada and Crandall 1998). The strength of the phylogenetic signal was assessed via nonparametric bootstrapping (1000 pseudo-replicates) and posterior probabilities (20 million generations, sampling frequency = 1 every 1000 generations, burn-in value = 1000). Regardless of whether the tree was midpoint rooted (Fig. 1A) or rooted with two rhino sequences (Fig. 1B), the *Equus* (*Amerhippus*) sequences fall inside the caballine horse cluster with maximum bootstrap support and posterior probabilities. The *Equus* (*Amerhippus*) haplotype appears among typical caballine horse haplotypes in median-spanning network analyses (NETWORK 4.2 software available at http://www.fluxus-engineering.com; Bandelt et al. 1999) but four steps from the nearest *Hippidion* relative (Fig. 2). Similarly, the HVR-I sequence from the ACAD1652 specimen also unambiguously clusters (maximal bootstrap support and posterior probabilities) with previously reported *Hippidion* sequences, confirming the homogeneity of the *Hippidion* mtDNA gene pool (Fig. 1).

Our analysis provides the first genetic characterization of *Equus* (*Amerhippus*) fossils. This new sequence information definitively removes the possibility that previously examined *Hippidion* specimens were all misattributed *Equus* (*Amerhippus*) specimens. At the same time it casts doubt on the current taxonomic status of *Equus* (*Amerhippus*), as they were found to be members of the caballine horse lineage (rather than members of a distinct subgenus inside equids). Given that modern caballine horse breeds exhibit mitochondrial haplotypes found in *Equus* (*Amerhippus*), the two taxa should presumably be recognized as conspecific caballine equids. This finding supports calls for a taxonomic revision of the more than 50 recognized species of American Pleistocene equids (Azzaroli 1998) and the suggestion that only a few authentic species might have been present in Late Pleistocene America (Weinstock et al. 2005). The taxonomy of the genus *Equus* has been in disorder for several decades (Winans 1989), mainly because the interspecific variation in skeletal morphology is generally not much greater than the intraspecific variation. Consequently, none of the qualitative and quantitative differences that have been used to separate species the genus *Equus* are great enough to assign species unambiguously, and the majority of palaeontologists who have worked with this genus would agree that at least some, if not most, of the nominal species are of dubious validity.

According to currently available ancient DNA data, at least three equid lineages were present in America during the late Pleistocene, namely, caballine horses (sensu stricto), "stilt legged horses" (a group of caballine horses sensu lato named by Weinstock et al. [2005]) and hippidions (Orlando et al. 2003; Weinstock et al. 2005) (Fig. 1). These data suggest that temporal and regional variation in body size and morphological and anatomic features should be considered a sign of extraordinary plasticity within each of these lineages. Such environment-driven adaptative changes would explain why the taxonomic diversity of equids has been overestimated on morphoanatomical grounds.

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