American Journal of Botany

American Journal of Botany 97(9): 1579–1584. 2010.

BRIEF COMMUNICATION

Evidence of a high-Andean, mid-Holocene plant community: An ancient DNA analysis of glacially preserved remains[1]

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- *Premise of the study*: Around the world, tropical glaciers and ice caps are retreating at unprecedented rates because of climate change. In at least one location, along the margin of the Quelccaya Ice Cap in southeastern Peru, ancient plant remains have been continually uncovered since 2002. We used genetic analysis to identify plants that existed at these sites during the mid-Holocene.
- *Methods*: We examined remains between 4576 and 5222 yr old, using PCR amplification, cloning, and sequencing of a fragment of the chloroplast *trnL* intron. We then matched these sequences to sequences in GenBank.
- *Key results*: We found evidence of at least five taxa characteristic of wetlands, which occur primarily at lower elevations in the region today.
- *Conclusions*: A diverse community most likely existed at these locations the last time they were ice-free and thus has the potential to reestablish with time. This is the first genetic analysis of vegetation uncovered by receding glacial ice, and it may become one of many as ancient plant materials are newly uncovered in a changing climate.

Key words: ancient DNA; Andes; bofedal; Holocene; puna.

Historically, much of what we know about ancient plant communities comes from morphological examination of plant microfossils and macrofossils. Over the past 2 decades, however, we have gained the exciting ability to directly analyze genetic material of ancient plants, and this can add greatly to what we know about plant community change over long time scales. Ancient DNA (aDNA) analysis of plants, although not common in the literature, has now successfully been used to characterize Holocene and Pleistocene vegetation (Kuch et al., 2002; Willerslev et al., 2003), to examine the history of domestication in crops (Allaby et al., 1999; Jaenicke-Despres et al., 2003), and to track genetic change in wild populations (Raniello and Procaccini, 2002; Tani et al., 2003). Ancient plant DNA is sometimes preserved in wood or other monotypic archaeological remains, but it also can exist in bulk samples that contain a mix of taxa, such as lake sediments, rodent middens, coprolites, and permafrost, peat, or ice cores (Willerslev et al., 1999; Willerslev et al., 2004; Gugerli, et al., 2005; Willerslev et al., 2007; Gilbert et al., 2008). Ancient DNA is of low quality because it becomes increasingly fragmented over time as a result of hydrolysis and oxidation. DNA naturally preserved in cold and/or dry environments, however, now has been successfully extracted from specimens upward of 500 000 yr old, with the theoretical limit of preservation estimated at about 1 million years (Willerslev et al., 2004; Willerslev et al., 2007). Ancient DNA fragments can be amplified with PCR or can be directly sequenced with sequencing-by-synthesis technology (e.g., Shapiro et al., 2004; Noonan et al., 2006). Modern, intact DNA is heavily favored over fragmented aDNA during amplification, so great care must be taken to avoid and identify potential modern contaminants of ancient genetic material.

In this study, we used a PCR-based approach to isolate sequences from ancient plant remains recently uncovered by the retreating Quelccaya Ice Cap in southeastern Peru. The rapid retreat of this Andean ice cap—recently as fast as ~60 m per yr for its largest outlet glacier, Qori Kalis—has been well documented over the past 4 decades and is closely linked to modern climate warming (Thompson et al., 2006). As it retreats, *rooted* plant remains have been uncovered at sites along the central-western margin, near what are now meltwater-filled lakes located at elevations near 5200 m a.s.l. (Table 1). Buffen et al. (2009) radiocarbon-dated the remains at two sites to roughly 4700 and 5100 calibrated yr before present (cal. y.b.p.) and remains embedded in sediments at a third site to between roughly 5200 and 7000 cal. y.b.p., thus providing an estimate of when these areas were last ice-free. The burial, under ice, of these plants marks an abrupt mid-Holocene transition of the regional climate to likely cooler and wetter conditions, which allowed the ice cap to rapidly advance, cover, and preserve existing plants. Other documented cases exist of retreating ice uncovering

[1] Manuscript received 12 February 2010; revision accepted 6 July 2010.

The authors thank A. Talaba, L. Stenzler, I. Lovette, M. Rasmussen, M. Arakaki, J. Walters, M. Carling, and A. Chang for all their generous help and advice on this project. This research was funded by the Andrew W. Mellon Foundation and the Cornell Department of Ecology and Evolutionary Biology.

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doi:10.3732/ajb.1000058

TABLE 1. Location and age of plant remains containing ancient DNA.

Collection location, elevation, and coordinates	Sample no.	Collection year	Calibrated 1σ age range (cal. y.b.p.)
Base Camp Lake, 5210 m (13°55.76'S, 70°51.09'W)	1	2007	5166–5277 (5222)
	2	2005	5055–5188 (5122)
North Lake, 5208 m (13°54.95'S, 70°50.71'W)	3	2005	4953–5052 (5003)
	4	2005	4524–4628 (4576)
	5	2005	4689–4762 (4726)
Boulder Lake Sequence, 5143 m (13°56.01'S, 70°51.54'W)	6	2006	5119–5185 (5152)

Note: Buffen et al., 2009, data reproduced with permission. Cal. y.b.p. = calibrated yr before present.

vegetation preserved in-place where it once grew (Bergsma et al., 1984), but we are aware of no genetic studies of such material.

Initial visual inspection of the remains from Peru indicated that much of the material might be composed of *Distichia muscoides* (Juncaeae), a cushion-forming rush species common to wetland communities (bofedales) above 3500 m in the region (Young et al., 1997; Squeo et al., 2006). Bofedales are diverse communities and include many plants from the species-rich families Poaceae and Asteraceae among others (León and Young, 1996); however, no other species could be clearly distinguished by direct examination of the remains. To address the question of whether a community of plants existed at these locations the last time they were ice-free, we cloned and sequenced aDNA fragments from five samples of the remains found at the soil surface and from one sample of similar age that was preserved in a nearby sedimentary deposit (Table 1).

MATERIALS AND METHODS

Ancient plant material—The plant remains we analyzed were collected between 2005 and 2007 at three sites located within a span of ~2 km, along the retreating central-western edge of the Quelccaya Ice Cap in the southeastern Peruvian Andes. (See Buffen et al., 2009, for details; a sample of *Distichia* remains is archived at the Universidad Nacional Mayor de San Marcos herbarium in Lima, Peru; collection number 196741 of L. Thompson et al., 01.) The area in which these ancient plants were found has been exposed over approximately the past 10 yr, and the immediate surroundings harbor only sparse patches of early colonizing vegetation (K. Young et al., University of Texas at Austin, unpublished data). Specimens at two of the sites—North Lake and Base Camp Lake (NL and BCL)—were found as large, *rooted* mats at the soil surface (Fig. 1B). Samples were taken from the interior layers of each mat to avoid collection of material that might be contaminated from the outside environment. Ancient plant material was also found embedded in a clastic sedimentary sequence, the Boulder Lake Sequence (BLS), exposed at a nearby site. Outer surfaces were removed and discarded before sample collection at the BLS to avoid potentially contaminated material. As described in Buffen et al. (2009), samples were radiocarbon-dated (Table 1) at either the Center for Accelerator Mass Spectrometry at the Lawrence Livermore National Laboratory (Livermore, California, USA) or the National Ocean Sciences Accelerator Mass Spectrometry Facility of the Woods Hole Oceanographic Institution (Woods Hole, Massachusetts, USA). If sufficient material was available, samples were dated multiple times at one or both facilities. The ^{14}C ages were calibrated using the CALIB 5.0.1 radiocarbon calibration program (Stuiver and Reimer, 1993) with the southern hemisphere calibration data set SHCal04. Of the total specimens collected by Buffen et al. (2009), we obtained plant sequence information from two NL samples, three BCL samples, and one sample from the BLS (490–505 cm depth, Table 1).

DNA extraction and amplification—One or two DNA extractions (and subsequent PCR amplifications) were carried out on each sample under a reverse-flow hood in a clean laboratory facility dedicated solely to aDNA processing at the Cornell Laboratory of Ornithology (Ithaca, New York, USA). Before this work, no plant material had been processed in that laboratory. PCR amplification of the DNA was carried out using three different primer sets (in separate reactions): (1) degenerate primers ITS1+2 (5′-GCGGAAGGATCATTGTCR-3′ and 5′-GAGCCKAGATATCCGTTGY-3′), designed to amplify an approximately 250-bp section of the first nuclear ribosomal internal transcribed spacer region; (2) *trnL*(*c*/*d*); and (3) *trnL*(*g*/h). These latter two were developed by Taberlet et al. (2007) to amplify a 254- to 767-bp fragment and a 10- to 143-bp fragment of the chloroplast leucine transfer RNA (*trnL*) intron, respectively. The short *trnL* fragment is nested within the large *trnL* fragment. In simulations, the *trnL*(*g*/*h*) primers amplify 11 404 sequences across 310 plant families listed in GenBank (National Center for Biotechnology Information, Bethesda, Maryland, USA) and identify 80% of these sequences unambiguously to the correct family (Taberlet et al., 2007).

Plant tissue was pulverized with sterile equipment under liquid nitrogen, and DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Valencia, California, USA). DNA was amplified with Platinum Taq Polymerase (Invitrogen, Carlsbad, California, USA) or JumpStart Taq Polymerase (Sigma, St. Louis, Missouri, USA), 1 μL DNA and 1 μL bovine serum albumin per 20 μL PCR reaction, 250–375 μM primer, and 2.5 mM or 4 mM $MgCl_2$, respectively. Amplification was carried out for 55–60 cycles. Tissue-free extraction and PCR controls were run in parallel. PCR product was purified using the Qiagen PCR cleanup kit and cloned into the TOPO TA vector (Invitrogen). A total of 578 clones were PCR screened for the presence of an insert, and 316 of these were cleaned via ExoI-SAP reaction and sequenced using BigDye v3.1 Terminator Cycle Sequencing (ABI, Foster City, California, USA).

Sequence identification—High-quality **(**clearly readable), trimmed sequences of 15 bp or longer were assembled into contigs by using the built-in algorithm of CodonCode Aligner (CodonCode Corporation, Dedham, Massachusetts, USA) with minor editing. Contig consensus sequences and unique unaligned sequences were compared with sequences in the GenBank environmental (env) and nonredundant (nr/nt) sequence databases in batches using P-BLAST homology search via the Cornell University BioHPC computing cluster. For sequences >20 bp (191 sequences), the search was conducted with the BLASTn algorithm with a relaxed *E*-value cutoff of 10^{-2}. Sequences 15–20 bp (10 sequences) were subjected to BLAST (Altschul et al., 1990) comparison with manual Web-based BLASTn search with parameters adjusted for short input sequences. For the *trnL*(*g*/*h*) sequence group, plant and cyanobacterial sequences and their five top-scoring species-level hits (including ties) were aligned by using ClustalX (Thompson et al., 1997), and then uncorrected pairwise sequence similarity for all pairs was calculated using PAUP* version 4.1 (Swofford, 2002) with gaps treated as missing data (Table 2).

RESULTS AND DISCUSSION

Genetic analysis allowed us to isolate and identify many sequences from the ancient Peruvian plant material. This proved a useful way to identify ancient plant taxa in the samples that were otherwise almost entirely unidentifiable by eye. Overall, we isolated 201 high-quality (clearly readable) sequences from 6 ancient plant samples. (Sequences from Table 2 are deposited in the EMBL Nucleotide Sequence Database. European Bioinformatics Institute, Cambridge, UK, under accession numbers FN994781 to FN994811.) Among three primer sets, amplification was best with the *trnL* primers, which target high-copy plastids, and it was poorest when targeting the lower copy number nuclear ITS region. In the ITS sequence set, 14 of 16 isolated sequences were unique (30 to 389 bp long); all yielded high-scoring matches to microorganisms commonly found in soil and water (data not shown) and are all thus likely to be modern contaminants. We isolated 100 sequences from the chloroplast *trnL*(*c*/*d*) region, 56 of which were unique, ranging from 21 to 389 bp long. Similarly, in this set all but one sequence matched modern contaminants (Fig. 1A). Sequences from the shortest DNA region, *trnL*(*g*/*h*), were of notably different composition than those from the other two sets. Twenty-eight of 84 total sequences were unique,

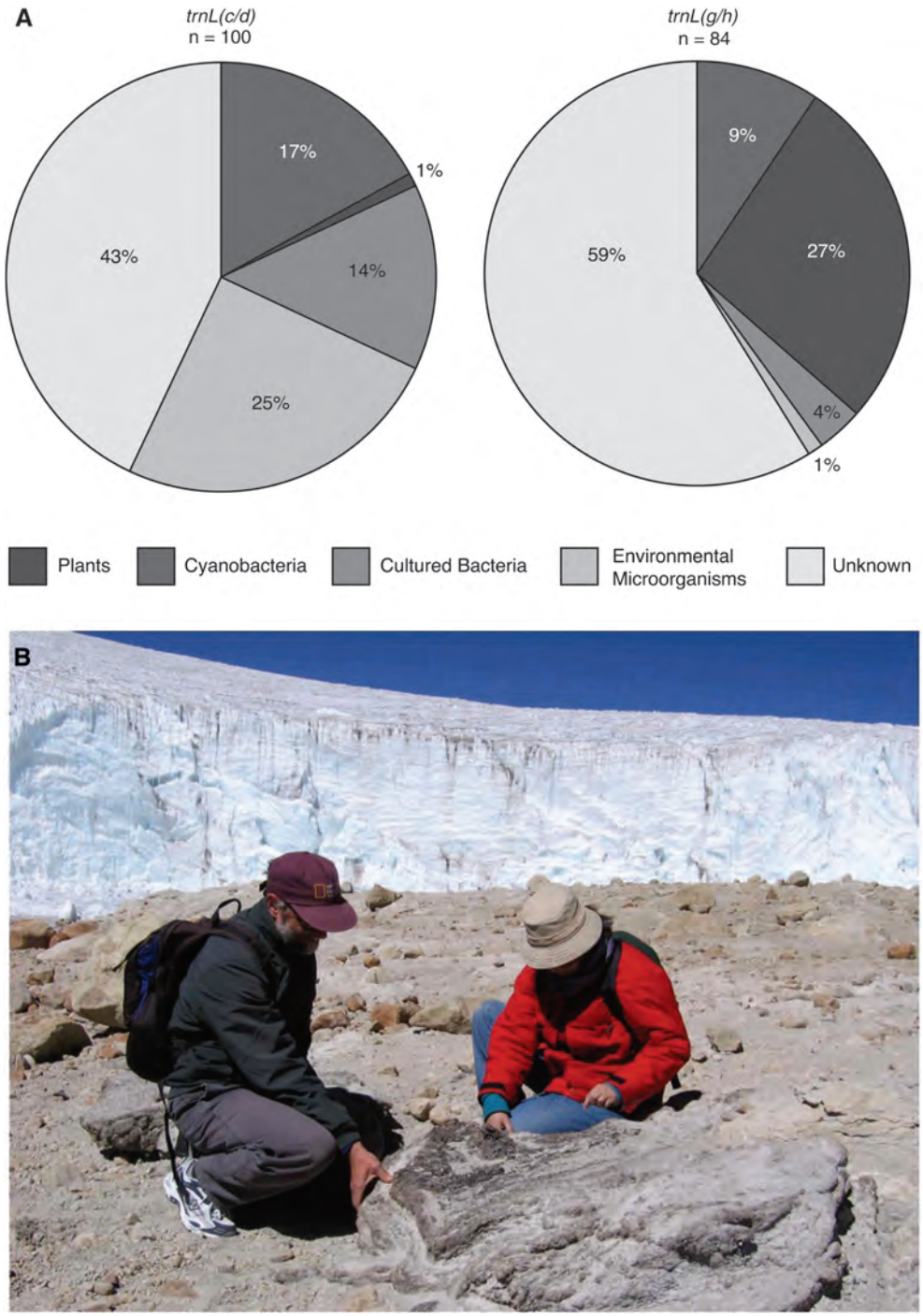

Fig. 1. (A) Categorization of sequences amplified with the *trnL* primer sets. Sequences were categorized by the identity of their top three species-level BLAST (Altschul et al., 1990) hits before a cutoff significance *E* value of 10^{-2}. Environmental microorganisms are defined as hits to metagenomic libraries of various water, soil, and other environmental samples. Unknown sequences had no significant hits. (B) Image of two researchers by one of the large mats of ancient plant material found at the Base Camp Lake site.

and 27% were from plants (Fig. 1A). The plant sequences comprised at least 7 unique taxa, of which 6 are present only among sequences from the short *trnL*(*g*/*h*) region and not the larger *trnL*(*c*/*d*) region in which it is nested. This inverse-PCR relation is consistent with the pattern of amplification obtained from highly fragmented aDNA pools (Handt et al., 1994; Deguilloux et al., 2002).

Ancient plant taxa—Despite its short length, the *trnL*(*g*/*h*) sequence region allowed us to readily distinguish these plant taxa from cyanobacteria in the samples as well as from each other at the family, and sometimes the genus, level (Table 2). The plant sequences we identified among these remains span a range of families, which indicates a diverse plant community existed at these high-elevation sites ~5,000 yr ago. Furthermore,

TABLE 2. Percent sequence identity between *trnL*(*g*/*h*) DNA sequences and top BLAST hits.

		Extracted Sequences							
		Sequence[a]	A (14)	B (2)	C (1)	D (1)	E (1)	F (1)	G (8)	H (3)
		Length (bp)	50	49	23	53	53	52	83	54
Group	Sequence Match	Sample no.	1, 4, 6	6	1	4	5	4	1	2, 3
Juncaceae	*Oxychloe* (2 species)		100%	39%	70%	50%	48%	33%	30%	36%
	Distichia muscoides		100%	39%	70%	50%	48%	33%	30%	36%
	Patosia clandestina		100%	39%	70%	50%	48%	33%	30%	36%
	Juncus prominens		94%	39%	78%	50%	48%	35%	28%	38%
	Juncus maritimus		94%	39%	83%	50%	48%	33%	30%	36%
	Juncus (4 other species)		94%	35%	70%	51%	49%	35%	31%	37%
Asteraceae	*Senecio* (4 species)		48%	100%	35%	46%	46%	68%	33%	44%
	Monticalia arbutifolia		48%	100%	35%	46%	46%	68%	33%	44%
	Misbrookea strigosissima		48%	100%	35%	46%	46%	68%	33%	44%
	Taraxacum (2 species)		48%	100%	35%	46%	46%	68%	33%	44%
	Eriochlamys sp.		49%	100%	35%	45%	45%	68%	33%	45%
Bryaceae	*Bryum mildeanum*		70%	27%	100%	30%	30%	26%	26%	35%
	Imbribryum alpinum		70%	27%	100%	30%	30%	26%	26%	35%
Poaceae	*Merxmuellera guillarmodae*		50%	52%	30%	100%	92%	44%	35%	33%
	Trisetum spicatum		50%	52%	30%	100%	92%	44%	35%	33%
	Agrostis vinealis		50%	52%	30%	100%	92%	44%	35%	33%
	Sphenopholis obtusata		50%	52%	30%	100%	92%	44%	35%	33%
	Koeleria pyramidata		50%	52%	30%	100%	92%	44%	35%	33%
	Trisetum sp.		50%	52%	30%	100%	92%	44%	35%	33%
	Deschampsia (3 species)		48%	55%	30%	91%	100%	42%	36%	36%
	Koeleria macrantha		48%	55%	30%	91%	100%	42%	36%	36%
	Festuca arundinacea		50%	55%	30%	98%	94%	46%	37%	35%
	Lolium multiflorum		50%	55%	30%	98%	94%	46%	37%	35%
Rutaceae	*Swinglea glutinosa*		33%	68%	26%	44%	42%	100%	33%	38%
	Oxanthera (2 species)		33%	68%	26%	44%	42%	100%	33%	38%
	Microcitrus (4 species)		33%	68%	26%	44%	42%	100%	33%	38%
	Merope angulata		33%	68%	26%	44%	42%	100%	33%	38%
	Fortunella margarita		33%	68%	26%	44%	42%	100%	33%	38%
	Citrus medica		33%	68%	26%	44%	42%	100%	33%	38%
Cyanobacteria	*Nostoc* sp. A		34%	39%	26%	32%	34%	31%	94%	33%
	Nostoc sp. B		36%	39%	26%	32%	32%	33%	94%	37%
	Nostoc symbiont A		34%	39%	26%	34%	36%	31%	92%	31%
	Nostoc symbiont B		34%	39%	26%	34%	36%	31%	92%	31%
Picea sp.	*Picea* (7 species)		36%	55%	35%	33%	35%	38%	31%	100%

Note: Shown are all plant and cyanobacterial *trnL*(*g*/*h*) sequences (A through H) and their percent similarity to their five top-scoring BLAST hits (Altschul et al., 1990), including ties, up to a significance cutoff *E* value of 10^{-2}. Each row represents a single sequence that may be common to more than one species within a genus, as indicated. Gaps were treated as missing data. Sequences are deposited in the EMBL database under accession numbers FN994781 to FN994811.

[a] Numbers in parentheses indicate the number of times the sequence occurred among sequenced clones.

all the sequences were from plant groups that are found in bofedal wetland communities today. The highest number of ancient plant sequences (Sequence A, Table 2) matched *Distichia muscoides* (the plant visually identified among the remains). However, these sequences also match the species' very close South American relatives among the Juncaceae, *Oxychloe* spp. and *Patosia clandestina*. (For a subset of plant samples, we attempted amplification of another 100-bp region that is polymorphic between these species, but we were unsuccessful.) In addition to *Distichia*, we detected sequences from at least one moss species from the highly diverse *Bryum/Imbribryum* group (Bryaceae). Present both in the wetlands and in dry areas at high elevations are mosses, including members of the Bryaceae, Amblystegiaceae, Fissidentaceae, and Grimmiaceae families. We also detected members of the two most species-rich families in the high Andes: Poaceae and Asteraceae. Within Poaceae, we identified at least two unique sequences that matched almost exclusively members of the subfamily Pooideae. This group is represented in the high Andean wetlands by genera such as *Agrostis*, *Calamagrostis*, and *Festuca* (Young et al., 1997). Two of our sequences also matched members of the subfamily Asteroideae within the Asteraceae. This subfamily is represented in bofedales today by *Erigeron*, *Senecio*, and *Xenophyllum* (K. Young et al., University of Texas at Austin, unpublished data). A single sequence (Sequence F, Table 2) matched members of the Rutaceae, a family that is not known today at high elevations in this region, only among lowland taxa (e.g., *Zanthoxylum*, 79% identity). Therefore, Sequence F may have resulted from miscoding lesions or PCR error, or it may represent an unknown member of the high-elevation flora.

The source of the ancient sequences may include the structural tissues, seeds/spores, or pollen of ancient plants within the mats; however, the sequences are likely to be very local in nature. Although pollen is widely deposited across the Quelccaya Ice Cap by wind, we did not detect sequences of taxa whose pollen is most abundant in surface snow on the ice cap (Reese and Liu, 2002) and in sediment cores from lower-elevation lakes in the region (Bush et al., 2005). The ancient taxa we identified in our samples match only taxa most prevalent in high-elevation wetlands today.

Wetland plant communities are the most diverse ecosystems above 4000 m in the Andes. Although not present at the study site, in the vicinity they reach 5200 m where freshwater is abundant in small meltwater lakes. The taxa present within the ancient remains indicate that not only cushion plants like *Distichia* but also a diverse wetland community existed at the sites approximately 5000 yr ago. This points toward the perhaps cyclical reestablishment of bofedal vegetation in some locations as climate fluctuates on millennial timescales in the high Andes. Primary succession after deglaciation has been studied infrequently (but see Jones and Henry, 2003); however, results here emphasize its potentially important, recurrent role in the persistence of integrated plant communities on timescales reaching 5000 yr or more. This type of vegetation is crucial for the livelihood of pastoralist communities (Postigo et al., 2008) and has been linked to the development of neotropical camelid domestication (Baied and Wheeler, 1993). In the present day, increased water supply and subsequent expansion of bofedal pasturelands have been observed by pastoralists living at 4000 m as well as detected via remote sensing (Postigo et al., 2008). The ability of not one species but a community to reestablish at the edge of its present altitudinal range may be of great importance as the climate warms in the coming decades.

Efficacy of the* trnL *primers for future aDNA analysis—In addition to yielding information about an ancient plant community, this analysis revealed both the advantages and disadvantages of using the *trnL* primers for analyzing ancient plant remains. To date, this primer set has been used infrequently in aDNA analysis (but see Willerslev et al., 2007). However, it was chosen for use here because for bofedal species, more sequences from this genetic region are available in GenBank than from other genetic regions. Although we were able to detect and categorize highly fragmented plant sequences using this primer set, its efficiency in isolating plant sequences from bulked samples is somewhat hampered because it regularly amplifies the *trnL* intron from cyanobacteria that have a common ancestor with the chloroplasts of modern plants. Cyanobacteria made up almost 14% of the sequences amplified in this study. Although macroscopic, free-floating cyanobacteria in the genus *Nostoc* (Table 2) do occur among the cushions of *Distichia* in the waters of high-Andean wetlands (Young et al., 1997), the modern origin of at least some of the sequences from cyanobacteria is supported by the fact that they occur in both the long- and short-region *trnL* sequence sets. Cyanobacteria also are known colonizers of recently deglaciated soils (Nemergut et al., 2007; Schmidt et al., 2008).

Other contaminants easily picked up by the *trnL* primers are sequences from *Picea* (spruce). It is not likely that members of this genus existed in southern Peru in the recent past because in the present day, *Picea* are restricted entirely to the northern hemisphere (Ran et al., 2006). In a comparison of Sequence H (Table 2) and a close South American family, Podocarpaceae, the two were not significantly similar. *Picea* also occurs as the lone plant sequence in the longer *trnL*(*c*/*d*) sequence set (Fig.1). *Picea* was identified very frequently with these primers in a study of degraded fecal DNA (Valentini et al., 2009) and in negative controls from at least one other aDNA study (Willerslev et al., 2007). We did not detect *Picea* in extractions performed on paper products previously used in the laboratory and thus hypothesize that the source of this sequence is more likely infrequent contamination from pollen carried in from outside during the time DNA extractions were conducted.

This study also highlights the continued need for increased plant and environmental genetic sequencing and annotation. Just over 50% of the sequences we obtained across all three primer sets yielded no significant matches at a relaxed *E*-value significance cutoff of 10^{-2}. This is common even in aDNA studies of nonbulk samples (Noonan et al., 2005; Green et al., 2006). More sequences might be identifiable if GenBank or other public databases were to contain broader coverage of sequence information for barcoding markers from plants as well as microorganisms. A greater portion of the information preserved in aDNA pools might be decipherable if a comprehensive database of regionally specific barcodes existed for use in plant species identification, similar to the current system for animal taxa (Ratnasingham and Herbert, 2007). Effort continues on this front despite many inherent difficulties (Kress et al., 2005; Hollingsworth et al., 2009).

Conclusion—Tropical glaciers and ice caps are rapidly receding because of climate warming, and although this may not bode well for many modern ecosystems, there nevertheless exists the exciting prospect of plant and other ancient remains (e.g., Rollo et al., 2000) being uncovered in the coming decades. As demonstrated here, aDNA analysis has the power to add to what we can initially learn about the past from traditional paleobotanical analyses. The *trnL* primers provide an imperfect yet valuable tool for identifying ancient plants among glacially preserved remains. In this case, we can infer that not only one species but a community of plants occurred at a high-elevation location ~5000 yr ago under likely warmer conditions. These taxa all occur in bofedal wetland communities today, demonstrating the ability of at least a portion of them to persist together as a community under climate change.

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