



# Larch Cellulose Shows Significantly Depleted Hydrogen Isotope Values With Respect to Evergreen Conifers in Contrast to Oxygen and Carbon Isotopes

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The analysis of the stable isotope of the tree-ring cellulose is an important tool for paleo climatic investigations. Long tree-ring chronologies consist predominantly of oaks and conifers in Europe, including larch trees (*Larix decidua*) and cembran pines (*Pinus cembra*) that form very long tree ring chronologies in the Alps and grow at the treeline, where tree growth is mainly determined by temperature variations. We analyzed  $\delta^{13}\text{C}$ ,  $\delta^{18}\text{O}$  and  $\delta^2\text{H}$  isotopes in the cellulose extracted from tree-rings of wood samples collected at high altitude in the Swiss and Tyrol Alps, covering the whole Holocene period. We found that larch cellulose was remarkably more depleted in deuterium than that of cembran pine, with mean  $\delta^2\text{H}$  values of  $-113.4 \pm 9.7\text{‰}$  for larch and of  $-65.4 \pm 11.3\text{‰}$  for cembran pine. To verify if these depleted values were specific to larch or a property of the deciduous conifers, we extended the analysis to samples from various living conifer species collected at the Bern Botanical Garden. The results showed that not only the larch, but also all the samples of the deciduous larch family had a cellulose composition that was highly depleted in  $\delta^2\text{H}$  with regard to the other evergreen conifers including cembran pine, a difference that we attribute to a faster metabolism of the deciduous conifers. The  $\delta^{18}\text{O}$  values were not statistically different among the species, in agreement with the hypothesis that they are primary signals of the source water. While the  $\delta^{13}\text{C}$  values were slightly more depleted for larch than for cembran pine, likely due to metabolic differences of the two species. We conclude that the deciduous larch conifers have specific metabolic hydrogen fractionations and that the larch unique signature of  $\delta^2\text{H}$  is useful to recognize it from other conifers in subfossil wood samples collected for paleoclimatic studies. For climate information the absolute  $\delta^2\text{H}$  values of larch should be considered carefully and separate from other species.

**Keywords:** stable isotopes, deuterium, larch, conifer, cellulose

## INTRODUCTION

The stable isotope ratios of carbon, oxygen and hydrogen have been studied in various components of plants, including bulk wood samples, lignin, whole leaves and leaf waxes (Borella et al., 1998; Borella et al., 1999; Loader et al., 2003; Kahmen et al., 2011; Kimak and Leuenberger 2015), and those analyzed in tree ring samples were successfully used for the reconstruction of past climate conditions, thus widening the field of dendroclimatology (Borella et al., 1998; Leuenberger 1998; McCarroll and Loader 2004; Treydte et al., 2007; Frank et al., 2015). Tree ring samples are made of cellulose, hemicelluloses and lignin (Khezami et al., 2005) and cellulose is the most used material for isotope ratio studies (Borella et al., 1998; Borella et al., 1999; Loader et al., 2003; McCarroll and Loader 2004). It is a carbohydrate that contains the stable isotope ratios of carbon ( $\delta^{13}\text{C}$ ), oxygen ( $\delta^{18}\text{O}$ ) and hydrogen ( $\delta\text{D}$  alias  $\delta^2\text{H}$ ) (Cormier et al., 2018) that carry climate information and can act as climate proxies. In dry alpine sites the  $\delta^{13}\text{C}$  values are controlled mainly by stomatal conductance, which is linked to summer moisture stress and thus to precipitations (Gagen et al., 2004). Early studies noted strong relationships between cellulose  $\delta^{18}\text{O}$  and mean annual temperature and humidity (Libby et al., 1976; Burk and Stuiver 1981). More recently in relatively humid locations tree ring  $\delta^{18}\text{O}$  values have been used to reconstruct precipitation (Brienen et al., 2012; Boysen et al., 2014), whereas in other locations tree-ring  $\delta^{18}\text{O}$  reflected a combination of vapor pressure deficit, relative humidity and sunshine (Roden and Ehleringer 2007; Xu et al., 2011; Boysen et al., 2014; Hartl-Meier et al., 2014; Labuhn et al., 2014). The  $\delta^2\text{H}$  values have been measured in tree rings for paleo climatological investigations for forty years (Schiegl 1974), but their interpretation still remains complex. The interest in  $\delta^2\text{H}$  is stimulated by recent developments in isotope ratio mass spectrometry combined with equilibrium methods that allow the measurement of carbon, oxygen and hydrogen isotope ratios at the same time (Filot et al., 2006; Loader et al., 2014). This led to results that indicated that  $\delta^2\text{H}$  in plant cellulose is determined by 1) the  $\delta^2\text{H}$  value of the water source, 2) the water evaporation in the leaf that enriches heavier water isotopes in the liquid phase and 3) the biosynthetic isotopic fractionation between leaf water and the final organic compounds like cellulose, that includes many complex biochemical processes (Cormier et al., 2019). Altogether, the deuterium fractionation is related to both environmental and physiological factors (Augusti 2007).

The oxygen and hydrogen in the plant cellulose originate mainly from meteoric water and the fractionation processes during pre-photosynthetic, photosynthetic and post-carboxylation reactions affects both  $\delta^{18}\text{O}$  and  $\delta^2\text{H}$ , but in a different way (Yakir et al., 1990). Leaf evapotranspiration is expected to enrich heavy isotopes in leaf water (Nabeshima et al., 2018), but some enzymes of glucose biosynthesis are selective for the stable H isotopes and imprint a specific Deuterium ( $^2\text{H}$ ) abundance in each H group bound to the C of cellulose (Augusti et al., 2006). It should be noted that the analytical approach of cellulose used here (the cellulose pyrolysis) detects all hydrogens, but the exchangeable hydrogens are equilibrated with a water of known hydrogen isotope ratio

(Filot et al., 2006). The non-exchangeable hydrogen isotope ratio is then calculated from the measured and the equilibrated non-exchangeable hydrogens. The specific  $\delta^2\text{H}$  in each H bound to the C of cellulose documents a distinct biochemical history (Augusti 2007) that showed minor differences between spruce and oak (Augusti et al., 2008). However, the isotopomer variations seem to have a minor effect on total variation of  $\delta^2\text{H}$  in cellulose, and no specific studies on larch isotopomers have been reported so far.

In the last decade various studies have been published on deuterium abundance in cellulose from different tree genus, mainly from *Quercus*, *Fagaceae* (Kimak et al., 2015), *Picea* (Gori et al., 2013), *Pinus*, *Pinales* (Nabeshima et al., 2018), *Larix* (Hafner et al., 2011) and *Abeis* that all use C3 photosynthesis mechanism (Tang et al., 2000; McCarroll and Loader 2004; Szczepanek et al., 2006).

One study found that in the early annual growing season the cellulose of leaves of deciduous oak and beech was enriched ( $-20\text{‰}$ ), while that of the evergreen taxus was more depleted ( $-100\text{‰}$ ) (Kimak et al., 2015). Similar results were found in tree rings cellulose from oak and beech, with higher  $\delta^2\text{H}$  values ( $-10\text{‰}$ ) in the early annual growing season than in the late growing season ( $-50\text{‰}$ ), possibly due to the use of stored carbohydrates (Nabeshima et al., 2018). Thus,  $\delta^2\text{H}$  values change during the tree growth phases. The heterotrophic metabolism causes a 2H (D) an enrichment of cellulose higher than that of autotrophic metabolism, probably due to a higher recycling of compounds in the Calvin and tricarboxylic acid cycles that is associated with a more complete exchange of C-bound H with the surrounding and enriched foliar water (Ziegler 1995; Nabeshima et al., 2018; Cormier et al., 2019). The effect of metabolism on  $^2\text{H}$  fractionation in cellulose is supported also by the finding that C4 plants are more  $^2\text{H}$ -enriched than C3 plants (Ziegler et al., 1976). The different photosynthesis systems and anatomies of C3 and C4 plants influence  $^2\text{H}$ -biological fractionation via C-bound H exchanges with water in the different anatomical compartments (Zhou et al., 2016).

The data reported above indicate that factors other than climate affect the isotopic fractionation signal of tree ring wood, and they should be identified for their robust use in paleo dendroclimatology. To this aim we have measured  $\delta^2\text{H}$ ,  $\delta^{18}\text{O}$  and  $\delta^{13}\text{C}$  isotopes in the cellulose of 4399 subfossil samples of 120 conifers cembra pine and larch and of one spruce collected at 12 different sites in Switzerland, Austria and Italy. They cover 8930 years and were collected in the project the Eastern Alpine Conifer Chronology for paleoclimatic studies (Nicolussi et al., 2009). With the aim to recognize the non-climatic signals in the dataset we investigated, in this study, site influences and species differences regarding isotope compositions. These investigations were extended to many living conifers sampled at the Bern Botanical Garden.

## MATERIALS AND METHODS

### Sampling Sites in the Alps

The wood samples belong to the Eastern Alpine Conifer Chronology Dataset, the collection and preparation of which

**TABLE 1** | Characteristics of the 12-sampling sites, number of trees and samples (each sample constitutes of a block of 5 years). Specie code: larch: LADE, cembran pine: PICE, spruce: PCAB.

Site	Coordinates	Aspect	Elevation (m)	Species	No. of isotope samples	No. of trees
FPCR*	7°33' E/ 46°04' N	WSW	1965	PICE	155	4
VRR*	9°51' E/ 46°26' N	E	2158	LADE	172	4
				PICE	140	4
UZAR*	8°17' E/ 46°34' N	SSO	1977	PICE	136	4
MIS	10°37' E/ 46°54' N	N	2252	LADE	25	1
				PICE	55	1
AHST	12°07' E/ 47°03' N	SE	1995	LADE	49	2
				PICE	33	1
MAZB	10°57' E/ 46°35' N	N	2125	LADE	185	6
				PICE	54	1
MAZE	10°57' E/ 46°35' N	N	2125	LADE	180	5
				PICE	187	8
MAZF	10°57' E/ 46°35' N	N	2105	LADE	97	2
				PICE	31	1
MM	7°55' E/ 46°02' N	NNE	1995	LADE	282	6
				PICE	353	6
TSC	9°53' E/ 46°24' N	NW	2162	LADE	249	6
				PICE	338	6
UA	8°13' E/ 46°34' N	E	1950	LADE	524	17
				PICE	70	2
ULFI	10°50' E/ 46°28' N	N	2110	LADE	1066	23
				PICE	152	4
ZER	7°47' E/ 46°03' N	N	2315	LADE	59	1
				PICE	17	1
HIB	12°15' E/ 46°54' N	E	2140	LADE	45	1
				PICE	36	1
Botanical garden bern	7°26' E/ 46°57' N	Different	480	—	—	—

\*The recent site.

have been described earlier (Nicolussi et al., 2009). Part of the dataset includes samples from living trees that cover the last 190 years, from 1820–2010 A.D. These tree ring samples were collected at three upper tree-line sites at the proximity of glacier sites at elevations above 1900 m (Ziehmer et al., 2018). These three sites are: Val Roseg (VRR) where samples of four cembran pines and four larch trees were collected, valley d'Hérens (FPCR) four larches and at Grimsensee (UZAR) four cembran pine trees were sampled (Table 1). The other part of the dataset consists of subfossil wood samples from the dominant tree-line species, the deciduous European larch (*Larix decidua* Mill) and the evergreen cembran pine (*Pinus cembra* (L.)) with the inclusion of a single tree of the evergreen spruce (*Picea abies* (L.) H.Karst). They cover the last 9000 years, the whole Holocene, and were collected at 12 different sites in the European Alps which cover a SW-NE transect, at an elevation range of 1,930 to 2,400 m a.s.l. with different aspects. The site locations are shown in Figure 1 and their characteristics are listed in Table 1. Each species and site are represented by four trees. The samples we analyzed were wood blocks of tree-rings covering five consecutive years (Ziehmer et al., 2018).

## Wood Samples From Bern Botanical Garden

We sampled tree branches at the Botanical Garden in Bern, some of which were dead. The bark and the cambium were removed from the wood before preparing the samples for analysis. A small wood amount (about 50 mg) was chosen for the extraction of the

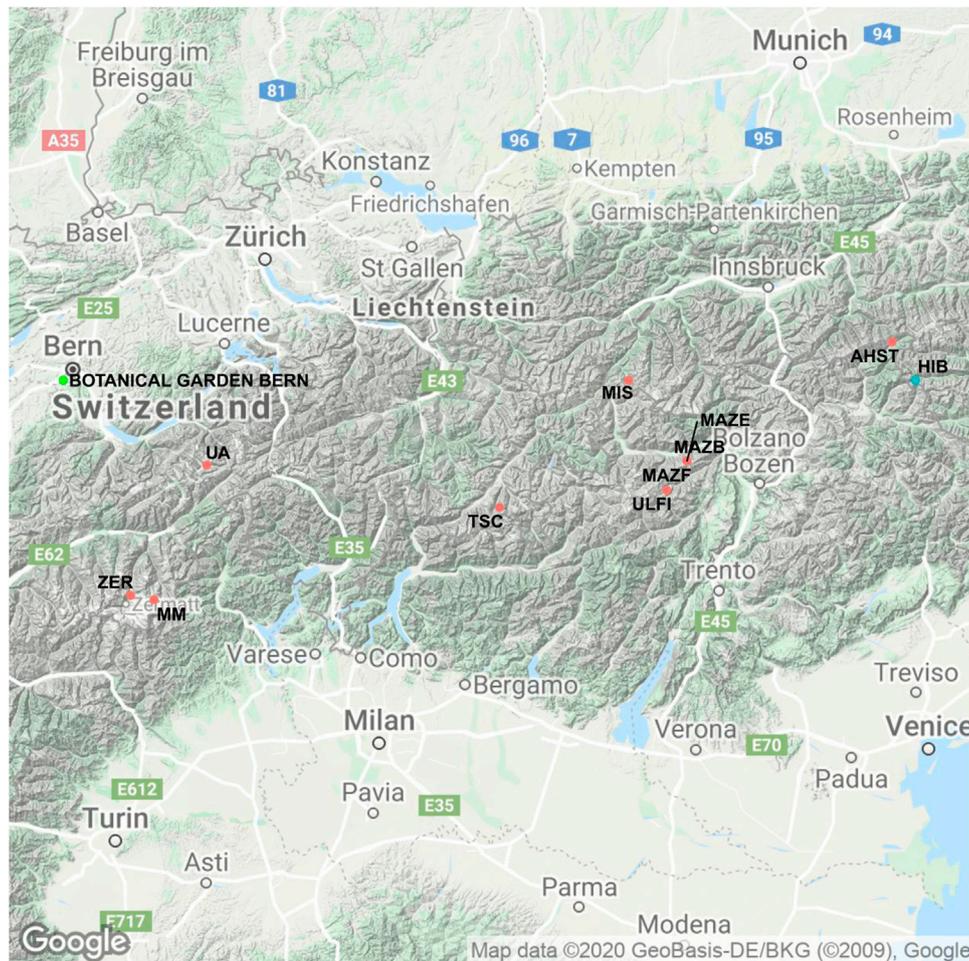
cellulose and the isotope ratio analysis was done as described below. Each sample was measured at least twice. The samples included 20 different conifer species and one of the broad leaf gymnosperm *Ginkgo biloba*. The larch family was represented by six different species: *Larix decidua*, *L. decidua* subsp. *polonica*, *L. sibirica*, *L. laricina* and one species of *Pseudolarix amabilis*. The evergreen conifers included eight different species of pinus (*Pinus bungeana*, *P. nigra*, *P. sylvestris*, *P. cembra*, *P. mugo*, *P. speuce*, *P. ponderosa*, *P. wallichiana*). The other evergreen conifers were *Cephalotaxus harringtonii*, *Cedrus atlantica*, *Cupressus nootkatensis*, *Taxus baccata* and *Tsuga Canadensis*.

## Sample Wood Dating

Calendar-dated tree-ring width series of Holocene wood sections are available at the Institute of Geography of the University of Innsbruck, where also the Eastern Alpine Conifer Chronology (EACC) has been established (Nicolussi et al., 2009). For isotope and cellulose analyses, the calendar-dated discs were cut into five-year blocks.

## Stable Isotope Analysis

The isotope measurements were carried out using wood blocks of consecutive five tree rings. The separation of wood samples into five-year blocks was performed at the University of Innsbruck, Austria. The procedure of cellulose extraction and the calculation of the cellulose content (cellulose dry weight/ wood dry weight) (Ziehmer et al., 2018) as well as the triple-isotope analysis were



**FIGURE 1** | Map of the 12 collection sites of the trees described in **Figures 2,3**. The sites are located in Switzerland and Tyrol and their codes used in **Figure 2** are shown. In blue are sites at which larch and cembra pine were collected, in green where cembra pine and spruce were collected, in black the sites of the modern (living) trees (with the exception of VVR, which is as site with both living and subfossil samples), in light-green the location of the Botanical Garden in Bern is illustrated. Details of site characteristics and of sample collection is given in **Table 1**. (© Google Maps 2020)

described before (Loader et al., 2015). Briefly, we used conventional Isotope Ratio Mass Spectrometry (Isoprime 100) coupled to a pyrolysis unit (HEKAtech GmbH, Germany), which is similar to the previously used TC/EA (for technical details see (Leuenberger 2007)). This approach was extended to measurements of non-exchangeable hydrogen of alpha-cellulose using the on-line equilibration method (Filot et al., 2006; Loader et al., 2015). The results are reported in per mil (‰) relative to the Vienna Pee Dee Belemnite (VPDB) for carbon and to Vienna Standard Mean Ocean Water (VSMOW) for hydrogen and oxygen (Coplen 1994). We applied two-point calibrations using Merck cellulose and IAEA-CH-6 crystalline sugar for carbon and oxygen isotopes as well as Merck cellulose and IAEA-CH-7 PE polyethylene foil for hydrogen isotopes. Wei Ming 101 cellulose was used as target material. Merck and Wei Ming 101 cellulose are well characterized internal laboratory standard materials. The precision of the measurement was  $\pm 3.0\text{‰}$  for hydrogen,  $\pm 0.3\text{‰}$  for oxygen and  $\pm 0.15\text{‰}$  for carbon (Loader et al., 2015).

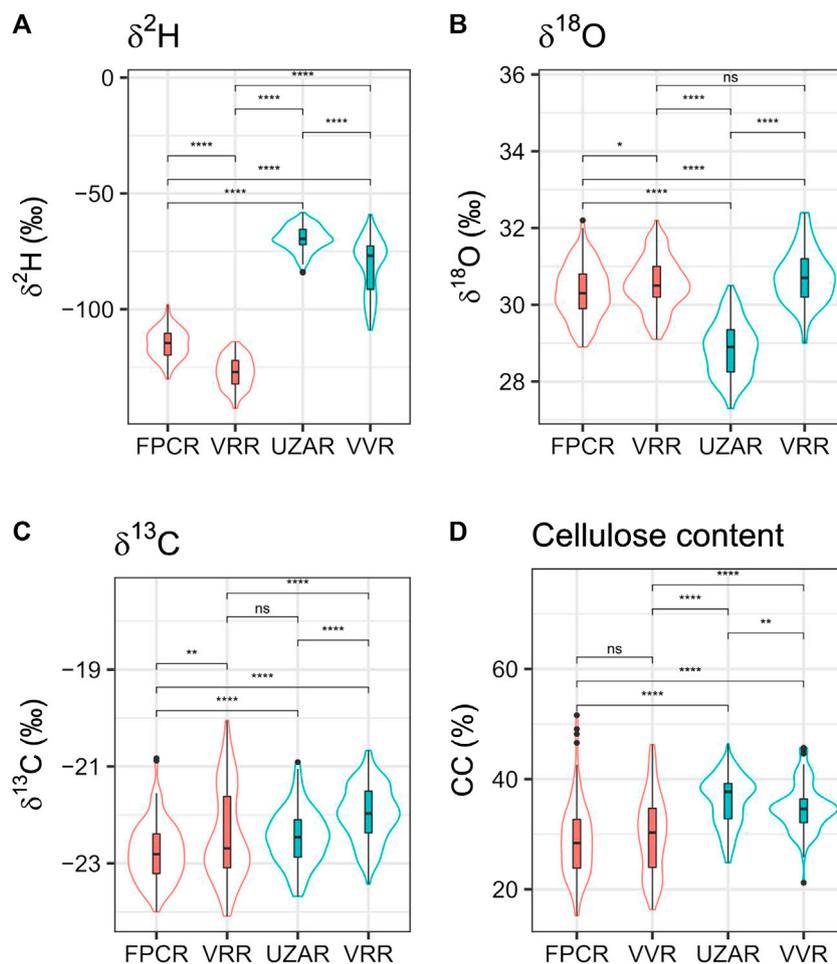
## Statistical Analysis

Statistical analysis was performed using the R software and was aimed at verifying differences between the groups. The type of data distribution was analyzed with the Shapiro-test (Shapiro and Francia 1972). To verify the equality between the groups we used pairwise comparisons by the Wilcoxon test (Wilcoxon et al., 1970). The density of the data is shown by the violin contours in **Figure 2** (Hintze and Nelson 1998).

## RESULTS

### Stable Isotopes and Cellulose Content in Living Conifers (1820–2010 A.D)

We first analyzed a subset of the samples of the project the Eastern Alpine Conifer Chronology for paleoclimatic studies (Nicolussi et al., 2009) represented by living trees grown in the last 190 years. They originate from eight larches and eight cembra pines, collected at three sites (VVR, FPCR and



**FIGURE 2** | Isotopes in the cellulose of living trees from Alpine sites. They are presented as boxplot with median and limits of 90 and 95 percentile, contoured by the violins plots that show the density distribution of the data used (Hintze and Nelson 1998). Mean values of  $\delta^2\text{H}$ ,  $\delta^{13}\text{C}$ ,  $\delta^{18}\text{O}$  and cellulose content of cembran pine and larch trees dated 1820–2010 A.D. and collected at three sites in the Alps: Valley d’Hérens (FPCR), Val Roseg (VRR) and Grimsensee (UZAR). The values of each site are based on four different trees whose wood has been sampled in blocks covering 5 years. At VRR both larch (red) and cembran pine (blue) were collected, while at FPCR and UZAR only one species was collected, respectively larch and cembran pine. The box plots show the median, SD and spread, the surrounding violin plot shows the sample density (Hintze and Nelson 1998). The statistical difference calculated with Wilcoxon test are indicated above the horizontal lines: ns, non-significant;  $p > 0.05$ , \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ , \*\*\*\* $p \leq 0.0001$ . LADE FPCR n samples: 132 n trees: 4LADE VRR: n sample s: 135 n trees: four PICE VRR n sample s: 125 n trees: 4PICE UAZR n sample s: 135 n trees: 4.

UZAR). The results, shown in **Figure 2**, are separated for species and for collection sites to illustrate and evaluate the effect of these two factors on isotope signatures.

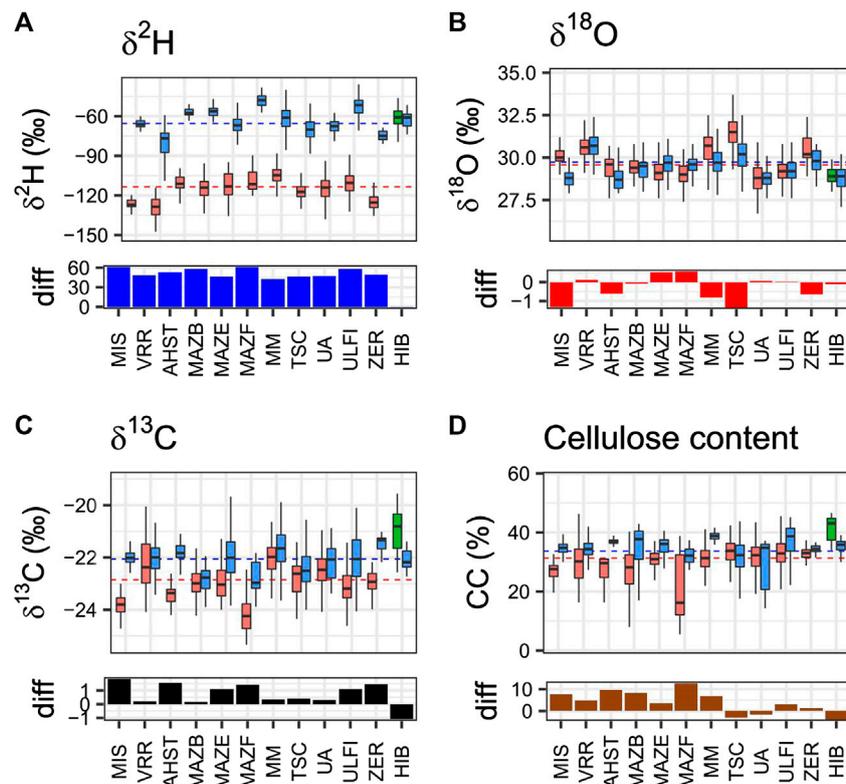
### $\delta^2\text{H}$ Data

The  $\delta^2\text{H}$  data (**Figure 2A**) show a major difference between larch and cembran pine irrespective of the collection site. The mean larch value (4 trees at each site) at VRR site was  $-127.2 \pm 6.6\text{‰}$  and at FPCR site was  $-114.9 \pm 6.3\text{‰}$ , while the mean cembran pine value at the UAZR site was  $-69.5 \pm 11.0\text{‰}$  and at VRR site  $-80.9 \pm 11.8\text{‰}$ . The VRR cembran pine group shows a tail in the lower part, which is due to a single tree with a mean value of  $-96.8\text{‰}$  that is more negative than the other three trees with mean values of  $-69.1$ ,  $-73.7$ ,  $-76.1\text{‰}$ . This made the distribution of the  $\delta^2\text{H}$  non-normal in the VRR cembran pine group, while it

was normal in the other three groups. The data indicate that the  $\delta^2\text{H}$  values follow mainly species type.

### $\delta^{18}\text{O}$ Data

The  $\delta^{18}\text{O}$  data (**Figure 2B**) show that the density distribution and the means of the larch at the FPCR and at the VRR sites are very similar and are also similar to those of the cembran pine at the VRR site, but significantly different from those of the cembran pine at the UZAR site. A Wilcoxon test indicated a marginally significant difference between two larch groups ( $p = 0.025$ ), contrarily, the difference between the two cembran pine groups was highly significant ( $p < 2 \times 10^{-16}$ ). The values of the cembran pine group from VRR were not significantly different from those of the larch from the same VRR site ( $p = 0.06$ ) but were significantly different from the larch values at the



**FIGURE 3** | Isotopes in the cellulose of sub-fossil Holocene trees. Tree samples were collected at 12 different sites in the Alps. Site abbreviations are indicated below the plots. Samples were dated by dendrochronological tools and the extracted cellulose analyzed for  $\delta^2\text{H}$ ,  $\delta^{18}\text{O}$  and  $\delta^{13}\text{C}$  isotope ratio variations and for their cellulose content. Each box represents median and limits of 25th and 75th percentile of each specie in each site, and the species are depicted in different colors: larch in red, cembran pine in blue and the single spruce in green. The horizontal black dashed line are the total mean values of cembran pine and larch. The histograms under the box plots show the per mil difference (diff) between the means, larch minus cembran pine (larch minus spruce for the HIB site). AHST: LADE n samples: 49 n trees: 2—PICE: n sample: 33 n trees: 1LADE: MAZB n sample: 185 n trees: 6—PICE n sample: 54 n trees: one LADE: MAZE n sample: 180 n trees: 5—PICE n sample: 187 n trees: eight LADE: MAZF n sample: 97 n trees: 2—PICE n sample: 31 n trees: one LADE: MIS n sample: 25 n trees: 1—PICE n sample: 55 n trees: one LADE: MM n sample: 282 n trees: 6—PICE n sample: 353 n trees: six LADE: TSC n sample: 249 n trees: 6—PICE n sample: 338 n trees: six LADE: UA n sample: 524 n trees: 17—PICE n sample: 70 n trees: two LADE: ULFI n sample: 1066 n trees: 23—PICE n sample: 152 n trees: four LADE: VRR n sample: 172 n trees: 4—PICE n sample: 140 n trees: four LADE: ZER n sample: 59 n trees: 1—PICE n sample: 17 n trees: one PCAB: HIB n sample: 45 n trees: 1—PICE n sample: 36 n trees: 1.

FPCR site ( $p = 6.4 \times 10^{-5}$ ). The data indicate that the  $\delta^{18}\text{O}$  values follow mainly the site and not the tree species.

### $\delta^{13}\text{C}$ Data

The  $\delta^{13}\text{C}$  values of larch tended to be more negative than those of cembran pine (Figure 2C). The dispersion of the larch group values at VRR site was larger than that of larch group at FPCR site, but the means are similar with no significant differences. Also, the cembran pine values at the two regions were not significantly different. The only non-significant difference (Wilcoxon test:  $p = 0.455$ ), although marginal, between the groups was observed for larch at VRR site and cembran pine at UZAR site. These values showed to be affected only to a minor extent by geography and species.

### Cellulose Content

The cellulose content values of the four groups are shown in Figure 2D. The value distribution is more dispersed in the larch groups than in the cembran pine groups, independent from the site origin. A non-significant difference is found with the

Wilcoxon test between the two larch groups ( $p = 0.133$ ). The cellulose did not seem to confer major information and was not considered in the following part of the study.

## Stable Isotopes of Subfossil Holocene Conifers

In order to verify whether the differences of living trees from Alpine sites shown above are not only due to the specific sites and restricted to the recent period, we extended the analysis to the complete wooden material of the Eastern Alpine Conifer Chronology (Nicolussi et al., 2009) collected at independent sites of the Alps, with the exclusion of the living trees of Figure 1. It consisted of >4000 wood samples from 120 trees (Table 1) and the sites of sample collection as listed in Figure 1.

### $\delta^2\text{H}$ Data

The  $\delta^2\text{H}$  values of the samples of the two species collected at the 12 sites are shown in Figure 3A as box-plots. It confirms an evident difference between the more negative values of the deciduous larch and the less negative values of the evergreen

cembran pine samples, with no overlap for samples collected at the same site. The total mean for larch is  $-113.4 \pm 9.7\text{‰}$  and that for cembran pine  $-61.4 \pm 11.4\text{‰}$ , values that are very close to those presented in **Figure 2**. An exception was the subfossil from spruce tree (*Picea abies*, PCAB) found at the HIB site the identity of which was initially unclear, since it is not easy to distinguish it from larch specimens by microscopic wood anatomy (Schoch et al., 2004). The  $\delta^2\text{H}$  values of these samples were in the range of those of the cembran pine (green in **Figure 3A**) and overlapped with those of the cembran pine that were collected at the same site, with no statistical difference ( $p = 0.74$ ) between the two. They are trees of different species and different time periods, altogether the differences between larch and cembran pine are always in the range 40 to 50‰, while that at HIB between cembran pine and spruce was close to 0‰.

### $\delta^{18}\text{O}$ Data

The  $\delta^{18}\text{O}$  values from each site are shown in **Figure 3B**. The total mean for larch is  $29.6 \pm 1.1\text{‰}$  and that for cembran pine  $28.9 \pm 0.5\text{‰}$ . A difference between the two species is not evident at sites VRR, HIB, MAZB, UA and ULFI. However, at the site ZER the two species showed a marginally significant difference (difference of the mean values of  $0.6\text{‰}$ ,  $p = 0.007$ ), whereas at the sites MIS, AHST, MAZE, MAZF, MM the differences were highly significant (difference of the mean values ok  $-1$  or  $0.6\text{‰}$   $p < 0.001$ ). Boxplots of the 12 sites partially overlap, MIS and TSC are the only sites for which we found a difference of the mean values larger than 1‰. In MAZE, MAZAF, UA, ULFI, VRR sites the mean of larch was smaller than for cembran pine, for the remaining sites it was higher. The differences between the two species are in the range of 0.6 to  $-1\text{‰}$  and do not show a common trend. The trees of some sites show values that are rather distant from the mean values and this is probably due to factors linked to the location (elevation, latitude, longitude, aspect) and others that influence the tree metabolism (e.g., soil composition), it can be noted that at sites where  $\delta^{18}\text{O}$  values are below total mean the corresponding  $\delta^2\text{H}$  are above or vice versa, suggesting that the two isotopes are subjected to distinct influences and that soil water is not the primary driver.

### $\delta^{13}\text{C}$ Data

The  $\delta^{13}\text{C}$  values of the trees are shown in **Figure 3C**. The total mean for larch is  $-22.6 \pm 0.8\text{‰}$  and that for cembran pine  $-22.2 \pm 0.6\text{‰}$ . The difference of the values between the two species is significant at all sites with the exception of MAZB site ( $p = 0.05$ ) and of UA ( $p = 0.001$ ). The dotted line indicates the total mean value and most of the cembran pine values (blue boxplots) are above or close to the mean value, while the larch values (red boxplots) are below or close to it. In fact, for the sites where both species are present, the larch is always more depleted than the cembran pine. The difference plot shows that the only negative column is at the site HIB, spruce shows significantly less depleted values than cembran pine, while the oxygen values are similar. This might be due to some characteristics of the site, like the aspect, that are known to affect temperature-controlled changes in assimilation regulated by enzymatic isotopic fractionation processes ( $\delta^{13}\text{C}$ ) (Esper et al., 2018).

## Stable Isotopes in Contemporary Conifers

The above data showed that larch has  $\delta^2\text{H}$  values of cellulose that are much lower than those of cembran pine and spruce (**Figures 2,3**). In order to verify if this difference is attributed to the metabolism of the evergreen conifers versus that of deciduous larch, we looked for a larger number of conifer species. The Bern Botanic Garden provided us with samples of six different species of larch (deciduous), one pseudo-larch (deciduous), and 13 different species of evergreen conifers including pinus, taxus and cedrus, and the gymnosperm, broadleaf, deciduous *Ginkgo Biloba*. We processed the samples and analysed their isotope content as describe above. **Figure 4** shows the results, in red are the deciduous conifers (larch and pseudo-larch), in green the evergreen conifers and in blue the values of *Ginkgo Biloba*, the horizontal lines are the mean value of each group and the dashed lines shows the interval of the standard deviation.

### $\delta^2\text{H}$

The  $\delta^2\text{H}$  values of the deciduous larch group (mean  $-91.8 \pm 7.6\text{‰}$ ) is well separated from that of evergreen conifers (mean  $-51.1 \pm 12.3\text{‰}$ ) (**Figure 4**). This confirms that the deciduous conifer trees are strongly depleted in comparison with the evergreen ones with no overlap between the two groups and a highly significant difference ( $p = 1.92 \times 10^{-5}$ ). The values of *Pseudolarix amabilis* (tree #7 in **Figure 4**) are in full agreement with those of the other larches (#1–6). While the values of the *Cedrus atlantica* (#8), *Cupressus nootkatensis* (#10) and *Taxus baccata* (#19) are in agreement with those of the *Pinus* (#11–18), which are all evergreen conifers. *Ginkgo biloba* (#21) does not belong to the conifer order and is deciduous, but its values are close to those of the evergreen conifers.

### $\delta^{18}\text{O}$

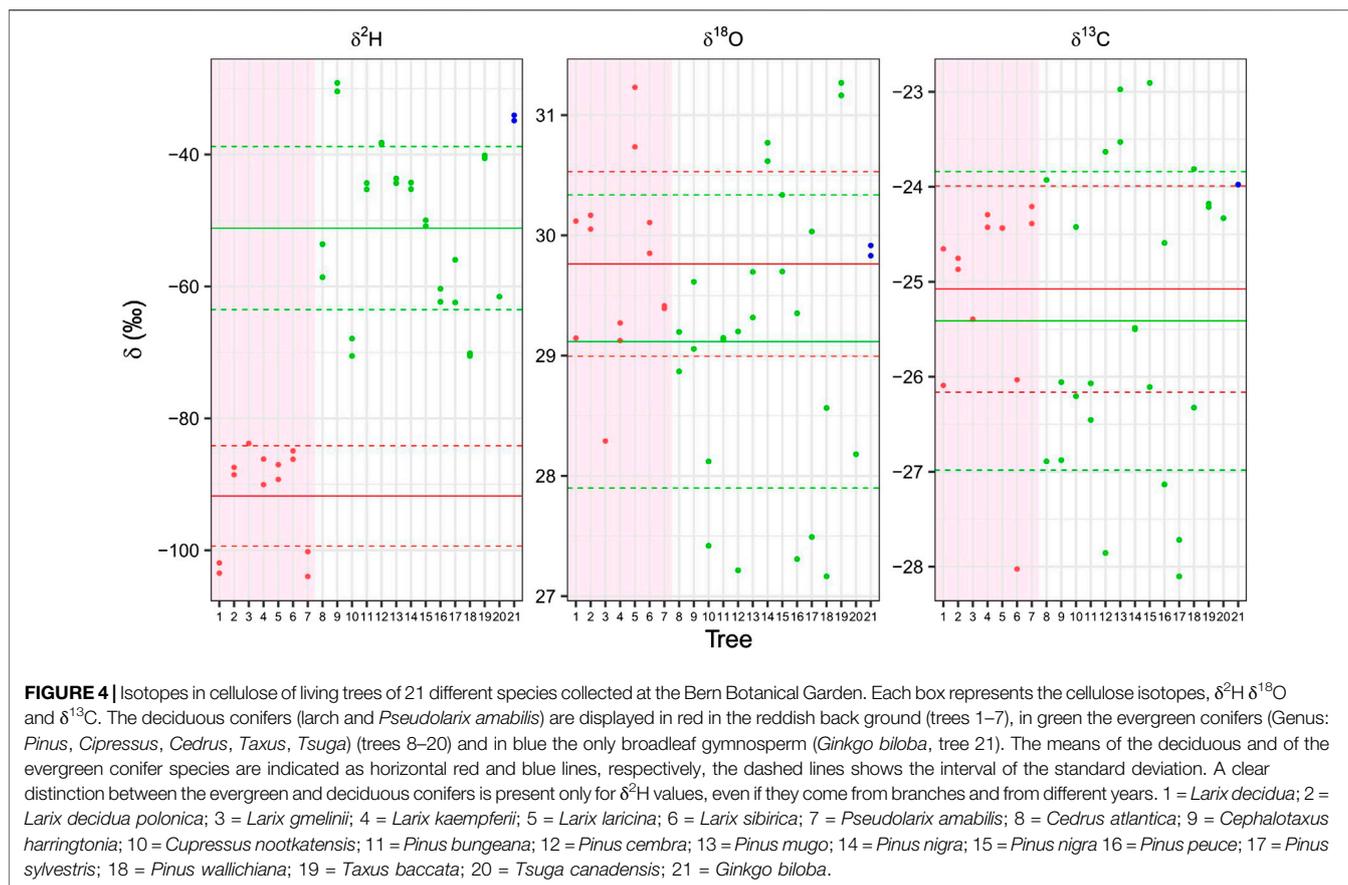
The difference between the  $\delta^{18}\text{O}$  value of the deciduous conifers ( $29.8 \pm 0.8\text{‰}$ ) and that of the evergreen conifers ( $29.1 \pm 1.2\text{‰}$ ) was not statistically significant (**Figure 4**). The distance between the two means was barely significant ( $p = 0.084$ ). The absolute  $\delta^{18}\text{O}$  and  $\delta^2\text{H}$  values of samples from the botanic garden conifers are less depleted from the ones of the of the Alpine trees, this was attributed to higher depletion of the heavy isotopes of hydrogen and oxygen of source water at high elevations (Kern et al., 2014).

### $\delta^{13}\text{C}$

Mean  $\delta^{13}\text{C}$  values show no statistically significant difference between the deciduous ( $-25.1 \pm 1\text{‰}$ ) and the evergreen groups ( $-25.4 \pm 1.5\text{‰}$ ) (**Figure 4**).

## DISCUSSION

A major finding of present work is the much higher depletion of deuterium in the cellulose of the deciduous larches compared to the evergreen conifer cembran pine growing at the same site. The  $\delta^2\text{H}$  values of larch that we found are in good agreement with the only previous measurement of the hydrogen isotope in larch cellulose (Hafner et al., 2011). The difference between  $\delta^2\text{H}$  of larch and cembran pine has been observed in eight trees covering the



last 190 years, grown in Val Roseg valley, Grimselstausee and Val d'Herens valley (**Figure 2**) and the 120 trees covering the whole Holocene (**Figure 3**). The difference is large, with a  $\delta^2\text{H}$  mean of  $-127\text{‰}$  for larch and  $-81\text{‰}$  for the cembran pine and no overlapping values. This is a non-climatic signal and was not observed in the other two stable isotopes we analyzed,  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$ , the values of which were similar in the two conifer species.

All but one of the trees of the Eastern Alpine Conifer Chronology were larch and cembran pine, and thus it was unclear whether the observed differences were specific to the two species or caused by different physiological processes of evergreen and deciduous conifers. The absence of difference between cembran pine and *Picea abies* in the site HIB is surprising as they are of different species and originate from different time periods, the cembran pine covered the period 5695–5516 B2K, some 1500 years before the spruce tree that covered the 4000–3766 B2K period. Thus, we measured conifer samples from the Bern Botanical Garden and the results showed that the cellulose of all the larch species including the *Pseudo-larix amabilis* had more depleted  $\delta^2\text{H}$  values than that of the evergreen conifers (**Figure 4**). The deciduous non-conifer *Ginkgo biloba* resulted to have  $\delta^2\text{H}$  value in the range of the evergreen ones. The range of  $\delta^2\text{H}$  values of the evergreen conifers ( $-30$  to  $-70\text{‰}$ ) is similar to the ones reported in deciduous broadleaf trees such as oak ( $-75$  and  $-45\text{‰}$ ) (Szczechpanek et al., 2006; Etien et al., 2009), and in the conifers *Pinus sylvestris* (Gori et al., 2013) and spruce

( $-20$  and  $-50\text{‰}$ ) in three regions that cover a vast geographical area with different conditions (Loader et al., 2008). These values are quite distinct from the  $\delta^2\text{H}$  values we found in the larch family with a mean of  $-120\text{‰}$  and range  $-150$  to  $-86\text{‰}$ . Thus, all the deciduous conifers (larch and pseudo larches), present  $\delta^2\text{H}$  values that are very different from those of the evergreen conifers. This can be used for the identification of larch from spruce wood that have similar wood anatomic characteristics and are therefore very difficult to distinguish in subfossil specimens (Schoch et al., 2004). Moreover, our data show that for paleoclimatic use of isotopic dendrochronology the  $\delta^2\text{H}$  values of larch cellulose must not mixed with those of evergreen conifers, but can be used separately or after applying suitable corrections or scaling.

The major  $\delta^2\text{H}$  difference indicates that deciduous and evergreen conifer handle hydrogen in a different way, while the similar  $\delta^{18}\text{O}$  values indicate that they handle oxygen in the same way. The soil water is the source of the two elements and their isotopes, and its uptake by the roots does not cause fractionation of both isotope compositions (White et al., 1985; Ehleringer and Dawson 1992; Dawson and Ehleringer 1993; Lin and Sternberg 1993). Evapotranspiration in the leaves causes enrichment in similar extent of the two heavy isotopes (Flanagan et al., 1991; Yakir 1992; McCarroll and Loader 2004; Studer et al., 2015), thus we can conclude that this does not cause the difference of  $\delta^2\text{H}$  between the two species but leaving the  $\delta^{18}\text{O}$  in the two species unchanged (**Figure 2**). The difference will

occur in some subsequent biological steps, likely at carboxylation or post-carboxylation levels that might differ in deciduous and evergreen conifers.

Deciduous larches are characterized by a short photosynthesis period that involves a higher photosynthesis capacity to compete with the surrounding evergreen trees (Tranquillini 1962; Tranquillini 1979). Consequently, they have faster rates of photosynthesis and net assimilation and a more efficient carboxylation system than evergreen conifers (Sweet and Wareing 1968; Larcher 1969; Fry and Phillips 1977; Gowin et al., 1980). The biochemical steps that amplify  $\delta^2\text{H}$  depletion when photosynthetic activity is high have been identified (Cormier et al., 2018): when the supply of photosynthetic carbohydrates is low, the produced organic compounds are relatively  $^2\text{H}$ -enriched (Cormier et al., 2018), when the photosynthetic activity is high the produced organic compounds are relatively  $^2\text{H}$ -depleted (Yakir 1992). Moreover, larch is known to have a lower heterotrophic metabolism than the surrounding evergreen conifers (Vaganov et al., 2009; Rinne et al., 2015).

In cembran pine heterotrophism increase with the altitude (Wieser et al., 2019). The finding that the  $\delta^2\text{H}$  difference between larches and evergreen conifers is lower in Bern (~500 m elevation) than at >2000 m of tree-line (40‰ in **Figure 4** vs 52‰ in **Figure 3**) support the hypothesis that heterotrophism affect  $\delta^2\text{H}$  but the difference between the two metabolisms is maintained.

Regarding the  $\delta^{13}\text{C}$  values they were all within the range of  $-23\text{‰}$  to  $-28\text{‰}$ , as previously described in C3 plants (Sternberg and DeNiro 1983; Leuenberger 1998). This value in leaf sugar is controlled mainly by the ratio  $c_i/c_a$  ( $c_i$  being the  $\text{CO}_2$  concentration in the intercellular space of leaves and  $c_a$  the  $\text{CO}_2$  concentration of the atmosphere), which is related to photosynthetic water-use efficiency, WUE, (also called transpiration efficiency) (Farquhar and Sharkey 1982). In our results  $\delta^{13}\text{C}$  values of larch were consistently slightly more negative than those of cembran pine (**Figure 3**), in agreement with previous data showing that larch species have more negative carbon values than the evergreen conifers because of their lower water-use efficiency (WUE) (Gower and Richards, 1990; Kloeppel et al., 1998). Altogether the results indicate that neither the species nor geography have major effect on  $\delta^{13}\text{C}$  signature.

Regarding the  $\delta^{18}\text{O}$  values we found no evident difference between the two species, in accordance with the interpretation that these values are more sensitive to the source water signal than to other external variables, such as relative humidity and vapor pressure deficit. We found a  $\delta^{18}\text{O}$  difference between the sites in the Swiss and the Tyrolian Alps, likely due to the source water, the  $\delta^{18}\text{O}$  of which is known to be linked to the altitude and to a continental effect (Rozanski et al., 1993). This indicates that  $\delta^{18}\text{O}$  values of different conifers species can be used together for paleoclimatic studies, after taking into account the geographical effects.

## CONCLUSION

We show that the deciduous larch family of trees presents unique highly negative  $\delta^2\text{H}$  values compared to evergreen conifers. We

attributed the difference to a higher photosynthetic and metabolic activity of larch that drive a reduced exchange of  $\delta^2\text{H}$  sucrose with the surrounding enriched leaf water leading to  $\delta^2\text{H}$  depletion of the photosynthetic products, in comparison with cembran pine and more in general with the evergreen conifers. No significant differences of  $\delta^{18}\text{O}$  values were found between trees grown at the same site and under similar conditions, but differences were found among trees grown at different sites. Minor more negative  $\delta^{13}\text{C}$  values of larch were consistently found than those of the cembran pine, but no evident driver of the difference could be identified. We also conclude that for paleoclimatic studies, for  $\delta^2\text{H}$  values the evergreen and deciduous conifers should be kept separately or normalized for the tree species, while  $\delta^{18}\text{O}$  does not seem to be affected by tree species, but by the source water of each site. Regarding  $\delta^{13}\text{C}$  we do not have yet a clear driver of the difference in carbon isotope. We suggest the determination of the  $\delta^2\text{H}$  values as tool for distinguishing larch samples from those of other conifers, such as spruce, which are difficult to identify in subfossil wood.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

## AUTHOR CONTRIBUTIONS

TA and MZ performed the stable isotope analyses, TA drafted the first version of the manuscript. KN collected the samples and made the cross dating. ML contributed to the evaluation of the results. ML, KN, and CS conceived of the presented idea. All authors provided comments to improve the manuscript.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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