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Carbon, hydrogen and oxygen stable isotope ratios of whole wood, cellulose and lignin methoxyl groups of *Picea abies* as climate proxies

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**RATIONALE:** Carbon, hydrogen and oxygen (C, H and O) stable isotope ratios of whole wood and components are commonly used as paleoclimate proxies. In this work we consider eight different proxies in order to discover the most suitable wood component and stable isotope ratio to provide the strongest climate signal in *Picea abies* in a southeastern Alpine region (Trentino, Italy).

**METHODS:** $\delta^{13}C$, $\delta^{18}O$ and $\delta^2H$ values in whole wood and cellulose, and $\delta^{13}C$ and $\delta^2H$ values in lignin methoxyl groups were measured. Analysis was performed using an Isotopic Ratio Mass Spectrometer coupled with an Elemental Analyser for measuring $^{12}C/^{13}C$ and a Pyrolyser for measuring $^2H/^1H$ and $^{18}O/^{16}O$. The data were evaluated by Principal Component Analysis, and a simple Pearson’s correlation between isotope chronologies and climatic features, and multiple linear regression were performed to evaluate the data.

**RESULTS:** Each stable isotope ratio in cellulose and lignin methoxyl differs significantly from the same stable isotope ratio in whole wood, the values begin higher in cellulose and lignin except for the lignin $\delta^2H$ values. Significant correlations were found between wood and the cellulose fractions for each isotope ratio. Overall, the highest correlations with temperature were found with the $\delta^{18}O$ and $\delta^2H$ values in whole wood, whereas no significant correlations were found between isotope proxies and precipitation.

**CONCLUSIONS:** $\delta^{18}O$ and $\delta^2H$ values in whole wood provide the best temperature signals in *Picea abies* in the northern Italian study area. Extraction of cellulose and lignin and analysis of other isotopic ratios do not seem to be necessary.

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extractation to be necessary only for some tree species, since stable isotope ratios differ according to site features and tree species.

A further problem with cellulose is that some of the hydrogen atoms undergo exchange with xylem water and then with the atmosphere. Most investigations have therefore focused on nitrocellulose, which contains only non-exchangeable hydrogen, although the extraction and preparation method is even more time-consuming and labour-intensive. The development of any new methods to simplify sample preparation would therefore be of great help.

Recently, it was suggested that stable hydrogen isotope values of lignin methoxyl groups in tree rings might be a source of climate information and could serve as a paleoclimate proxy. Conspicuous $^2$H depletion of wood methoxyl groups relative to source water has been observed with a uniform isotopic fractionation (mean $-216 \pm 19\%$) recorded with respect to meteoric water over a range of $^{13}C$ values from +20 to $-110\%$, indicating that the methoxyl groups reflect the isotope values of the meteoric source water of woody plants. Lignin methoxyl groups are considered to be stable, i.e. the hydrogen atoms of the methoxyl moiety do not exchange with those of plant water during metabolic reactions in the plant. Thus, the initial $^{13}C$ value of the methoxyl groups of lignin in woody tissue at formation is retained throughout the lifetime of the tree and in preserved tissue.

The main aim of this study is to develop a simple model to accurately estimate climate proxy information from *Picea abies* (L.) Karst. in the south-eastern Alps. We used a series of $^{13}C$, $^{18}O$ and $^{2}H$ values of bulk wood and two wood constituents (cellulose and methoxyl groups of lignin) in order to find the most suitable combination of wood component and stable isotope ratio for obtaining the strongest climate signal. The originality of our study lies mainly in the simultaneous analysis of eight different proxies in ten trees at three different sites with annual resolution. We consider this multi-proxy to be a powerful technique for extracting climatic information from tree-ring isotope chronologies.

Our main questions are:

(1) Is wood component extraction necessary for climate reconstruction from *P. abies* in the south-eastern Alps and, if so, what is the best wood component?

(2) How strong are the various climate proxy signals?

(3) Can the proxies be combined to build a better model for estimating climate?

### EXPERIMENTAL

**Samples, field sampling and dendrochronological methods**

Field sampling was carried out on mature Norway spruce stands at three different elevations in the south-eastern Alps: Baselga (900 m a.s.l.; 46°4’48”N, 11°3’33”E), Val Maggiore (1300 m a.s.l; 46°17’32”N, 11°36’49”E) and Cermis (1900 m a.s.l; 46°15’0”N, 11°29’43”E).

The climate features are typical of the southern alpine region with cold winters, mild summers and frequent precipitation mostly concentrated in the summer months (June to September). The climate is cold temperate and the vegetation season depends on elevation and date of snow melt, varying between 120 days (at the Cermis stand) and 150 days (at the Baselga stand).

All the sites are located on slopes ranging from 30% to 50%, have a northern aspect, and are within a distance of 25 km from each other (Fig. 1). The bedrock is porphyric granite, partially covered with morainic material and podzolic soils. The three stands are dense and homogeneous. All the forests within which they lie are characterised by several large, even-aged, homogeneous group of trees.

A total of ten mature *P. abies* trees were selected at each site. Care was taken to select only dominant trees growing in similar light and soil moisture conditions in plots measuring 100 m x 100 m. Four cores were extracted at 90° from each other using a 0.5 cm diameter Pressler increment borer; the pith was reached in at least one of the four cores. To prevent isotopic contamination, no lubricants, markers or sandpaper were used during field sampling and dendrochronological measurements.

The cores of all trees were prepared with a core-microtome and standard dendrochronological methods were used to cross date. Ring-width measurements on each core were made to the nearest 0.01 mm using LINTAB measuring equipment (Frank Rinn, Heidelberg, Germany). Raw Tree-Ring-Widths (TRW) of all curves were plotted, cross-dated visually and then cross-dated statistically by: (a) Gleichläufigkeit, i.e. the percentage agreement in the signs of first-differences of two time series; and (b) a t-test, which determines the degree of correlation between the curves. Cross-dating quality was checked using the COFECHA programme as a standard dendrochronological protocol for quality control.

Average tree ages at the sites were over 100 years at Baselga, 150 years in Val Maggiore and 230 years at Cermis.

**Sample preparation**

After being dated, all the cores were prepared for isotopic analysis. Although some authors argue that latewood gives the strongest climatic signal, others have found good signals using the whole tree ring, especially with conifer. As the *P. abies* growing at Cermis presented very narrow rings with indistinguishable latewood bands, whole annual ring was used for all wood samples. Annual rings from each core were carefully separated with a razor under a binocular microscope at the early and late-wood border. To minimise the juvenile effect observed in young trees, only rings formed during the mature growth phase were analysed (e.g. after 60 years at the high elevation site at Cermis, after 40 years at medium elevation in Val Maggiore, and after 30 years at low elevation at Baselga).

Since isotopic signals vary around the circumference of the tree, we pooled the ring samples from the four radii. Finally, all rings from a given year at each site (ten sampled trees per site) were pooled to reduce isotope variability between trees. Care was taken to use the same dry weight in order to prevent the isotopic value being biased by the relative mass contribution of the tree rings (larger tree rings affect isotope values more than small ones). Overall, we separated 150 tree rings at Cermis (from 1860 to 2009), 110 in Val Maggiore (from 1900 to 2009) and 75 at Baselga (from 1935 to 2009).
Dried samples of whole wood were ground (<0.1 mm) in a centrifugal mill (ZM200, Retsch GmbH, Hann, Germany) and all wood samples were split into three subsamples. The first group of subsamples did not undergo any treatment (whole wood); the second was used for cellulose extraction, and the third for lignin methoxyl group extraction. Cellulose was extracted according to a slightly modified version of the method described by Loader and co-authors. In brief, the samples were washed twice in 5% NaOH solution for 2 h at 60°C in order to remove fats, oils, resins and hemicellulose. In a second step, the lignin was removed with a 7% NaClO2 solution for a minimum of 36 h at 60°C. Since this solution is only reactive for about 10 h, it was changed and refilled daily as necessary. After each treatment the samples were washed three times with boiling distilled water and dried for 48 h at 60°C. No further preparation was needed to measure the stable carbon and hydrogen isotope values of the lignin methoxyl groups. The dried and ground wood samples were analysed directly using the HI method as described below and by Keppler et al. and Greule et al.

**Isotope analysis**

Overall, we analysed eight isotopic proxies for each sample: $\delta^{13}C$, $\delta^{18}O$ and $\delta^2H$ values in whole wood, $\delta^{15}N$, $\delta^{18}O$ and $\delta^2H$ values in cellulose and $\delta^{13}C$ and $\delta^2H$ values in lignin methoxyl groups. No analysis of $\delta^2H$ values in lignin methoxyl groups for the years 1900–1932 in Val Maggiore was performed due to the small amount of wood material. Sub-samples of 0.3–0.35 mg of cellulose and whole wood were weighed and placed in tin capsules for $\delta^{13}C$ analysis and in silver capsules for $\delta^{18}O$ and $\delta^2H$ analysis. All the samples were then oven-dried (80°C) to remove water vapour, and stored in a desiccator until analysis. The analysis was performed using Isotope Ratio Mass Spectrometry (IRMS) with a Finnigan DELTA XP isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany) coupled with an Elemental Analyser (Flash EA1112, Thermo Scientific) to measure $^{13}C/^{12}C$ ratios, and a Pyrolyser (Finnigan™ TC/EA, High-Temperature Conversion Elemental Analyser, Thermo Scientific) to measure $^2H/^1H$ and $^{18}O/^{16}O$ ratios. To separate the gases, the EA was fitted with a Porapack Q GC column (3 m; 6 × 4 mm, o.d./i.d.) and the Pyrolyser with a molecular sieve 5 Å (0.6 m) GC column. The devices were interfaced with the isotope ratio mass spectrometer through a dilutor (ConFlo III, Thermo Scientific) for dosing the sample and reference gases, and fitted with an autosampler (Finnigan AS 200, Thermo Scientific). An appropriate cover on the pyrolyser autosampler allowed continuous flushing with nitrogen to guarantee dryness of the samples during measurement.

For $^{12}C/^{13}C$ measurements, the tin capsules were inserted into the EA where they were quantitatively burnt to CO2 and H2O (H2O removed using Mg(ClO4)2 filter in the presence of O2 and CuO). The CO2 was flushed into the mass spectrometer through the ConFlo III device, where the content of the different isotopomers ($m/z$ 44 and 45) was determined. For $^{18}O/^{16}O$ and $^2H/^1H$ analysis, the capsules were dropped into the pyrolyser tube where at a temperature of 1450°C the oxygen and hydrogen atoms of the sample were quantitatively converted into CO and H2, respectively, at a temperature of 1450°C, then flushed into the mass spectrometer. The isotope ratio mass spectrometer first measured
2H/H and then, following the magnet jump, 18O/16O. The H3 factor, which allows correction of the contribution of [H3]+ to the m/z 3 signal,[48] was checked and found to be lower than 9. The isotopic ratio values are expressed in δ‰ (= [(Rsample − Rstandard)/Rstandard] * 1000, where R is the ratio between the heavier isotope and the lighter one) against international standards (Vienna-Pee Dee Belemnite (V-PDB) for δ13C values, and Vienna-Standard Mean Ocean Water (V-SMOW) for δ2H and δ18O values). To calculate the δ‰ values, working in-house standards for proteins were used calibrated against international reference materials: L-glutamic acid USGS 40 (IAEA-CH-3, IAEA-CH-4, and IAEA-CH-6 (IAEA) for 13C/12C; and benzoic acid (IAEA-601) for 18O/16O in casein.

The δ2H/1H values of whole wood and cellulose were corrected against an internal standard with an assigned value of δ2H (−113‰), according to the comparative equilibration technique.[41]

The hydrogen and carbon isotope signatures of lignin methoxyl groups were measured using CH3I released following treatment of the dried and ground wood samples with HI, as previously described by Greule and co-authors (for δ2H measurements, see,[25] for δ13C measurements, see[26]). In brief, hydriodic acid (0.5 mL, 55-58%) was added to the sample in a crimp caps containing PTFE-lined butyl septa (thickness 0.9 mm) and incubated for 30 min at 130°C. After being heated, the vials were allowed to equilibrate at room-temperature (22 ± 0.5°C, air-conditioned room) for at least 30 min before a headspace sample (10–90 μL) was removed and directly injected directly into the gas chromatography–combustion/thermal conversion–isotope ratio mass spectrometry (GC-C/TC-IRMS) system.

To determine the δ13C and δ2H values we used an HP 6890N gas chromatograph (Agilent, Santa Clara, CA, USA) equipped with an A2005 autosampler (CTC Analytics, Zwingen, Switzerland), coupled to a DeltaS XP isotope ratio mass spectrometer (ThermoQuest Finnigan, Bremen, Germany) via an oxidation reactor (for δ13C values) [ceramic tube (Al2O3), length 320 mm, 0.5 mm i.d., with Cu/ Ni/Pt wires inside (activated by oxygen), reactor temperature 960°C] or via a pyrolysis reactor (for δ2H values) [ceramic tube (Al2O3), length 320 mm, 0.5 mm i.d., reactor temperature 1450°C] and a GC Combustion III interface (ThermoQuest Finnigan). The gas chromatograph was fitted with a ZB-5 ms capillary column (Phenomenex, Torrance, CA, USA) (30 m × 0.25 mm i.d., δf 1.0 mm). The GC conditions for δ13C analysis were: split injection (split ratio 10:1), injector temperature 200°C; initial oven temperature at 30°C for 3.8 min, ramp at 30°C/min to 100°C. The carrier gas was helium at a flow rate of 1.8 mL/min.

The GC conditions for δ2H analysis were as follows: split injection (split ratio 4:1), injector temperature 200°C; initial oven temperature 30°C for 7 min, ramp at 40°C/min to 120°C with helium carrier gas at a constant flow rate of 0.6 mL/min. All the δ13C and δ2H values were normalised relative to V-PDB or V-SMOW using a CH3I standard. The δ13C value of CH3I was calibrated against international reference substances (IAEA-CH-4, IAEA-CH-7, NBS-22) using an online elemental analyser (EA)/IRMS system (IsoAnalytical Ltd., Sandbach, UK). The calibrated δ13C value vs. V-PDB for CH3I was −69.27 ± 0.05‰ (n = 15, 1σ).

The δ2H value of CH3I was also calibrated against international reference substances (IA-E002, IAEA-CH-7, NBS-22) using the EA/IRMS system described above. The calibrated δ2H value vs. V-SMOW was −179.0 ± 2.9‰ (n = 15, 1σ).[24] The standard was measured after every fourth sample injection.

The analytical precision (1 standard deviation) was <0.2‰ for δ13C values, 0.4‰ for δ18O values, and <2‰ for δ2H values.

### Atmospheric CO2 correction

Because fossil fuels are depleted in 13C, anthropogenic increases in CO2 concentrations in the atmosphere have lowered the δ13C values of air by about 1.5‰. Since fractionation is an additive process, this trend is reflected in most of the tree-ring δ13C series. The motive behind adjusting data for climate research is to remove this noise without weakening the climate signal, since these trends are unrelated to past climates.[4]

In our study, all the tree-ring δ13C series measurements were corrected to preindustrial atmospheric δ13C values by simple addition using data provided by Francey et al.[4,42] Since we pooled the tree rings formed during the mature growth phase, no other data treatment was necessary.[54,55]

### Meteorological data

Meteorological data were supplied by two weather stations located near the sites: (a) Paneveggio weather station: 1415 m a.s.l; 46° 18′33″N, 11° 44′50″E, about 5 km from the Val Maggiore stand and 15 km from the Cermis stand; and (b) Trento Laste weather station: 312 m a.s.l.; 46° 04′19″N, 11° 08′8″E, about 6 km from the Baselga stand[60] (Fig. 1). Homogenised and corrected climate data from 1958 onwards for temperature and precipitation obtained from these two weather stations[43] were used to remove undesired effects (e.g. long-term error or changes in metering practices) and to increase the strength of the climate signal.

Finally, to check for potential climatic divergence we compared the homogenised temperature data from the two weather stations with the high-resolution MODIS LST (Moderate Resolution Imaging Spectroradiometer Land Surface Temperature). MODIS LST data have been collected since 2002 and have been further developed by Neteler.[44,45] This dataset provides very accurate daily minimum and maximum temperatures in a GIS (Geographic Information System) format with a high resolution of 200 m pixels. Since the recorded temperature data were consistent between the two datasets (r = 0.80 at Cermeis; r = 0.83 at Val Maggiore; r = 0.79 at Baselga; p < 0.05) we assumed the homogenised temperatures from the two weather stations to be representative of our three stands.

For the study, we used mean monthly, seasonal (winter: 21 December to 20 March; spring: 21 March to 20 June; summer: 21 June to 20 September; autumn: 21 September to 20 December) and annual temperature and precipitation at each site during the 1958-2009 period.

During the study period, the mean annual precipitation was 1196 mm at the Paneveggio weather station and 931 mm at Trento Laste, while the mean annual temperatures were 6°C and 12°C, respectively. The coolest and warmest months were January and July, with mean minimum temperatures of
−7°C and −3°C at the Paneveggio and Trento Laste weather stations, respectively, and mean maximum temperatures of 25°C and 29°C.

Statistical analysis

Descriptive statistics and stable isotope time series were carried out for all the data: Cermis from 1860 to 2009; Val Maggiore from 1900 to 2009 (except for the δ2H values of the lignin methoxyl group); and Baselga from 1936 to 2009. The Pearson’s correlation coefficient (Cermis from 1860 to 2009; Val Maggiore from 1933 to 2009; and Baselga from 1936 to 2009) was calculated and used as the basis for exploring the relationships between the stable isotope chronologies.

The reliability of the isotopic parameters as climate proxies was only tested against the 1958–2009 period. Principal component analysis (PCA) was performed as a clustering technique to identify isotope values with similar patterns and to determine whether and how isotope values from the same wood component are related. PCA was performed on mean-centred and autoscaled data.

A simple Pearson’s correlation coefficient was calculated to determine the relationships between the isotope chronologies and climatic features, taking mean monthly, seasonal and annual temperatures and precipitation into account.

Multiple linear regression models were used to predict mean annual temperature. Six different models were built to discover which wood component (whole wood, cellulose, lignin methoxyl groups) best explains climate variation. Model parameters were the three isotope chronologies (δ13C, δ18O and δ2H values) and the three different wood components. The models were: (1) the full model, with all eight isotope proxies in all three wood components (ModelWLC); (2) a model excluding cellulose, with five isotope proxies in whole wood and lignin methoxyl fraction (ModelWL); (3) a model excluding lignin methoxyl, with six isotope proxies in whole wood and cellulose fraction (ModelWC); (4) only the three isotope proxies in whole wood (ModelW); (5) only the three isotope proxies in cellulose fraction (ModelC); and (6) only the two isotope proxies in lignin methoxyl (ModelL). An ANOVA (analysis of variance) was performed to compare ModelWLC with the other simpler models (i.e. ModelWL, ModelWC, ModelW, ModelC and ModelL) in order to identify the simplest efficient model.

Finally, in order to obtain the minimal adequate model, a further simplification was made by reducing the isotopic variable from the selected models by means of step-wise variable selection (p < 0.01). All statistical analyses were carried out with the R 2.11.0 programme.[46]

RESULTS AND DISCUSSION

Stable isotope chronologies

The time series of each isotope parameter for each of the three sites are shown in Fig. 2. The ANOVA showed that, for each isotope, the mean stable isotope ratio in extracted cellulose and lignin methoxyl groups differed significantly (p < 0.001) from that in whole wood. With the exception of the most recent period at Baselga, the δ13C values in extracted cellulose (δ13CC) and lignin methoxyl (δ13CL) were higher than the values in whole wood (δ13CW). On average, the δ13C values for whole wood were about 1.4‰ lower than those for cellulose at all three sites. Similar results have been reported by other authors.[16,47]
The $^18$O values in cellulose ($^18\text{O}_{\text{C}}$) were generally higher than those reported for whole wood ($^18\text{O}_{\text{W}}$), by an average of 3.9% at Cermis, 4.0% in Val Maggiore and 3.30% at Baselga. These results are consistent with the differences ranging from ca. 3% to 5.21%, reported by other researchers. The $^2$H values in lignin methoxyl (2$^\text{H}_1$) are always lower than in wood (2$^\text{H}_0$) and cellulose ($^2\text{H}_2$), the mean values begin at ~30% at Cermis and Baselga and ~308% in Val Maggiore. The highest values are found for cellulose fraction ($^2\text{H}_2$). These findings are consistent with previous results, where the $^2\text{H}_1$ values ranged from ~325% to ~153% and the $^2\text{H}_2$ values from 141% to ~296%. The differences between the stable isotope values for cellulose and lignin and those in whole wood are a consequence of the isotopic fractionations involved in the pathways that lead to their biosynthesis.

The relationship between the stable isotope ratio of each bioelement in whole wood and in the two subsfractions across the three sites was explored by means of the Pearson’s correlation coefficient. Table 1 summarises these results. The strongest correlations are between whole wood and the cellulose fraction for each isotope ratio. In agreement with previous studies, we found that the $^{13}$C values in whole wood were highly positively correlated with the $^{13}$C values in the cellulose fraction, suggesting similar sources of variability for these proxies. The $^{13}$C values in lignin methoxyl groups correlate to a lesser extent with $^{13}$C values in cellulose and $^{13}$C values in whole wood.

For the stable oxygen isotope ratios, the $^18\text{O}_{\text{W}}$ and $^18\text{O}_{\text{C}}$ values were highly ($p < 0.001$) correlated in Val Maggiore ($r = 0.75$) and at Baselga ($r = 0.67$) but less so at Cermis ($r = 0.67$), the correlation coefficients being much higher than those found by other authors. Our results agree with data published by Barbour and co-authors and Sidorova and co-authors. They reported a significant positive relationship between Pinus and Quercus ($r = 0.89$, $p < 0.001$) and Larix cajanderi Mayr ($0.63$, $p < 0.05$, during the 1904–2004 period), suggesting that similar climatic information is stored in these different wood components. The coefficients of correlation between the $^2\text{H}_1$ and $^2\text{H}_2$ values were always positive, and higher than those calculated for $^2\text{H}_2$ values: the $r$ values ranged from 0.66 (Val Maggiore, $p < 0.001$) to 0.88 (Baselga, $p < 0.001$).

### Relationship between climate data and stable isotope values

To obtain a general insight into the explanatory variables, a PCA analysis was performed on data from the stable isotope series within the common period (1958–2009). The loading

<table>
<thead>
<tr>
<th>Table 1. Correlation matrix of the eight proxies at each site. Isotope chronologies were: 1860–2009 at Cermis, 1933–2009 in Val Maggiore, 1936–2009 at Baselga</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CERMIS</strong></td>
</tr>
<tr>
<td>$^{13}\text{C}_{\text{W}}$ &amp; 0.91***</td>
</tr>
<tr>
<td>$^{13}\text{C}_{\text{L}}$ &amp; 0.72***</td>
</tr>
<tr>
<td>$^{18}\text{O}_{\text{W}}$ &amp; 0.28***</td>
</tr>
<tr>
<td>$^{18}\text{O}_{\text{C}}$ &amp; 0.41***</td>
</tr>
<tr>
<td>$^2\text{H}_{\text{C}}$ &amp; 0.12</td>
</tr>
<tr>
<td>$^2\text{H}_{\text{L}}$ &amp; 0.30***</td>
</tr>
<tr>
<td>$^2\text{H}_{\text{W}}$ &amp; 0.23***</td>
</tr>
<tr>
<td><strong>VAL MAGGIORE</strong></td>
</tr>
<tr>
<td>$^{13}\text{C}_{\text{W}}$ &amp; 0.88***</td>
</tr>
<tr>
<td>$^{13}\text{C}_{\text{L}}$ &amp; 0.57***</td>
</tr>
<tr>
<td>$^{18}\text{O}_{\text{W}}$ &amp; 0.19</td>
</tr>
<tr>
<td>$^{18}\text{O}_{\text{C}}$ &amp; 0.21</td>
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<tr>
<td>$^2\text{H}_{\text{C}}$ &amp; 0.27*</td>
</tr>
<tr>
<td>$^2\text{H}_{\text{L}}$ &amp; 0.38***</td>
</tr>
<tr>
<td>$^2\text{H}_{\text{W}}$ &amp; 0.55***</td>
</tr>
<tr>
<td><strong>BASELGA</strong></td>
</tr>
<tr>
<td>$^{13}\text{C}_{\text{W}}$ &amp; 0.83***</td>
</tr>
<tr>
<td>$^{13}\text{C}_{\text{L}}$ &amp; 0.24**</td>
</tr>
<tr>
<td>$^{18}\text{O}_{\text{W}}$ &amp; 0.39***</td>
</tr>
<tr>
<td>$^{18}\text{O}_{\text{C}}$ &amp; 0.61***</td>
</tr>
<tr>
<td>$^2\text{H}_{\text{C}}$ &amp; 0.63***</td>
</tr>
<tr>
<td>$^2\text{H}_{\text{L}}$ &amp; 0.38**</td>
</tr>
<tr>
<td>$^2\text{H}_{\text{W}}$ &amp; 0.55***</td>
</tr>
</tbody>
</table>

*p < 0.05; **p < 0.01; ***p < 0.001

$^{13}\text{C}_{\text{W}}$: carbon isotope values in whole wood; $^{13}\text{C}_{\text{L}}$: carbon isotope values in cellulose; $^{13}\text{C}_{\text{L}}$: carbon isotope values in lignin methoxyl groups; $^{18}\text{O}_{\text{W}}$: oxygen isotope values in whole wood; $^{18}\text{O}_{\text{C}}$: oxygen isotope values in cellulose; $^2\text{H}_{\text{W}}$: hydrogen isotope values in whole wood; $^2\text{H}_{\text{C}}$: hydrogen isotope values in cellulose; $^2\text{H}_{\text{L}}$: hydrogen isotope values in lignin methoxyl groups.
plot is given in Fig. 3: the variance explained by the first principal component is 39.2% (PC 1), while the second principal component (PC 2) accounts for 29.6% of the variance. The $\delta^{18}O$, $\delta^{13}C$ and $\delta^2H$ values of the various wood components clustered together; within each isotope series, cellulose and whole wood have the closest relationship. These findings agree with the good relationship found between the $\delta^{13}C$ and $\delta^{18}O$ values in different wood components for the complete time series (Table 1). The stable isotope values in lignin methoxyl are less highly correlated with the other components and therefore appear to be influenced by different environmental and biochemical factors.

In order to test their reliability as climate proxies, each stable isotope value of each component was separately correlated to the mean temperature and precipitation at each site. We accepted only a significance level of $p < 0.01$. The mean annual and mean seasonal climate variables are shown as predictors. For precipitation, we report only the mean autumn precipitation at Cermis because we found no correlations ($p < 0.01$) in the other cases. The coefficients of correlation between climate variables and isotope series are given in Table 2. Looking at the results from the three sites, we can see that the correlation is significant ($p < 0.01$) for all the proxies, with the exception of the $\delta^{13}C_{W}$ and $\delta^{18}O_{W}$ series. The strongest correlation was obtained using the $\delta^{2}H_{W}$ value as a predictor for annual temperature ($p < 0.001$). Significant relationships were also found between the $\delta^{13}C_{L}$ and $\delta^{18}O_{W}$ series and various seasonal temperatures. The $\delta^{18}O_{C}$ series are related only to mean annual and mean seasonal temperature in Val Maggiore and to mean annual temperature at Baselga, but the correlation coefficients are always lower than those of the $\delta^{18}O_{W}$ series with the same climate proxies.

The $\delta^{13}C_{L}$ series are only negatively correlated with annual and some seasonal temperatures at Baselga. We do not have an explanation for this but it should be borne in mind that stable isotope values of different wood fractions depend not only on the species, but also on climatic features at the stand.

![Figure 3. Biplot of the PCA analysis reporting the isotope proxies in whole wood ($\delta^{13}C_{W}$, $\delta^{18}O_{W}$, $\delta^{2}H_{W}$ values), cellulose ($\delta^{13}C_{C}$, $\delta^{18}O_{C}$, $\delta^{2}H_{C}$ values) and lignin methoxyl groups ($\delta^{13}C_{L}$, $\delta^{2}H_{L}$ values) for the period 1958-2009. Axis labels report the percentage of variance expressed by each component.](image)

**Table 2. Coefficients of correlation between isotope proxies and climate features for the three sites with respect to the 1958–2009 period**

<table>
<thead>
<tr>
<th>Site</th>
<th>C.F.</th>
<th>Period</th>
<th>$\delta^{13}C_{W}$</th>
<th>$\delta^{13}C_{C}$</th>
<th>$\delta^{13}C_{L}$</th>
<th>$\delta^{18}O_{W}$</th>
<th>$\delta^{18}O_{C}$</th>
<th>$\delta^{2}H_{W}$</th>
<th>$\delta^{2}H_{C}$</th>
<th>$\delta^{2}H_{L}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CERMIS</td>
<td>t</td>
<td>ANNUAL</td>
<td>0.57***</td>
<td>0.69***</td>
<td>0.36**</td>
<td>0.63***</td>
<td>0.46***</td>
<td>0.38**</td>
<td>0.49***</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>WINTER</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>SPRING</td>
<td>0.48***</td>
<td>0.46***</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>SUMMER</td>
<td>0.38**</td>
<td>0.49***</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>AUTUMN</td>
<td>-0.37**</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>VAL MAGGIORE</td>
<td>t</td>
<td>ANNUAL</td>
<td>0.60***</td>
<td>0.57***</td>
<td>0.62***</td>
<td>0.45***</td>
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<tr>
<td></td>
<td></td>
<td>WINTER</td>
<td></td>
<td>0.37**</td>
<td>0.43***</td>
<td>0.47***</td>
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<tr>
<td></td>
<td></td>
<td>SPRING</td>
<td>0.45***</td>
<td>0.42**</td>
<td>0.54***</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>SUMMER</td>
<td>0.53***</td>
<td>0.44**</td>
<td>0.39**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BASELGA</td>
<td>t</td>
<td>ANNUAL</td>
<td>-0.61***</td>
<td>0.43***</td>
<td>0.37**</td>
<td>0.75***</td>
<td>0.69***</td>
<td>0.52***</td>
<td>0.45**</td>
<td>0.59**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WINTER</td>
<td>-0.61***</td>
<td></td>
<td>0.43***</td>
<td>0.52***</td>
<td>0.45**</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>SPRING</td>
<td>-0.61***</td>
<td></td>
<td>0.47***</td>
<td>0.40**</td>
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<tr>
<td></td>
<td></td>
<td>SUMMER</td>
<td>-0.48***</td>
<td></td>
<td>0.38**</td>
<td>0.59**</td>
<td>0.59**</td>
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<tr>
<td></td>
<td></td>
<td>AUTUMN</td>
<td>-0.48***</td>
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</tbody>
</table>

**p < 0.01; ***p < 0.001**

$\delta^{13}C_{W}$: carbon isotope values in whole wood; $\delta^{13}C_{C}$: carbon isotope values in cellulose; $\delta^{13}C_{L}$: carbon isotope values in lignin methoxyl groups; $\delta^{18}O_{W}$: oxygen isotope values in whole wood; $\delta^{18}O_{C}$: oxygen isotope values in cellulose; $\delta^{2}H_{W}$: hydrogen isotope values in whole wood; $\delta^{2}H_{C}$: hydrogen isotope values in cellulose; $\delta^{2}H_{L}$: hydrogen isotope values in lignin methoxyl groups; C.F.: Climatic factor; t: temperature; p: precipitation

*Average daily temperatures and precipitation were used for the periods: 21 December to 20 March (winter), 21 March to 20 June (spring), 21 June to 20 September (summer), 21 September to 20 December (autumn).
level and on the geographical origin of the wood.\textsuperscript{[19,23]} Contrary to what would be expected from previous reports,\textsuperscript{[4,8]} we found no significant correlations between stable isotope ratios and precipitation ($p < 0.01$), except for the $\delta^{13}C$ values and mean autumn precipitation at Cermis ($-0.37$, $p > 0.01$), but it is not strong enough to suggest precipitation as a primary control. This is consistent with the findings of Warren \textit{et al} \textsuperscript{[53]} who demonstrated that $\delta^{13}C$ values are highly correlated with precipitation only at dry sites. It should be remembered that the $\delta^{13}C$ values in tree rings are not directly linked with precipitation but are instead influenced by those environmental factors which affect plant physiological processes (e.g. stomatal conductance and the rate of photosynthesis), with stomatal conductance having the greatest influence.\textsuperscript{[4]} We therefore focused on temperature as the primary environmental/climatic factor affecting the stable isotope values of tree rings in this region.

Although correlations vary in strength between sites, the patterns are quite similar, suggesting the absence of a strong influence at stand level. Overall, the highest correlations between temperature and the eight stable isotope series/components were found for the $\delta^{2}H_W$ and $\delta^{18}O_W$ values, suggesting that these two parameters are a suitable source of climate information. The $\delta^{2}H_W$ values are related to mean annual temperature and mean winter, spring and summer temperatures, while the $\delta^{18}O_W$ values exhibit a similar correlation only during the growing season (spring and summer). Even if the variability in $\delta^{2}H$ values is controlled by the same factors influencing the $\delta^{18}O$ values,\textsuperscript{[4,52]} and is mainly determined by the isotopic composition of the soil water and by leaf water, it appears that other fractionation effects are also involved. Although hydrogen isotope behaviour has been modelled,\textsuperscript{[53]} very few data are available for this proxy\textsuperscript{[4]} and further studies are required.

To enhance the common climate signal and reduce non-climate noise,\textsuperscript{[4]} a multi-proxy approach was taken which made use of multiple linear regression models using the eight isotopic chronologies as variables. In order to define the best wood component for climate reconstruction in this region, six models were built using either isotope proxies in single wood components or in combinations of different wood components. Only mean annual temperature was used as a variable since this parameter exhibited the strongest correlations with the stable isotope data. The results are reported in Table 3. Each multivariate model was assessed using all the normal procedures, including checks on the homoscedasticity of the residuals, and leverages. In all cases, assumptions of linear regression were met. Scatter graphs (Fig. 4) show the relationship between the predicted mean annual temperature and the measured mean annual temperature for the three sites.

<table>
<thead>
<tr>
<th>Table 3. $R^2$ values of the multiple linear regression. Mean annual temperature was used as a predictor variable at the three sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site</td>
</tr>
<tr>
<td>CERMIS</td>
</tr>
<tr>
<td>VAL MAGGIORE</td>
</tr>
<tr>
<td>BASELGA</td>
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</tbody>
</table>

$^{**}p < 0.01; \, ***p < 0.001$

Each model was run with $\delta^{13}C$, $\delta^{18}O$ and $\delta^{2}H$ values in the various wood components.

- Model$_{WLC}$: full model with 3 kinds of wood components (whole wood, cellulose and lignin methoxyl groups);
- Model$_{WL}$: two wood components (whole wood and lignin methoxyl groups);
- Model$_{WC}$: two wood components (whole wood and cellulose);
- Model$_{W}$: single wood component (whole wood);
- Model$_{C}$: single wood component (cellulose);
- Model$_{L}$: single wood component (lignin methoxyl groups).

Figure 4. Scatter graphs of the predicted mean annual temperature vs measured mean annual temperature of Minimal Adequate Model at Cermis (a), Val Maggiore (b), and Baselga (c). The equation for the line of the best fit is computed from a multiple linear regression. $R^2$ and MSE are reported for each plot.
As reported in a previous study, we found that a multi-proxy approach enhanced the climate signal. McCarroll and co-authors state that the key to this lies in combining independent proxies or proxies that are not very similar. This leads to an improved estimate of climate signal since the proxies are not subject to similar sources of “error”. In our study, we improved the effective correlation by combining the proxies, but the best result was obtained at the Baselga stand (Table 3).

It is worth noting that this stand yields the best predictive model when both wood and lignin methoxyl groups are used (the $R^2$ value rises from 0.55 to 0.74). Therefore, combining these proxies improves temperature prediction.

The best temperature prediction was found at Baselga: both ModelWL and ModelWLC have an $R^2$ of 0.77 ($p < 0.001$). At each site, both ModelWL and the simpler ModelW have the same adjusted $R^2$ value and, in Val Maggiore, the simplest ModelW has the same adjusted $R^2$ as ModelWL. When single wood components are examined, ModelW is found to have higher $R^2$ values than those of the combined wood fractions.

In order to more efficiently test the difference between the models, an ANOVA was performed to compare ModelWL with the other simpler models. The results of the ANOVA are shown in Table 4 and they clearly show the absence of differences between ModelWL and ModelW at any site ($p < 0.001$). The simpler ModelW could therefore save two degrees of freedom without being significantly worse than the more complex model. Moreover, the simplest ModelW does not differ from ModelWL in Val Maggiore or at Cermis, while ModelL and ModelC always differed significantly from ModelWL ($p < 0.05$) at all three sites. Therefore, we selected ModelW at Cermis and in Val Maggiore, and ModelWL at Baselga, as adequate models to predict mean annual temperature.

The selected models were further simplified in order to obtain minimal adequate models. The results are reported in Table 5. An ANOVA confirmed that model simplification was completely justified at Cermis and in Val Maggiore, where it was possible to build the minimal adequate model using only the $\delta^{13}C_W$ and $\delta^{18}O_W$ series for both sites. On the other hand, only the $\delta^{13}C_W$ series was not significant at Baselga, and the minimal adequate model had four proxies ($\delta^{13}O_W; \delta^{2}H_W; \delta^{13}C_L; \delta^{2}H_L$ values).

**CONCLUSIONS**

We can conclude from our results that the stable isotope ratios in whole wood samples preserve the best temperature signal and that it is not necessary to include cellulose, at least as far as temperature reconstruction with *P. abies* in the south-eastern Alpine study area is concerned.

Our findings appear to disagree with those of many other studies which report that the stable isotope values of cellulose (mainly for $\delta^{18}O$) yield the best temperature information. For lignin methoxyl groups, only the $\delta^{13}C$ values (and only at the low altitude site) turned out to improve temperature prediction, although it has been suggested that the $\delta^2H$ values of lignin methoxyl groups could be used as a paleoclimate proxy.
proxy. One possible explanation for the different results might be that our study was carried out in a cold temperate region where precipitation is not an environmental constraint, whereas most previously published studies were carried out in Mediterranean areas where precipitation is a limiting factor. Our trees may therefore have responded in a different way to the climatic variables and we are aware that our results may not be reproducible in other regions with different climatic conditions.

With respect to isotope proxies, $\delta^2$H values in wood have the highest correlations with seasonal and mean annual temperatures, $\delta^{18}$O values correlate highly only with mean annual temperature, and $\delta^{13}$C values in lignin methoxyl groups correlate with mean annual temperature at the lower altitude location. Proxy selection allowed us to find the best-fit model to predict mean annual temperature: combining the $\delta^{18}$O and $\delta^2$H values in whole wood we obtained the minimal adequate model to reconstruct mean annual temperature at two of the three sites, while at the other site the $\delta^{13}$C and $\delta^2$H values of lignin methoxyl groups are also necessary. The current results will be useful in the selection of appropriate wood components and stable isotope proxies for future paleoclimate isotopic research. Moreover, our simplified model saves a considerable amount of time given that extraction of lignin methoxyl groups is simpler and faster than cellulose extraction.

Further studies are required to improve our knowledge of the complex mechanisms of fractionation, especially where the physiological processes determining the origin of isotope signatures in the different wood components are concerned, as has already been pointed out.\cite{4,8}

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REFERENCES


C, H and O stable isotopes of tree rings as climate proxies


