Sample preparation of ring-less tropical trees for $\delta^{18}$O measurement in isotope dendrochronology

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ABSTRACT Radial variation of $\delta^{18}$O before and after cellulose extraction was assessed in Acacia auriculiformis, Eucalyptus camaldulensis, and Celtis timorensis growing in northeastern Thailand to examine the necessity of extracting $\alpha$-cellulose to detect annual rings from trees without visible rings. Optimum sampling resolution to detect peaks in the radial variation of $\delta^{18}$O values was also examined. Cored samples were sectioned into 0.2 mm thickness in the radial direction. Each circular section sliced from a wood core sample were divided along wood grain into two semicircular sections, both of which were located at the same radial and longitudinal positions, and were side-by-side tangentially. One half was used for bulk analysis and the other for extraction. Peak positions were assigned from the seasonal variation of $\delta^{18}$O. The $\delta^{18}$O values cyclically changed in both bulk wood and $\alpha$-cellulose. The correlation coefficient between bulk wood and $\alpha$-cellulose $\delta^{18}$O was high in every species, and the offset was almost constant across the radial position. The mean cycle length of one sample was longer than those of the other two samples, although annual increment based on dendrometer monitoring was smaller than those of the other two samples. That is, the seasonal variation in $\delta^{18}$O values recorded in the xylem was not completely detected because of low amplitude or insufficient radial resolution. Therefore, we concluded that $\alpha$-cellulose extraction is unnecessary for annual ring detection. It is necessary to determine an appropriate sampling resolution based on growth rate for effective peak detection.

Key words: Annual ring detection, Stable oxygen isotope, Dendrochronology, Seasonal tropics, Cellulose extraction

INTRODUCTION

Most trees in the tropical region do not have annual rings because the seasonality of climatic factors is less pronounced through the year. In trees that have anatomically visible growth rings, these rings can be used for dendrochronology with some verification method, such as cambial marking (Worbes 1995). However, in trees without visible rings, definition of the growth zone, which is the region between two adjacent growth boundaries, is needed before application of dendrochronological methods. In this case, the radial variation of anatomical features, such as vessel lumen size and frequency (Ohashi et al. 2014), chemical element concentration (Hietz et al. 2015), or isotope ratios (Poussart et al. 2004) have been used to define the growth zone.

The stable oxygen isotope ratio ($\delta^{18}$O) in tree-ring cellulose is mostly determined by $\delta^{18}$O of source water (usually precipitation) and relative humidity during growth (Roden et al. 2000). Therefore, $\delta^{18}$O in the tree-ring cellulose should vary along with the seasonal variation of $\delta^{18}$O in precipitation and/or relative humidity even with the trees without visible boundaries. For example, Poussart et al. (2004) found that $\delta^{18}$O variation of Podocarpus neriifolius in Thailand and Samanea saman in Indonesia was mainly caused by seasonal change in relative humidity. Ohashi et al. (2016) analyzed $\delta^{18}$O of eight trees in Brazil and they attributed the seasonal variation in $\delta^{18}$O of trees to $\delta^{18}$O of precipitation.

Sample preparation in isotope dendrochronology, especially for a high-resolution analysis, is problematic. Dendrochronological studies using $\delta^{18}$O in wood usually analyze cellulose, one of the three major chemical components in wood (McCarroll and Loader 2004). Each component (cellulose, hemicellulose, and lignin) of wood has its own $\delta^{18}$O value (Wilson and Grinsted 1977), and the percentage of each component can be different at stem position or among individuals. Thus, several studies have
tested the necessity of cellulose extraction in dendrochronological studies. Despite the variations in percentage and its $\delta^{18}O$ value among each wood component, whole wood samples show similar $\delta^{18}O$ trend as in the $\alpha$-cellulose samples within a tree (Mischel et al. 2015), and what is more, whole wood samples including both sapwood and heartwood may exhibit better correlation with the climate factors than $\alpha$-cellulose samples (Gori et al. 2013). There are two procedures to extract $\alpha$-cellulose: extraction after dividing (Loader et al. 1997) and extraction before dividing (Kagawa et al. 2015). Each tree ring is subdivided into thin sections with radial thickness ranging from 25 $\mu$m (Poussart and Schrag 2005) to 60 $\mu$m (Pons and Helle 2011), depending on the growth rate, to detect annual rings based on the radial variation of $\delta^{18}O$. Therefore, the number of samples to be analyzed can be overwhelming, and the extraction step could be a major bottleneck, if the former procedure is selected. If the latter procedure is selected, the number of samples to be extracted becomes fewer, but the chemical treatment shrinks the sample lath and makes dividing difficult. Moreover, sampling resolution may become insufficient because samples cannot be divided into as finer subdivisions as in the former method. In case of annual ring detection, if the radial variation of $\delta^{18}O$ trend in $\alpha$-cellulose samples is the same as the samples before extraction, the cellulose extraction step could be skipped and the time required for sample preparation would be reduced significantly. Most of the studies comparing the trend between bulk and $\alpha$-cellulose deal with inter-annual variation. Szymczak et al. (2011) found that Pinus nigra from the island of Corsica exhibited a high correlation ($r = 0.77$) between $\alpha$-cellulose and the whole wood. Weigt et al. (2015) analyzed five tree species (Fagus sylvatica, Quercus robur, Picea abies, Abies alba, and Pseudotsuga menziesii) in Germany and reported that the whole wood and the $\alpha$-cellulose carried environmental signal at similar strengths. However, few studies have dealt with intra-annual variation. For example, Pons and Helle (2011) compared the seasonal variation of $\delta^{18}O$ in the whole wood and $\alpha$-cellulose of Carapa guianensis and Goupia glabra in central Guyana; the correlation was 0.55 and 0.77, respectively, and the $\delta^{18}O$ values of whole wood were almost parallel to that of $\alpha$-cellulose.

Analysis at a high time-resolution is necessary for detecting seasonal variation of $\delta^{18}O$ in trees, and samples must be subdivided into thin sections with sufficient time-resolution. However, cellulose extraction is labor-intensive work, especially for a large number of samples. If this extraction step can be eliminated, the analytical time can be greatly reduced. Whether the $\delta^{18}O$ chronology of whole wood samples parallels to that of $\alpha$-cellulose samples must be tested to determine the necessity of $\alpha$-cellulose extraction. In this study, we compared the radial variation of $\delta^{18}O$ before and after cellulose extraction to evaluate the necessity of extracting $\alpha$-cellulose to detect annual rings from trees with no visible rings. Moreover, we examined the optimum sampling interval of analytical samples that achieves sufficient time resolution to detect peaks in the radial variations of $\delta^{18}O$ values.

MATERIALS AND METHODS

Study site

The study was conducted in plantations of Acacia auriculiformis (AA) and Eucalyptus camaldulensis (EC), and a natural forest (FOII) in Sakaerat Silvicultural Research Station (14°28′06.1″N, 101°54′15.0″E), Nakhon Ratchasima Province, northeastern Thailand. Three sites (AA, EC, and FOII) were located within 1 km of each other, and there was little difference in climatic conditions among them. The air temperature was measured every hour during research period, and mean annual temperature was 22.1, 23.4, and 22.9°C in 2011, 2012, and 2013, respectively. Annual precipitation was 1155, 1132, and 1419 mm in 2011, 2012, and 2013, respectively (Sakaerat Environmental Research Station 2016). Fig. 1 shows precipitation from September 2011 to December 2013. In this region, dry season usually starts in November and continues until April. Fig. 2 shows the monthly change in the stable oxygen isotope ratio (%) of the precipitation in Bangkok (IAEA/WMO 2016), approximately 180 km southwest of Sakaerat. The $\delta^{18}O$ value of precipitation is increasingly negative from February to October, then increases until January. The lowest $\delta^{18}O$ value is observed at the end of the wet season.

Samples

One tree from each site, A. auriculiformis from AA (AA2-3), E. camaldulensis from EC (EC5-3), and Celtis timorensis tree from FOII (FOII11-3) was sampled in February 2014. Diameter at breast height (DBH) of each tree was 22.7, 22.1, and 26.9 cm, respectively. Wood core samples (5 mm in diameter, 3-4 cm in length) were collected from the trunk at 1.3 m above the ground with an incremental borer. Radial growth of sampled species in each site was monitored with dendrometer bands every two
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Serial tangential sections were prepared from each core sample at 0.2 mm thickness in the radial direction, starting from cambium, with a sliding microtome. The sections were placed in acetone for 30 minutes to remove the glue (cyanoacrylate adhesive) that was used to prevent breakage of the core during sectioning. Each circular section sliced from a core sample was divided along wood grain into two semicircular sections, both of which were located at the same radial and longitudinal positions, and were side-by-side tangentially. One half was used for bulk analysis (bulk sample) and the other was used for extraction (extracted sample). Holocellulose was prepared from the extracted samples, and then every other holocellulose section was selected to prepare $\alpha$-cellulose section (Fig. 4).

Chemical treatment

We followed the methodology described in Loader et al. (1997) for $\alpha$-cellulose extraction. We omitted extractive removal treatment because we used sapwood whose extractive content is negligible. Firstly, lignin was removed with sodium chlorite solution. Samples were processed in a mixture of sodium chlorite (10 g), acetic acid (5 mL), and deionized water (350 mL) at 70°C for one hour, after which samples were washed with deionized water. These steps were repeated four times and the final products were labeled as holocellulose. Secondly, half of the selected holocellulose samples were further treated with sodium hydroxide solution (17.5 % wt/wt) at 80°C for one hour to remove hemicellulose. Later, samples were washed with deionized water, and the final product was labeled as $\alpha$-cellulose. Both holocellulose and $\alpha$-cellulose sample were dried at 65°C overnight. Dry weights of samples before and after the chemical treatments were measured and the yields of holocellulose and $\alpha$-cellulose were calculated.

We used a Teflon vessel (8 mm external diameter, 150 mm length) with a polyethylene filter (average pore size 40 µm) (Harada et al. 2014) or a glass tube (8 mm external diameter, 5 cm length) with glass wool as the reaction tube. The samples were put into the tubes and the tubes were placed in an airtight beaker. Extraction was conducted in a temperature-controlled oven.
Stable oxygen isotope analysis

A 100–200 μg portion of each sample was weighed and wrapped in a 7 mm × 7 mm silver sheet that was cut out from silver foil (150 mm × 150 mm, 4 μm thick, Nitto Kagaku Co Ltd., Nagoya, Japan). The oxygen isotope ratios were measured using a continuous flow isotope ratio mass spectrometer (Delta V Advantage; Thermo Fisher Scientific, Waltham, MA, USA) coupled with a high-temperature combustion elemental analyzer (TC/EA; Thermo Fisher Scientific, Waltham, MA, USA). Obtained results were expressed in delta (δ) notation in per mil (‰) units relative to Vienna standard mean ocean water (VSMOW). The oxygen isotope ratios of samples were calibrated with Merck cellulose. Standards were measured every nine samples. Standard deviation (SD) of the repeated measurements of standards within a sequence was less than 0.34 ‰.

Data processing

We obtained regression line between distance from cambium (explanatory variable) and δ¹⁸O values (dependent variable) by fitting Kernel regression. We assume that neighboring samples have close δ¹⁸O values. The regression line can follow all data points because degrees of freedom can be same as the number of samples.
Kernel regression was used to detect any trend in seasonal variation in the sequential $\delta^{18}O$ values. Kernel ridge regression is ridge regression combined with the kernel method. Ridge regression is a regression analysis with regularization. Without regularization, parameters of linear regression are obtained by minimizing the residual sum of squares (the least square method). However, in ridge regression, the parameters are obtained by minimizing the residual sum of squares plus a penalty term for regularization which is the square of the $L^2$ norm of the parameter.

Given the explanatory variable $x = (x_1, x_2, \ldots, x_n)$ and the target variable $y = (y_1, y_2, \ldots, y_n)$, the kernel ridge regression assumes the following linear relationship between $x$ and $y$:

$$y_i = k(x_i, x)\theta + \epsilon$$

where $k(x_i, x)$ is the kernel function, $\theta$ is the coefficient, and $\epsilon$ is the noise term. In this study, $x$ is the distance from the cambium (mm) and $y$ is $\delta^{18}O$ value (‰). $\theta$ is determined to minimize the residual sum of squares plus a penalty term:

$$\sum_{i=1}^{n} (y_i - k(x_i, x)\theta)^2 + \alpha \left\| \theta \right\|^2$$

where $\alpha$ is the regularization parameter ($\alpha \geq 0$). As for our data, the radial basis function (RBF) kernel was used as the kernel function:

$$k(x, x') = \exp\left(-\gamma \|x-x'\|^2\right), \gamma = \sigma^{-2}$$

where $\sigma > 0$. The parameter of RBF kernel $\gamma$ and the regularization parameter $\alpha$ were selected by trying various sets of $\gamma$ and $\alpha$ to minimize the mean square error calculated by the leave-one-out cross-validation. Kernel ridge regression was performed by “scikit-learn” (Pedregosa et al. 2011) in Python. $\delta^{18}O$ values were obtained for every 0.05 mm by kernel ridge regression. Later, the position of peaks was determined. Peaks smaller than the threshold value were ignored. The threshold value was determined based on the SD of standards. Thus, if SD was less than 0.34‰ then the threshold value was $3\sqrt{\text{SD}^2 + \text{SD}^2} \approx 1.44$‰.

The differences in peak positions between the bulk samples ($P_b$) and extracted samples ($P_e$) were evaluated by the root mean squared error (RMSE). By assuming that bulk samples and extracted samples have $n$ peaks ($P_b = (P_{b1}, P_{b2}, \ldots, P_{bn})$, $P_e = (P_{e1}, P_{e2}, \ldots, P_{en})$), $P_{b1}$ and $P_{e1}$ were the closest peaks to cambium, RMSE was defined as follows:

$$\text{RMSE} = \sqrt{\frac{\sum_{i=1}^{n}(P_{bi}-P_{ei})^2}{n}}$$

### RESULTS

#### Offset of $\delta^{18}O$ values for extracted samples from bulk wood

The $\delta^{18}O$ values cyclically changed in both bulk wood and $\alpha$-cellulose whereas the offset was almost constant across the radial position within an individual core (Fig. 5). The mean offset of $\delta^{18}O$ values of $\alpha$-cellulose from bulk wood was $4.7 \pm 1.1$‰ in AA2-3, $3.2 \pm 0.6$‰ in EC5-3, and $3.1 \pm 0.8$‰ in FOII11-3. The mean offset of $\delta^{18}O$ values of holocellulose from bulk wood was $2.2 \pm 1.8$‰, $0.2 \pm 0.7$‰, and $1.3 \pm 1.4$‰ respectively. The yields (%) of holocellulose and $\alpha$-cellulose were 76–84% and 40–47%, respectively (Fig. 6). We omitted holocellulose from data analysis because the yield of holocellulose was too high and the offset was too low compared to Pettersen (1984) and Cullen and Macfarlane (2005), respectively.

#### Radial variation of $\delta^{18}O$

Cyclic changes in $\delta^{18}O$ values in the radial direction was observed in all trees, both from bulk wood and $\alpha$-cellulose (Fig. 5). The trend in bulk wood was similar to that of $\alpha$-cellulose, but the cycles were more conspicuous in bulk wood partly because of the higher time resolution. The correlation coefficient between $\alpha$-cellulose and bulk wood was 0.83, 0.90, and 0.77 for AA2-3, EC5-3, and FOII11-3, respectively.

The range of $\delta^{18}O$ variation (%) was similar among samples except for $\alpha$-cellulose of AA2-3 and bulk wood of FOII11-3 (Table 1). The range was wider in $\alpha$-cellulose of AA2-3 and narrower in bulk wood of FOII11-3.

#### Number of peaks and their positions

Fig. 7 indicates the positions of peaks detected based on predicted values by kernel ridge regression. Parameters and $R^2$ values for the regressions are listed in Table 2. In AA2-3, the peak located at 9.40 mm from cambium in $\alpha$-cellulose was not detected in bulk wood (Fig. 7 (a)). In EC5-3, two peaks located at 6.45 mm and 10.8 mm from
In FOII11-3, all the peaks detected in α-cellulose were also detected in bulk wood. Regarding the peaks both in α-cellulose and bulk wood, the RMSE was 0.13, 0.13, and 0.57 for AA2-3, EC5-3, and FOII11-3, respectively. The mean cycle length of FOII11-3 was 3.76 mm for bulk wood and 3.67 mm for α-cellulose, being longer than that of AA2-3 (2.13 mm for bulk wood, 1.90 mm for α-cellulose) and EC5-3 (2.77 mm for both bulk wood and α-cellulose).

Predicted values by kernel ridge regression were also obtained using bulk wood with 0.2 mm sampling interval.

![Fig. 5. Radial change of δ^{18}O values in bulk wood (circle) and α-cellulose (triangle).](image)

![Fig. 6. Mass fraction of α-cellulose and holocellulose. Mean values are represented by a white circle.](image)

| Sample ID | Type¹ | Mean (%) | SD (%) | Range (%) |
|-----------|-------|----------|--------|-----------|
| AA2-3     | B     | 23.65    | 1.27   | 5.53      |
|           | C     | 28.37    | 1.87   | 8.83      |
| EC5-3     | B     | 22.06    | 1.16   | 5.04      |
|           | C     | 25.27    | 1.37   | 5.19      |
| FOII11-3  | B     | 22.55    | 1.02   | 3.99      |
|           | C     | 25.68    | 1.25   | 5.02      |

¹B: bulk; C: α-cellulose.
and detected peaks were compared with peaks of 0.4 mm-interval sample to examine the effect of the difference in sampling interval on peak detection (Fig. 8). In AA2-3, all the peaks detected in 0.2 mm-interval sample were also detected in 0.4 mm-interval sample (Fig. 8 (a)). In EC5-3, two peaks located at 6.70 mm and 10.75 mm from cambium were not detected in 0.4 mm-interval sample (Fig. 8 (b)). In FOII11-3, one peak located at 1.75 mm from cambium was not detected in 0.4 mm-interval sample (Fig. 8 (c)).

**DISCUSSION**

**Offset and correlation between bulk samples and α-cellulose samples**

The offset values observed in this study were rather small, but within the range of values in Barbour et al. (2001), in which measured \( \delta^{18}O \) values were from *Quercus* and *Pinus* species grown on a wide range of source water \( \delta^{18}O \) values. In EC5-3, the offset values were smaller than the range (5.0–7.0 ‰) of other three *Eucalyptus* species (Cullen and Macfarlane 2005). This smaller offset was attributable to less lignin content (Fig. 6). More lignin content resulted in greater offsets because \( \delta^{18}O \) of lignin is smaller than that of α-cellulose (Cullen and Macfarlane 2005). The lignin content in softwoods is greater than in hardwoods. The lignin content of tropical hardwoods tends to be greater than that of temperate hardwoods (Pettersen 1984). Therefore, if the offset values are compared within a genus, the offset values of tropical trees are expected to be larger than those of temperate trees of the same genus.

The offset for AA2-3 was larger than that of EC5-3 and FOII11-3 especially in the inner part (Fig. 5). This difference was probably caused by both the difference in
assumed that they used source water with the same $\delta^{18}O$ value (Roden et al. 2000). Leaf water $\delta^{18}O$ value becomes increasingly positive when evaporation rate increases, and the $\delta^{18}O$ is reflected in the $\delta^{18}O$ value of $\alpha$-cellulose. Therefore, in the case of the inner part of AA2-3, we assume $\delta^{18}O$ values of $\alpha$-cellulose were greater probably because of high transpiration at the time of the wood formation, whereas $\delta^{18}O$ values of bulk wood were not so enriched probably because of higher lignin content.

Correlation coefficients in this study (0.83, 0.90, and 0.77 for AA2-3, EC5-3, and FOII11-3, respectively) were relatively higher than the values reported in previous studies. As for intra-annual variation, Pons and Helle (2011) studied Carapa guianensis Aublet and Goupia glabra Aublet in central Guyana. They reported that correlation coefficients were 0.55 and 0.77, respectively, which were lower than those from this study. As for inter-annual variation, Battipaglia et al. (2008) compared $\delta^{18}O$ in late wood of Fagus sylvatica and Acer pseudoplatanus in bulk wood and $\alpha$-cellulose. They reported that the correlation coefficients were lower than those of this study for F. sylvatica (0.46) and insignificant for A. pseudoplatanus. As far as ratios of wood components stay constant across different years of wood formation, $\delta^{18}O$ variation of whole wood is expected to be parallel to that of $\alpha$-cellulose. However, in the case of dendrochronological samples over wider time span, wood components could have changed through time, and this could result in a weak correlation between whole wood and $\alpha$-cellulose samples.

**Effect of $\alpha$-cellulose extraction on radial variation of $\delta^{18}O$**

Although the peak positions of bulk wood were the same as those of $\alpha$-cellulose in most cases, the peak detections sometimes failed. This failure is partly due to small radial growth rates or small amplitude of $\delta^{18}O$.

In AA2-3 and EC5-3, the number of peaks in bulk wood was fewer than that of $\alpha$-cellulose (Fig. 7) because signal amplitudes in $\alpha$-cellulose decreased in the bulk samples, or peak detection processes were affected by an outlier. In both cases, this would be solved by measuring the same samples multiple times or increasing sample size.

The failure of peak detection could happen if neighboring samples have a large difference in their $\delta^{18}O$ values. We need to adjust parameters for Kernel ridge regression or use other method to extract trend from data if such situation occurs. Moreover, before applying this
method to detect growth ring, we need to test the validity of the method by using trees forming annual rings.

Effect of sampling resolution on radial variation of $\delta^{18}O$

Some peaks would not be detected if sampling interval are too wide. The number of peaks of 0.4 mm-interval sample were less than that of 0.2 mm-interval sample in EC5-3 and FOII11-3 because of insufficient sampling resolution to capture the peaks (Fig. 8). Annual increment of wood should be divided into sufficient number of subsamples in order to detect seasonally change of $\delta^{18}O$.

Optimal resolution for annual ring detection depends on growth rate. Pons and Helle (2011) reported that certainty of the annual ring detection was better at high resolution (60 $\mu$m) than at low resolution (200 $\mu$m). Ohashi et al. (2014) studied annual ring detection using vessel features (vessel frequency and mean vessel lumen area), and reported that approximately 40–60% of the annual-ring width was suitable for sampling resolution which is defined as the product of radial interval and filter length of moving average. In this study, optimal sampling resolution could be smaller than 0.2 mm when we consider the growth rate estimated by the dendrometer monitoring.

Radial variation of $\delta^{18}O$ and its potential for annual ring detection

Seasonality in $\delta^{18}O$ values of precipitation was reflected in $\delta^{18}O$ values of xylem in most cases (Fig. 5), but sometimes it was absent. The mean cycle length of FOII11-3 was larger than those of AA2-3 and EC5-3 (Fig. 7) However, the annual increment based on dendrometer monitoring showed smaller values than those of other two tree species (Fig. 3). Therefore, seasonality in $\delta^{18}O$ values recorded in the xylem was not completely reflected because of low signal amplitude or insufficient sampling resolution.

The three studied species tended to grow fast in the wet season and slow in the dry season (Fig. 3), and the change in the $\delta^{18}O$ values of precipitation during the growing season was recorded in tree xylem. However, if the growing season was very short, the amount of change in the $\delta^{18}O$ values of precipitation would be very small and the amplitude should be also very small.

We need to cross-check the $\delta^{18}O$-dated tree age by other methods such as cambial marking (Ohashi et al. 2009) or radiocarbon dating (Worbes 1995) to verify the annual nature of the cycle in the $\delta^{18}O$ values in tree xylem. Another way to verify the annual nature is cross-dating using high correlation of $\delta^{18}O$ values among trees that grew in the same environment (Nakatsuka et al. 2004). Another possibility would be cross-dating the $\delta^{18}O$ chronology of the ring-less tropical tree by comparing with the $\delta^{18}O$ chronology of the tree forming annual rings such as teak (Tectona grandis). Moreover, the seasonality in the $\delta^{18}O$ values of precipitation should be monitored in the study site.

CONCLUSIONS

We compared radial variation of $\delta^{18}O$ values in tropical trees with no distinct annual rings before and after cellulose extraction to examine the necessity of $\alpha$-cellulose extraction for tropical dendrochronology. Cyclic variations in $\delta^{18}O$ values in the radial direction were observed in three tree species for both bulk wood and $\alpha$-cellulose samples. Correlations between bulk wood and $\alpha$-cellulose were high in every species, and the offset was almost constant across the radial position. Thus, we concluded that $\alpha$-cellulose extraction is unnecessary for annual ring detection. Moreover, appropriate sampling resolution, depending on the growth rate is necessary for effective peak detection.

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