



APPLICATION OF CONFOCAL LASER SCANNING MICROSCOPY IN DENDROCHRONOLOGY

UPORABA KONFOKALNE LASERSKE VRSTIČNE MIKROSKOPIJE V DENDROKRONOLOGIJ

Angela Balzano^{1*}, Klemen Novak¹, Miha Humar¹, Katarina Čufar¹

UDK 630*561.24:537.533.35
Original scientific article / Izvirni znanstveni članek

Received / Prispelo: 11. 11. 2019
Accepted / Sprejeto: 6. 12. 2019

Abstract / Izvleček

Abstract: We used the Confocal Laser Scanning Microscope (CLSM) Olympus LEXT OLS5000 for non-destructive observation and image analysis of wood anatomy traits in growth layers of tree species from different climatic zones. In European beech (*Fagus sylvatica*), where tree rings can generally be recognised, we discuss the changes in tree-ring structure due to adverse effects (insect attacks). Growth layers in Mediterranean Aleppo pine (*Pinus halepensis*) from south-eastern Spain are not always annual and contain numerous intra-annual density fluctuations (IADFs). Ocote pine (*Pinus oocarpa*) growing at high elevation in Honduras showed growth layers with clear growth ring boundaries and IADFs. In both pines, CLSM allowed us to recognise and measure tracheid parameters to define density fluctuations. In tropical true mahogany (*Swietenia macrophylla*) from Venezuela and cedrela (*Cedrela odorata*) from Costa Rica, we studied the growth layers with variable dimensions of vessels demarcated by marginal axial parenchyma.

Keywords: confocal laser scanning microscopy (CLSM), wood anatomy, wood identification, image analysis, dendrochronology, tree-rings, growth ring boundaries

Izvleček: Konfokalni laserski vrstični mikroskop (CLSM) Olympus LEXT OLS5000 smo uporabili za nedestruktivno proučevanje anatomskih znakov v prirastnih plasteh dreves iz različnih podnebnih pasov. Pri bukvi (*Fagus sylvatica*), kjer je na splošno mogoče prepoznati branike, CLSM omogoča študij anatomskih posebnosti zaradi škodljivih učinkov (npr. napadov žuželk). Prirastne plasti sredozemskega alepskega bora (*Pinus halepensis*) v jugovzhodni Španiji ne nastajajo vedno v skladu s koledarskim letom, zaznamujejo jih namreč tudi številna nihanja gostote (IADFs) znotraj posameznih plasti. Bor *Pinus oocarpa*, ki raste na visoki nadmorski višini v Hondurasu, izkazuje jasno razmejene prirastne plasti in pojav IADFs. V obeh borih smo s pomočjo CLSM prepoznali in izmerili dimenzije traheid za boljše določanje IADF in za boljše razmejitev prirastnih plasti. V lesu srednjeameriškega mahagonija (*Swietenia macrophylla*) iz Venezuele in cedrele (*Cedrela odorata*) iz Kostarike smo analizirali prirastne plasti, ki imajo spremenljive dimenzije trahej in so razmejene z marginalnim aksialnim parenhimom.

Ključne besede: konfokalna laserska vrstična mikroskopija, anatomija lesa, identifikacija lesa, analiza slike, dendrochronologija, branike, prirastne plasti, meje med prirastnimi plastmi

1 INTRODUCTION

1 UVOD

Comparative and quantitative wood anatomy combined with dendrochronology may provide information regarding wood anatomy related to species, provenance, as well as the time and conditions in which the wood was formed.

Research into wood anatomy allows the analysis of anatomical traits, which can reflect import-

ant environmental signals (Verheyden et al., 2004; García-González & Fonti, 2006; Campelo et al., 2010). Wood quality also depends on wood anatomy, which varies between and within species, individual trees and even growth layers.

Dendrochronology, as the study of tree rings (growth layers), is based on cross-dating, which helps to assign every tree ring to the calendar year when it was formed. This principle can be applied in trees from temperate environments, where the tree rings are generally annual. They usually contain early- and late-wood, and are demarcated by tree-ring boundaries.

¹ University of Ljubljana, Biotechnical Faculty, Department of Wood Science and Technology, Ljubljana, Slovenia

* e-mail: angela.balzano@bf.uni-lj.si

However, in Mediterranean and tropical environments it is often challenging to define tree rings and the boundaries between them, as their formation is not necessarily synchronised with calendar years (Cherubini et al., 2003; De Luis et al., 2011; Balzano et al., 2018, 2019a, b). Such growth layers typically contain numerous anomalies associated with intra-annual density fluctuations (IADFs) (Campelo et al., 2007; De Micco et al., 2016a) sometimes called false rings (e.g. Kaennel & Schweingruber, 1995).

To help with the identification of tree rings we often apply quantitative wood anatomy (QWA), which is based on the measurement of anatomical traits (for instance shape, dimensions and frequency of xylem elements, thickness of cell walls etc.) in relation to time (position in the growth layer). This is used to characterise the relationships between plant growth and various environmental factors.

Studying anatomical features requires time-consuming sample preparation for microscopy, i.e. the cutting of thin microscopic slides. Therefore, there is a search for methodologies which enable observation and analyses of microscopic structures without special specimen preparation (e.g., Fioravanti et al., 2016, 2017). One such method could be confocal microscopy, which has already been successfully applied in material and surface science, although to date there are few studies in the field of life sciences and wood anatomy or dendrochronology (e.g., Haag et al., 2018).

The objective of the present study is thus to evaluate the possibilities of applying a Confocal Laser Scanning Microscope (CLSM) Olympus LEXT OLS5000 for different case studies in dendrochronology and quantitative wood anatomy, and to evaluate its suitability in replacing classical methods based on the time-consuming preparation of thin sections and light microscopy.

2 MATERIALS AND METHODS

2 MATERIAL IN METODE

2.1 MATERIAL

2.1 MATERIAL

We studied wood structure on cores (5 mm in diameter) collected from trees with an increment borer. The cores were smoothed by sanding (with sandpaper grits from 80 to 280) to observe

the cross-sections of the wood to identify the growth layers and observe their wood-anatomical characteristics.

The investigated species were:

- 1) temperate European beech (*Fagus sylvatica* L.) from a site in Croatia ca. 45.42°, Longitude: 16.28°, Altitude: ca. 100 m) (Lukić, 2018),
- 2) Mediterranean Aleppo pine (*Pinus halepensis* Mill.) from Alicante in southern Spain (Latitude: 38.40°, Longitude: -0.44°, Altitude: ca. 70 m),
- 3) ocote pine (*Pinus oocarpa* Schiede ex Schltdl.) from Siguatepeque in Honduras (Latitude: 14.60°, Longitude: -87.83°, Altitude: ca. 1100 m), from high elevation in the tropical zone, collected by Jean Pierre Veillon in 1970 (Luna-Lugo, & Marcano-Berti, 2011),
- 4) Tropical true mahogany (*Swietenia macrophylla* King) from Ticoporo in Venezuela (Latitude 8.15°, Longitude -70.84°, Altitude: ca. 200 m) collected by Jean Pierre Veillon in 1969 and
- 5) cedrela (*Cedrela odorata* L.) from Turrialba in Costa Rica (Latitude 9.90°, Longitude -83.69°, Altitude ca. 700 m) collected by Jean Pierre Veillon in 1966.

2.3 CONFOCAL LASER SCANNING MICROSCOPY

2.3 KONFOKALNA LASERSKA VRSTIČNA MIKROSKOPIJA

The objects were observed with a Confocal Laser Scanning Microscope (CLSM) Olympus LEXT OLS5000 (Olympus Corporation Tokyo 163-0914, Japan) (Figure 1) with the following objectives: MPLFLN5x (numerical aperture 0.15, working distance 20 mm), MPLFLN10xLEXT (numerical aperture 0.3, working distance 10.4 mm), and LM-PLFLN20xLEXT (numerical aperture 0.45, working distance 6.5mm). The microscope is equipped with a 405 nm violet laser, which enables the best lateral resolution in range (0.12 µm), and allows the microscope to capture fine patterns and defects that conventional optical microscopes, white-light interferometers, or red laser-based microscopes are unable to detect.

First, we used optical systems for colour observation. The system acquired microscope colour images by illuminating the sample with the white light-emitting diode (LED) light source and imaging the reflected light with the complementary metal-oxide-semiconductor (CMOS) image sensor.

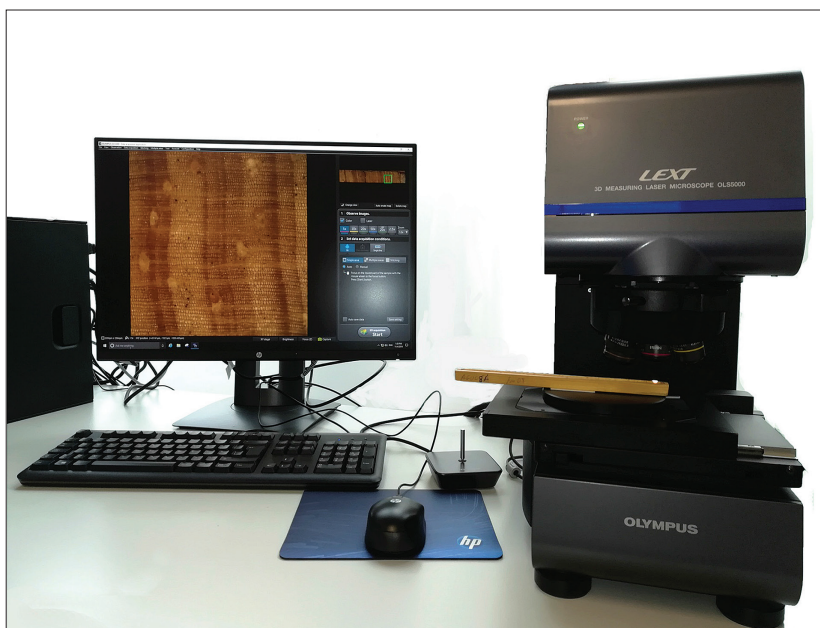


Figure 1. Workstation with a confocal laser scanning microscope LEXT OLS5000 3D (Olympus Corporation Tokyo 163-0914, Japan) equipped with an optical system for colour observation, image stitching, and image analysis software OLS5000.

Slika 1. Delo s konfokalnim laserskim vrstičnim mikroskopom LEXT OLS5000 3D (Olympus Corporation Tokyo 163-0914, Japonska), ki je opremljen z optičnim sistemom za opazovanje barvne slike, spajanje zajetih slik in programsko opremo za analizo slike OLS5000.

The objects were placed on the stage. By moving the stage, we used the system to create a panoramic macro map based on stitching images on the moving trace in real-time for an overview of the sample. Colour digital images obtained using the stitching function covered the entire length of the cores in order to identify the growth layers and perform tree-ring width measurements for dendrochronology. As the focal depth was relatively deep, we could use the colour images to observe some details or define the positions for detailed observations using the confocal laser scan.

High resolution laser confocal images were acquired at different focus positions to identify the minute structures and measure anatomical parameters. All the measurements were performed with the OLS5000 image analysis software.

3 RESULTS AND DISCUSSION

3 REZULTATI IN RAZPRAVA

3.1 DENDROCHRONOLOGY – GROWTH LAYERS AND ANOMALIES IN WOOD

3.1 DENDROKRONOLOGIJA – PRIRASTNE PLASTI IN ANOMALIJE V LESU

The presented examples show the potential of use of LEXT OLS5000 3D in different wood species, *Fagus sylvatica*, *Pinus halepensis*, *Pinus oocarpa*, *Swietenia macrophylla* and *Cedrela odorata*, with different wood anatomies and structures of the growth layers addressed for different research purposes.

The stitched images of polished increment cores based on combining the live observations (Figure 2) with the analysis of an acquired image enabled us to study the growth layers and growth ring boundaries at different magnifications.



Figure 2. Stitched images of increment cores acquired with a 5x objective at the resolution of 96 dpi. From top to bottom: *Fagus sylvatica*, *Pinus halepensis*, *Pinus oocarpa*, *Swietenia macrophylla* and *Cedrela odorata*.

Slika 2. Spojene slike izvrтков lesa, pridobljene z objektivom 5x povečave pri ločljivosti 96 dpi. Od zgoraj navzdol: *Fagus sylvatica*, *Pinus halepensis*, *Pinus oocarpa*, *Swietenia macrophylla* in *Cedrela odorata*.

At higher magnifications and resolutions, we could observe structural variations in the wood and apply image analysis. The detailed results for different wood species are shown in the next chapters.

3.2 TREE-RING ANOMALIES IN EUROPEAN BEECH

3.2 ANOMALIJE BRANIK V LESU BUKVE

European beech (*Fagus sylvatica*), as a typical temperate species, normally contains clear growth rings formed due to interruption of cambium activity during the cold period of the year (e.g., Prislan et al., 2013a, b). The investigated cores of *Fagus sylvatica* from a site near Petrinja in Croatia contained tree rings with numerous anomalies. This caused difficulties to correctly perform tree-ring width measurement and cross-dating (Lukić, personal communication). In 2013 numerous beech trees suffered an attack of gipsy moth (*Lymantria dispar*), which typically attacks oaks. In 2013 it caused also defoliation of beech (Lukić, 2018), and consequently an extremely narrow tree ring was formed (Figure 3). On the pictures observed under the stereomi-

croscope and on those obtained by flat-bed scanner, the tools usually applied in dendrochronology, the 2013 ring appeared pale and sometimes could not be recognised (Figure 3a). At higher magnification and resolution of CLSM, the tree ring and its structure could be identified. The 2013 ring showed early- and latewood and clear growth ring boundary (Figure 3d). Another extremely narrow tree ring was formed in 1966 (Figure 3c). It contains earlywood and narrow latewood and a clear growth ring boundary, which could indicate that cambial production was possibly interrupted at the end of June (Prislan et al., 2013a). The same species also had some very wide tree rings with intra-annual density fluctuations, like the 1984 tree ring, which at lower magnifications were erroneously interpreted as tree rings (Figure 3a, b), whereas higher magnification showed no growth ring boundary (Figure 3e).

The questions raised can as a rule be solved by dendrochronological cross-dating, while the application of CLSM enables better interpretation of the anomaly.

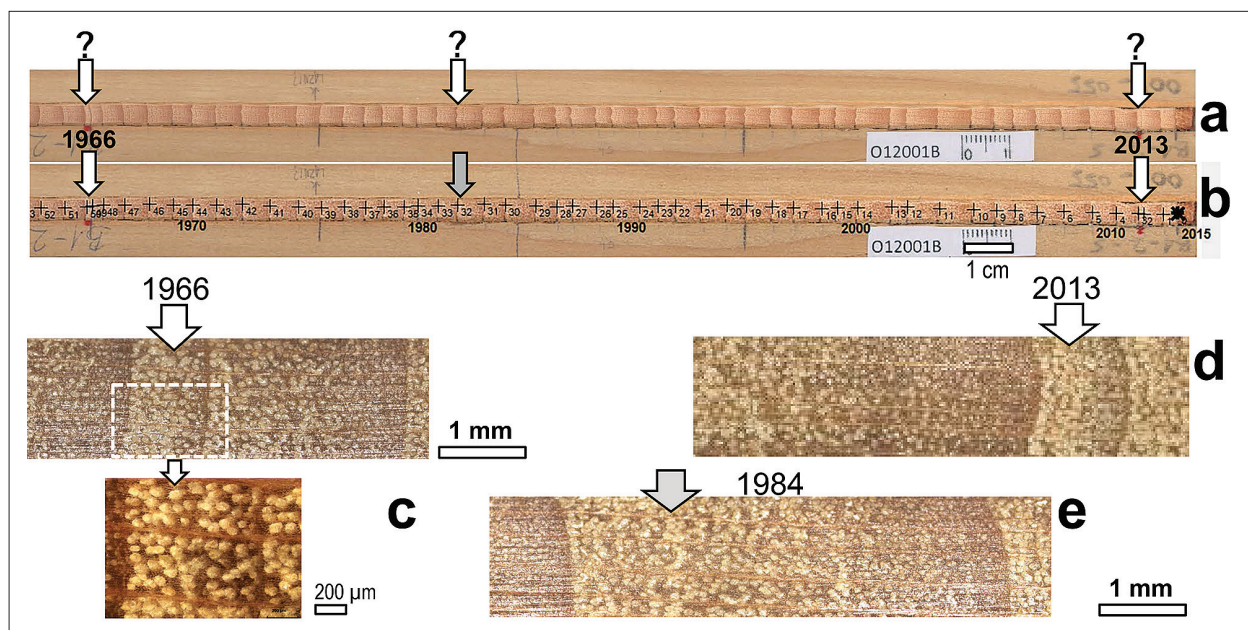


Figure 3. European beech (*Fagus sylvatica*): (a) a core scanned with a flat-bed scanner at 1200 dpi resolution with potentially very narrow rings (arrows), and (b) the same core after tree-ring width measurement in CooreRecorder and with cross-dating; (c, d, e) detailed views of individual rings captured by CLSM with tree ring 1966 at two magnifications (c), tree ring 2013 (d) and intra-annual density fluctuation in the 1984 ring (e).

Slika 3. Bukev (*Fagus sylvatica*): (a) izvrtek posnet s skenerjem z ločljivostjo 1200 dpi, z označenimi potencialno zelo ozkimi branikami (puščice) in (b) isti izvrtek po merjenju širin branik s pomočjo programa CooreRecorder in datiranju, (c, d, e) podrobna zgradba posameznih branik, posnetih s CLSM: branika 1966 pri dveh povečavah (c), branika 2013 (d) in gostotna variacija v braniki 1984 (e).

3.3 GROWTH RING ANOMALIES IN ALEPPO PINE 3.3 ANOMALIJE V PRIRASTNIH PLASTEH ALEPSKEGA BORA

Growth layers of Aleppo pine (*Pinus halepensis*) from Alicante in south-eastern Spain are often not annual and often contain numerous anomalies with intra-annual density fluctuations (IADFs) (Figure 4). They are characterised by latewood-like cells in earlywood (E-IADF), and earlywood like cells in different portions of latewood (L-IADF) (Figure 4). The species often have unclear growth ring bound-

aries (Figure 4b) which are a consequence of uninterrupted cambial production in winter (De Luis et al., 2007, 2011; De Micco et al., 2016a; Novak et al., 2016a; Balzano et al., 2018, 2019a, b), and lack of cambial dormancy in winter (Prislan et al., 2016). The species can also contain dark rings (Figure 4b), i.e. rings which contain only latewood (Novak et al., 2016a). Furthermore, the species contains numerous missing rings (Novak et al., 2011, 2016b). All these anomalies can only be detected with dendrochronological cross-dating.

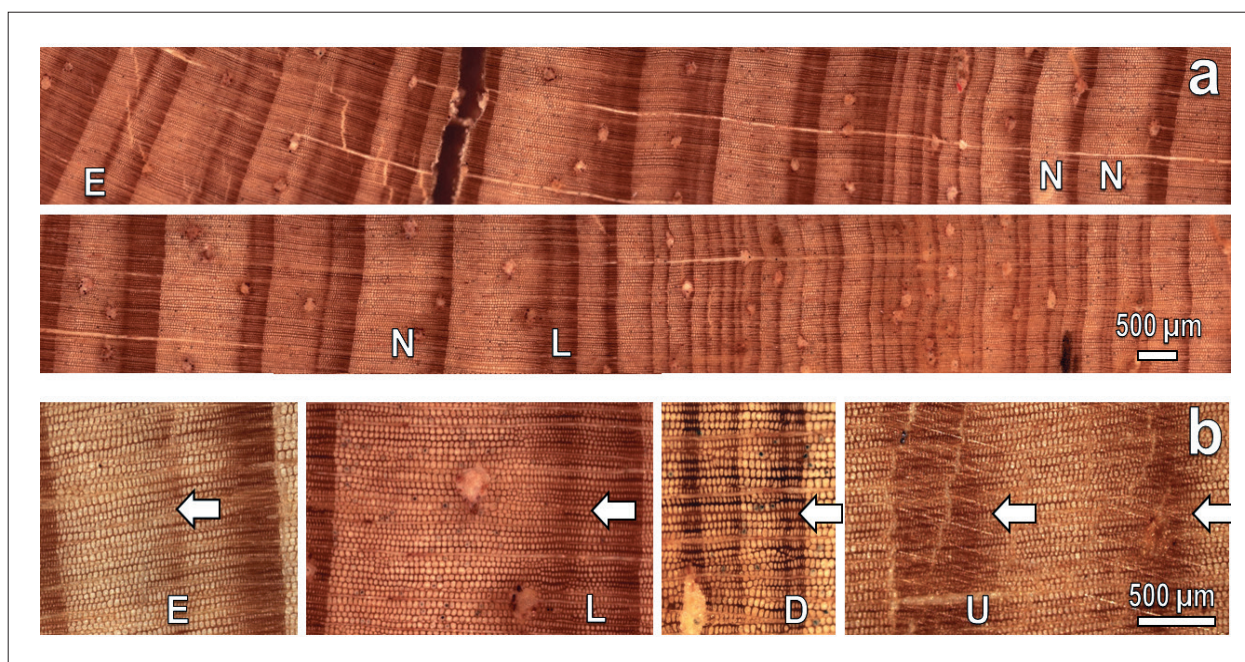


Figure 4. Mediterranean Aleppo pine (*Pinus halepensis*): (a) a core with “normal” rings (N) and a great variety of growth ring types and irregularities, and (b) a detailed view of growth rings containing E-IADF (latewood-like cells in earlywood) (E) and L-IADF (earlywood-like cells in latewood) (L); dark ring, composed of cells with latewood characteristics (D), and growth ring with unclear boundaries (U). Scale bars 500 μm.

Slika 4. Sredozemski alepski bor (*Pinus halepensis*): (a) pregled izvrtka, ki vsebuje »normalne« branike (N) in različne tipe anomalij v prirastnih plasteh ter (b) podrobni posnetki prirastnih plasti z gostotno variacijo tipa E-IADF (celice kasnega lesa v ranem lesu) (E) in L-IADF (celice ranega lesa v kasnem lesu) (L), temno braniko (D) in prirastno plastjo z nerazločnimi mejami (U). Merilne daljice 500 μm.

IADFs are usually first defined visually; however, their characteristics can be more precisely defined only by measuring the dimensions of tracheids or tracheid lumina, and the cell walls along the same radial in terms of tracheidogram (Vaganov, 1990). Such measurements are usually performed on thin cross-sections observed under a light microscope, after a time-consuming sample

preparation. The presented case study shows that it is possible to use CLSM and the associated image analysis system to obtain sufficient magnifications and resolutions, and thus to measure the tracheid parameters (Figure 5).

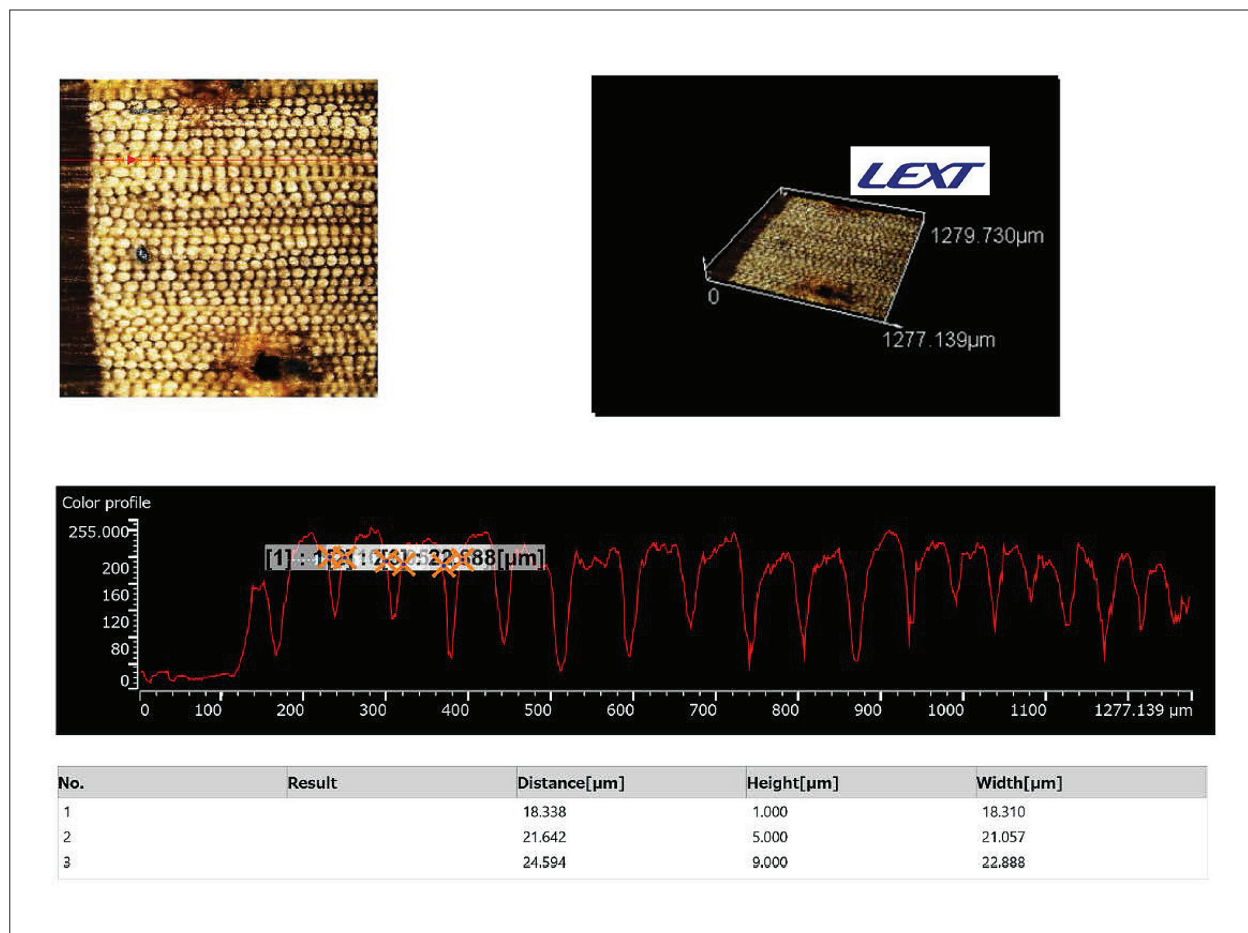


Figure 5. Wood of *Pinus halepensis* and measurement of cell wall thickness and dimensions of lumina (tracheids) of a radial row using Lext CLSM and image analysis software OLS5000. The laser system provides the intensity, colour and height of the image of the same object. Using the measurement option "profile" we can get the intensity, colour and height profiles in a chosen linear row and measure on it different parameters (here the diameter measurement of tracheids). The colour profile is also shown.

Slika 5. Les alepskega bora (*Pinus halepensis*): merjenje debelin celičnih sten in dimenzij lumnov (traheid) vzdolž radialnega niza traheid z uporabo CLSM Lext in programske opreme za analizo slike OLS5000. Laserski sistem zagotavlja intenzivnost, barvo in sliko po višini istega predmeta. Z možnostjo merjenja „profila“ lahko dobimo profile intenzitete, barve in višine v izbrani linearni vrstici in na njej izmerimo različne parametre (tukaj je prikazano merjenje premerov traheid). Prikazan je tudi barvni profil.

3.4 GROWTH LAYERS IN (*Pinus oocarpa*) FROM HONDURAS

3.4 PRIRASTNE PLASTI V BORU (*Pinus oocarpa*) IZ HONDURASA

Ocote pine (*Pinus oocarpa*), native to Central America, is poorly known in Europe. We inspected the wood structure of this species on cross-sections of the cores to evaluate the suitability for tree-ring analysis. Images at larger magnifications

showed that the species has tree rings with typical early- and latewood and clear tree-ring boundaries. Many rings contain E- or L- types of IADFs (Figure 6).

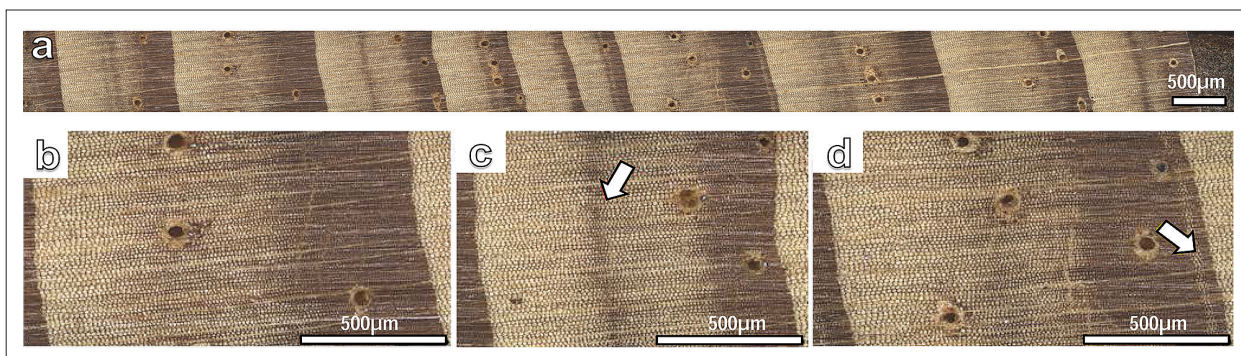


Figure 6. *Ocote pine* (*Pinus oocarpa*) from a high elevation site in Honduras: (a) overview of tree rings, (b) typical ring, (c) tree ring containing E-IADF (arrow), and (d) tree ring with L-IADF (arrow). Scale bars 500 µm.

Slika 6. Bor (*Pinus oocarpa*) z visokogorskega rastišča v Hondurasu: (a) pregled branik, (b) normalna branika, (c) branika, ki vsebuje gostotno variacijo tipa E-IADF (puščica) in (d) branika, ki vsebuje L-IADF (puščica). Merilne daljice 500 µm.

The first impression is that the structure of ocote pine tree rings is similar to that of tree rings of conifers (pines) growing in temperate environments. The investigated samples originated from the trees growing in Honduras, near Siguatepeque, ca. 1100 m above sea level, where the climate is characterised by three seasons: a wet and relatively cool season (between May and November) with sufficient rain to ensure vegetation, a colder interlude (November to February) with temperatures occasionally down to 8 °C, and a dry season (February to May), with temperatures of up to 32 °C (Wikipedia, 2019, El clima promedio en Siguatepeque, Honduras, 2019).

Although November to February temperatures are not very low, the clear growth ring boundaries could be a consequence of the interruption of cambial activity in winter. The change of seasons could explain the IADFs in the rings. Dendrochronological cross-dating supported by detailed anatomy studied with CLSM could help to explain the processes affecting IADF formation and interruption of cambial activity resulting in clear tree-ring boundaries.

3.5 GROWTH LAYERS IN TROPICAL TRUE MAHOGANY

3.5 PRIRASTNE PLASTI V TROPSKEM SREDNJEAMERIŠKEM MAHAGONIJU

True mahogany (*Swietenia macrophylla*) is a tropical hardwood species of the family Meliaceae that grows in Mexico, Central America, and tropical South America (except the Amazon basin). The species is also planted outside its natural range. It is listed in CITES, Annex II (Richter et al., 2017).

The wood of *Swietenia* contains heartwood which is brown to red-brown and can be differentiated from the sapwood. The air-dry density is 400–500–650 kg/m³ (Richter & Dallwitz, 2000). The species is characterised by interlocked grain and ribbon figures. The wood is diffuse-porous, vessels are of medium size with a tangential diameter in the range of 90–160–255 µm. The vessels regularly contain dark reddish-brown deposits. The fibres are as a rule septate and have medium-thick cell walls. The rays are multiseriate, mainly 1–2–4(–5) cells wide. Rays, axial parenchyma and vessel elements are storied, although storied rays cannot be observed in all specimens. Axial parenchyma is mainly banded. The bands are marginal (or seemingly marginal), mainly 4–8 cells wide and present growth ring boundaries. The wood also contains paratracheal axial parenchyma which is scanty to vasicentric.

The material presented in this study originates from Ticoporo near Barinas in Venezuela, which is characterised by a tropical climate with dry seasons (average annual temperature 27.6 °C, average temperature variation between the hottest and the coldest month less than 3 °C, average annual rainfall ca. 2500 mm) (Pereyra et al., 2005). The wood contains marginal parenchyma demarcating growth layers. It was found that the optimal technique and magnification should be used to recognise the growth layers (Figure 7). The dimensions of the vessels varied in the radial direction, as shown by image analysis of the CLSM system. This could be of help with defining the growth layers.

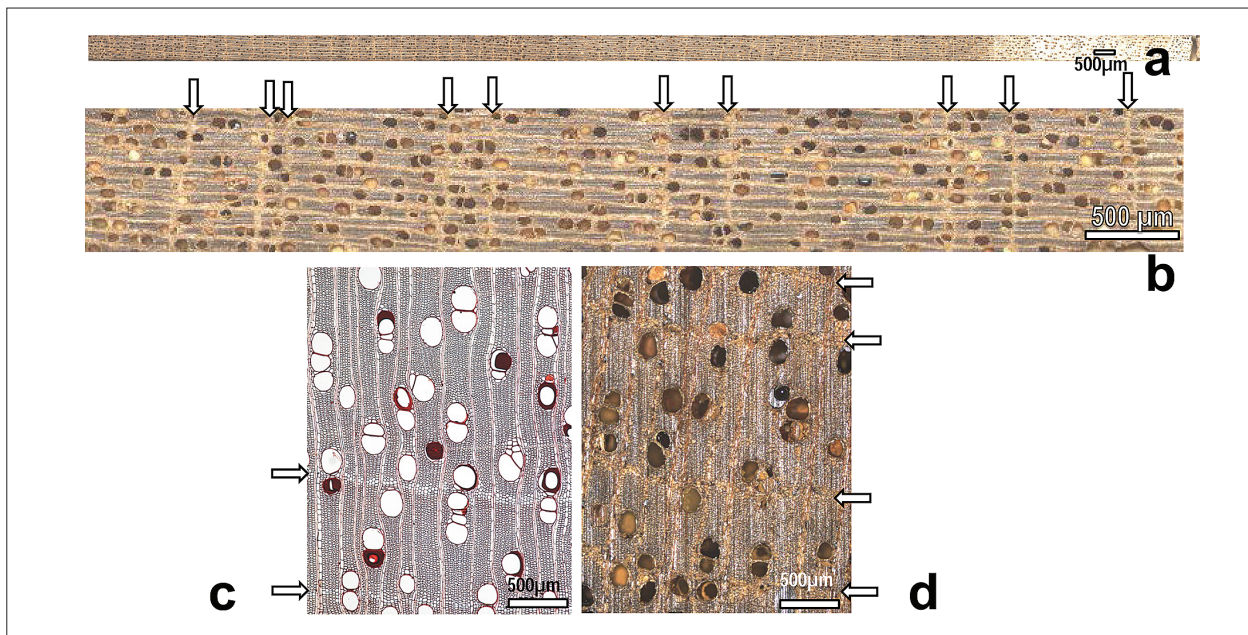


Figure 7. True mahogany *Swietenia macrophylla*: (a) stitched image of increment core acquired with CLSM with 5x objective at the resolution of 96 dpi, and (b) detail of the same image; (c) thin cross-section under the light microscope, and (d) wood at the same magnification observed with CLSM. Arrows show bands of marginal axial parenchyma. Scale bars 500 µm.

Slika 7. Pravi mahagoni *Swietenia macrophylla*: (a) spojena slika posnetkov prečnega prereza lesa iz izvrtka, pridobljena s CLSM, objektiv 5x povečave pri ločljivosti 96 dpi, in (b) podrobnosti iste slike; (c) prečni prerez tanke rezine pod svetlobnim mikroskopom in (d) les pri isti povečavi, posnet s CLSM. Puščice kažejo pasove marginalnega aksialnega parenhima. Merilne daljice 500 µm.

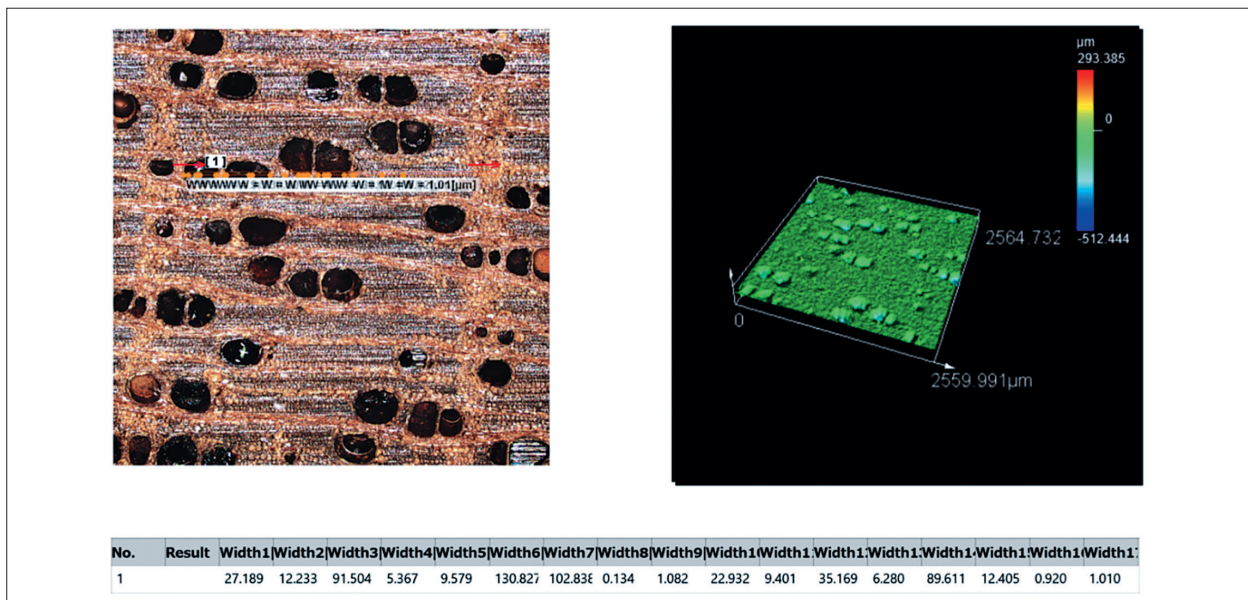


Figure 8. Measurement of vessel lumen diameter and area in *Swietenia macrophylla* in a linear row. Here are shown the colour profile and height profile obtained with a confocal laser scan.

Slika 8. Merjenje premerov in površine lumnov trahej v lesu mahagonija *Swietenia macrophylla*. Tu sta prikazana barvni profil in profil po višini objekta, pridobljena s konfokalnim laserskim skeniranjem.

Several studies have shown that dendrochronological methods might be possible for *Swietenia macrophylla* as the growth increments in the adult xylem are marked by terminal parenchyma bands and are annual (Dünisch et al., 2002, 2003). The same studies in *Swietenia macrophylla* in Brazil also showed that besides annual rings there are also non-annual increment zones, and false rings may occur. Furthermore, cambium showed dormant phases during the dry period in Brazil. Studies also indicated that the seasonal pattern of cambial growth of *Swietenia* and tropical tree species in general has to be analysed separately on each site before tree-ring chronologies can be established (Dünisch et al., 2002, 2003).

3.6 GROWTH LAYERS IN TROPICAL CEDRELA

3.6 PRIRASTNE PLASTI V TROPSKI CEDRELI

Cedrela, or Central American cedar (*Cedrela odorata*), is a hardwood from the Meliaceae family. The wood of *Cedrela* resembles that of *Swi-*

etenia (from the same family) and is of similar colour and density. The identification keys list only a few wood anatomical differences between the two taxa. *Cedrela* is generally semi-ring porous, has larger vessels, does not have storied structures and has a pleasant aromatic odour, whereas *Swietenia* is diffuse-porous, has medium-sized pores, storied structures and is odourless (Richter & Dallwitz, 2000).

The geographic distribution of *Cedrela* is Mexico and Central America, the Caribbean, and tropical South America. *Cedrela odorata* is on the CITES list of protected species, Appendix III (Richter et al., 2017).

Cedrela generally has distinct growth rings demarcated by marginal (or seemingly marginal) axial parenchyma bands, which are usually more than three cells wide (Richter & Dallwitz, 2000).

Detailed studies proved that also *cedrela* has growth layers which can be studied by dendrochronological methods (Dünisch et al., 2002, 2003). It was shown that the formation of increment zones

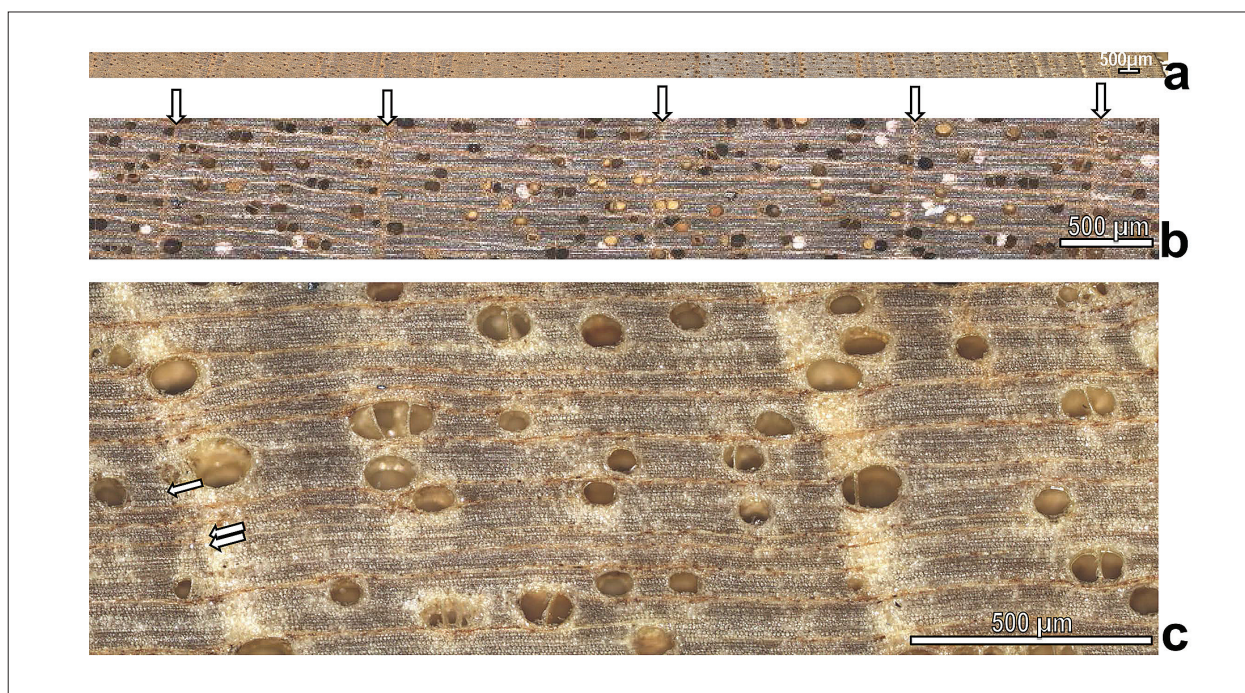


Figure 9. *Cedrela* (*Cedrela odorata*): (a) stitched image of increment core acquired with CLSM with 5x objective at the resolution of 96 dpi, and (b) details of the same image, arrows show growth ring boundaries; (c) detailed view of wood structure with bands of marginal axial parenchyma (double arrow) and fibres (arrow). Scale bars 500 µm. Slika 9. *Cedrela* (*Cedrela odorata*): (a) spojena slika posnetkov prečnega prereza lesa izvrtka, pridobljenih s CLSM, objektiv 5x povečave pri ločljivosti 96 dpi, in (b) podrobnosti iste slike, kjer puščice kažejo meje med prirastnimi plastmi; (c) prečni prerez lesa pri večji povečavi, kjer dvojna puščica kaže pas marginalnega aksialnega parenhima, enojna puščica pa vlakna. Merilne daljice 500 µm.

in cedrela was annual, and that annual growth increments are indicated by alternating fibre and vessel bands embedded in paratracheal parenchyma (Dünisch et al., 2002). Like in *Swietenia*, the cambium showed dormant phases during the dry period in Brazil (Dünisch et al., 2002, 2003).

The material presented in this study are the cores from trees from Turrialba in Costa Rica, at an altitude of ca. 700 m. We had an impression that the growth layers are less distinct than in *Swietenia* (Figure 9). Surface preparation of the cores was a possible reason that we could not find the alternating fibre and vessel bands embedded in paratracheal parenchyma reported by Dünisch and co-workers (2002).

In addition, less clear growth layers could be a consequence of climatic conditions in the area around Turrialba, which is characterised by significant rainfall, abundant even in the driest period. In Turrialba, the average annual temperature is 22.9 °C, and the average total annual rainfall is 2854 mm (Climate Turrialba, 2019).

4 CONCLUSIONS

4 ZAKLJUČKI

The case studies on cross-sections on wood of temperate European beech (*Fagus sylvatica*) from Croatia, Mediterranean Aleppo pine (*Pinus halepensis*) from Spain, ocote pine (*Pinus oocarpa*) from Honduras, as well as tropical true mahogany (*Swietenia macrophylla*) from Venezuela and cedrela (*Cedrela odorata*) from Costa Rica showed the potential of using a Confocal Laser Scanning Microscope (CLSM) Olympus LEXT OLS5000.

The optical system makes it possible to obtain colour images, while the option to create panoramic macro maps enables stitching of images, and therefore we can observe large portions of wood (i.e. the entire core). As the focal depth is relatively deep, it is possible to enlarge the colour images and observe small details as well as to define the positions for detailed observations using the confocal laser scanning mode. This all builds a system which enables non-destructive observation of detailed wood anatomy. Furthermore, the image analysis software OLS5000 enables the measurement of cell parameters.

Other advantages of CLSM are the possibility to work at ambient conditions on polished cross-sections under different magnifications, the ability to control the depth of field and collect serial optical sections from thick specimens.

The observation of the such details can significantly improve the recognition of growth layers and the boundaries between them, as well as help to study growth anomalies. This can vastly enhance the quality of dendrochronological studies.

The study presented here, which to the best of our knowledge is one of the first to apply this technique in the field of wood science comprehensively, helped us to evaluate the potential and the advantages of CLSM in comparison with classical wood anatomy and dendrochronology techniques.

5 SUMMARY

5 POVZETEK

Primerjalna in kvantitativna anatomija lesa v kombinaciji z dendrokronologijo lahko poda informacije o zgradbi lesa, ki so povezane z genetiko, zemljepisnim območjem, pa tudi s časom in razmerami, v katerih je les nastal. Raziskave anatomije lesa omogočajo analizo anatomskih znakov, ki lahko odražajo pomembne okoljske signale (Verheyden et al., 2004; García-González & Fonti, 2006; Campelo et al., 2010). Za proučevanje anatomskih znakov je običajno potrebna priprava in uporaba tankih mikroskopskih rezin za svetlobno mikroskopijo, kar pa je zelo zamudno. Zaradi tega iščemo metodologije, ki omogočajo opazovanje in analize mikroskopskih struktur brez posebne priprave vzorcev.

Cilj te študije je oceniti možnosti uporabe konfokalne laserske vrstične mikroskopije (CLSM) za različne študije v dendrokronologiji in oceniti primernost CLSM za nadomeščanje klasičnih metod, ki temeljijo na zamudni pripravi mikroskopskih rezin in svetlobni mikroskopiji.

Zgradbo lesa smo proučevali na izvrtkih premera 5 mm, odvzetih iz dreves s prirastoslovnim svedrom. Prečne površine na izvrtkih smo gladko zbrusili. V lesu različnih vrst iz različnih okolij smo proučevali prirastne plasti (branike), meje med njimi (letnice) (Torelli, 1990) in druge lesno-anatomske posebnosti.

Raziskali smo les naslednjih vrst: 1) navadna bukev (*Fagus sylvatica* L.) z rastišča na Hrvaškem

z zmerno klimo (Lukić, 2018); 2) sredozemski alepski bor (*Pinus halepensis* Mill.) z rastišča v Alicanteju na jugovzhodu Španije; 3) bor (*Pinus oocarpa* Schiede ex Schltdl) z rastišča z visoko nadmorsko višino v tropskem pasu v bližini kraja Siguatepeque v Hondurasu (Luna-Lugo, Marcano-Berti, 2011); 4) pravi mahagoni (*Swietenia macrophylla* King.) z območja Ticoporo v Venezueli s tropskim podnebjem in 5) cedrela (*Cedrela odorata* L.) iz kraja Turrialba v Kostariki s tropskim podnebjem.

Vse vzorce lesa smo opazovali s konfokalnim laserskim vrstičnim mikroskopom Olympus LEXT OLS5000 (Olympus Corporation Tokyo 163-0914, Japonska) (slika 1) z objektivni MPLFLN5x (numerična apertura 0,15, delovna razdalja 20 mm), MPLFLN10xLEXT (numerična apertura 0,3, delovna razdalja 10,4 mm) in MPLAPON20xLEXT (numerična apertura 0,6, delovna razdalja 1 mm).

Na osnovi posameznih slik, ki smo jih opazovali v živo, smo pripravili sestavljene slike celotnih izvirkov (slika 2), na katerih smo s programom za analizo slike lahko proučevali prirastne plasti in meje med njimi ter druge anatomske posebnosti pri različnih povečavah.

Raziskani izvrtki bukve (*Fagus sylvatica*) so vsebovali branike s številnimi anomalijami, ki so povzročile težave pri pravilnem merjenju širin branik in sinhroniziranju zaporedij širin branik. Leta 2013 so na proučenih drevesih zabeležili škode zaradi metulja gobarja (*Lymantria dispar*), ki sicer obžira hrastove liste (Lukić, 2018). Poškodbe listov bukve so imele za posledico nastanek izredno ozke branike v letu 2013 (slika 3). Branika 2013 je bila svetla (zaradi odsotnosti ali zelo ozkega pasu kasnega lesa) in je na mnogih vzorcih ni bilo mogoče prepoznati s pomočjo tehnik, ki jih običajno uporabljamo v dendrokronologiji (slika 3a). Pri večjih povečavah in ločljivosti je CLSM omogočil opazovanje anatomske zgradbe branike (slika 3d). Leta 1966 je tudi nastala zelo ozka branika (slika 3c), ki je vsebovala rani les in nekoliko kasnega lesa z jasno letnico, kar bi glede na dosedanje raziskave delovanja kambija pri bukvi (Prislan et al., 2013a) lahko pomenilo, da se je nastajanje lesa končalo že konec junija. Bukev je imela tudi nekaj zelo širokih branik z medletnimi nihanji gostote (IADF), kot na primer branika iz leta 1984. Te variacije smo pri manjših povečavah s stereo lupo pogosto napačno interpretirali kot branike (slika 3a, b), medtem ko so večje povečave s CLSM pokazale, da gre samo za gosto-

tne variacije (slika 3e). Prirastne plasti alepskega bora (*Pinus halepensis*) vsebujejo številne gostotne variacije (IADF), ki jih je starejša literatura poimenovala lažne branike (slika 4a, b), zato je na splošno težko ugotoviti, kaj je branika in ali je nastala znotraj istega koledarskega leta (De Luis et al., 2011; Novak et al., 2016a; Balzano et al., 2018, 2019). Vrsta ima pogosto nejasne meje med prirastnimi plastmi (slika 4b), ki so posledica neprekinjenega delovanja kambija tudi pozimi (Prislan et al., 2016). Pojavljajo se tudi »temne branike«, ki predstavljajo prirastne plasti, ki v osnovi vsebujejo le kasni les (Novak et al., 2016a) (slika 4c). Predstavljena študija kaže, da je mogoče uporabiti CLMS in pripadajoči sistem za analizo slike, za opazovanje lesa pri veliki povečavi in ločljivosti ter za merjenje dimenzij celic in celičnih sten (slika 5). Slednje omogoča izdelavo traheidograma (Vaganov, 1990), kar pripomore k prepoznavanju gostotnih variacij in rastnih anomalij.

Na posnetkih prečnih prereзов lesa bora (*Pinus oocarpa*) iz Hondurasa, z rastišča na visoki nadmorski višini 1100 m, ki se sicer nahaja v tropskem pasu, smo ob večjih povečavah lahko videli različne prirastne plasti. Te so imele zgradbo, tipično za branike lesnih vrst iz zmerne podnebja, ki vsebujejo rani in kasni les ter jasne letnice. Številne prirastne plasti vsebujejo tudi tipične gostotne variacije tipa E- in L-IADF (slika 6). Slednje so verjetno posledica menjave treh letnih časov na območju Siguatepeque v Hondurasu, z zelo različnimi temperaturami in padavinami. Dendrokronološko sinhroniziranje, podprto s podrobno analizo anatomske zgradbe s pomočjo CLSM, bi lahko pomagalo razložiti procese, ki vplivajo na nastajanje IADF in na prekinitev aktivnosti kambija, kar ima za posledico jasne meje med prirastnimi plastmi.

Predstavljamo tudi dendrokronološki potencial pravega mahagonija (*Swietenia macrophylla*) (Torelli, 1997, 2006), ki v našem primeru izvira iz tropskega območja v Venezueli. Prirastne plasti pri tem mahagoniju razmejujejo jasni pasovi marginalnega aksialnega parenhima. Posnetki s CLSM kažejo, da je za prepoznavanje prirastnih plasti treba izbrati optimalno (in ne nujno največjo) povečavo (slika 7). Slike kažejo, da premeri trahej od začetka do zaključka plasti upadajo in da jih je mogoče izmeriti s sistemom za analizo slike. Tudi spremljanje dimenzij trahej je v pomoč pri določanju prirastnih plasti.

Les cedrele (*Cedrela odorata*), iz družine Meliaceae, kamor spadajo tudi mahagoniji (Torelli,

1999), ima na splošno razločne prirastne plasti, razmejene s pasovi marginalnega aksialnega parenhima, ki so na splošno široki več kot tri celice (Richter & Dallwitz, 2000). Pri opazovanju lesa cedrele z rastišča v Kostariki smo imeli vtis, da so prirastne plasti manj izrazite kot pri proučevanem mahagoniju (slika 9). Površinska obdelava lesa, ki ima sicer nizko gostoto, je morda razlog, da nismo mogli jasno prepoznati izmenjave pasov vlaken in aksialnega parenhima, o katerih poročajo Dünisch in sodelavci (2002, 2003). Omenjeni raziskovalci so med drugim ugotovili tudi, da so prirastne plasti pri cedreli iz Brazilije bolj pravilne kot pri mahagoniju in da jih bolj upravičeno lahko imenujemo letne prirastne plasti (branike).

Predstavljene študije na primerih različnih lesnih vrst iz različnih okolij kažejo, da ima CLSM velik potencial in da omogoča preučevanje anatomske zgradbe lesa v povezavi s klasičnimi dendrokronološkimi tehnikami. Pri tem se izognemo zamudni pripravi mikroskopskih preparatov za svetlobno mikroskopijo, ki jih potrebujemo za klasičen pristop v anatomiji lesa. Naši rezultati so pokazali, da si od CSML lahko obetamo nadgradnjo dendrokronoloških študij rastnih anomalij v odvisnosti od časa.

Predstavljena študija je po naših podatkih ena prvih, ki predstavlja možnosti uporabe konfokalne laserske vrstične mikroskopije na področju anatomije lesa in dendrokronologije.

ACKNOWLEDGEMENTS

ZAHVALE

The study was supported by the Slovenian Research Agency (ARRS), program P4-0015 and Spanish Ministry of Economy project CGL2015-69985-R (CLIMED). The work of Klemen Novak was supported by contract no. C3330-19-952011 for co-financing the operation of the "Researchers-2.1-UL-BF-952011", by the Ministry of Education, Science and Sport of the Republic of Slovenia and EU European Regional Development Fund. The samples from South America were obtained in collaboration with Dr. Miguel F. Acevedo, and Dr. Giorgio Silvio Tonella Tognola, the samples from Croatia are part of doctoral work of Ivan Lukić. We thank Jože Planinšič and Luka Krže, for their support with the work in the laboratory. We are grateful to Paul Steed for English language editing.

REFERENCES

VIRI

- Balzano, A., De Micco, V., Merela, M., & Čufar, K. (2019a). Tree-rings in Mediterranean pines – can we ascribe them to calendar years? *Les/Wood*, 68 (1), 5-14.
- Balzano, A., Battipaglia, G., & De Micco, V. (2019b). Wood-trait analysis to understand climatic factors triggering intra-annual density-fluctuations in co-occurring Mediterranean trees. *IAWA Journal*, 1, 1-18.
- Balzano, A., Čufar, K., Battipaglia, G., Merela, M., Prislán, P., Aronne, G., & De Micco, V. (2018). Xylogenesi reveals the genesis and ecological signal of IADFs in *Pinus pinea* L. and *Arbutus unedo* L. *Annals of botany*, 121(6), 1231-1242.
- Campelo, F., Nabais, C., Gutiérrez, E., Freitas, H., & García-González, I. (2010). Vessel features of *Quercus ilex* L. growing under Mediterranean climate have a better climatic signal than tree-ring width. *Trees*, 24(3), 463-470. DOI: <https://doi.org/10.1007/s00468-010-0414-0>.
- Campelo, F., Nabais, C., Freitas, H., & Gutiérrez, E. (2007). Climatic significance of tree-ring width and intra-annual density fluctuations in *Pinus pinea* from a dry Mediterranean area in Portugal. *Annals of Forest Science*, 64(2), 229-238.
- Cherubini, P., Gartner, B. L., Tognetti, R., Braeker, O. U., Schoch, W., & Innes, J. L. (2003). Identification, measurement and interpretation of tree rings in woody species from Mediterranean climates. *Biological Reviews*, 78(1), 119-148.
- De Luis, M., Gričar, J., Čufar, K., & Raventos, J. (2007). Seasonal dynamics of wood formation in *Pinus halepensis* from dry and semi-arid ecosystems in Spain. *IAWA Journal* 28, 389–404.
- De Luis, M., Novak, K., Raventós, J., Gričar, J., Prislán, P., & Čufar, K. (2011). Climate factors promoting intra-annual density fluctuations in Aleppo pine (*Pinus halepensis*) from semiarid sites. *Dendrochronologia*, 29(3), 163-169.
- De Micco, V., Campelo, F., De Luis, M., Bräuning, A., Grabner, M., Battipaglia, G., & Cherubini, P. (2016a). Intra-annual density fluctuations in tree rings: how, when, where, and why? *IAWA Journal*, 37(2), 232-259.
- De Micco, V., Balzano, A., Čufar, K., Aronne, G., Gričar, J., Merela, M., & Battipaglia, G. (2016b). Timing of false ring formation in *Pinus halepensis* and *Arbutus unedo* in Southern Italy: outlook from an analysis of xylogenesi and tree-ring chronologies. *Frontiers in plant science*, 7, 705.
- De Micco, V., Carrer, M., Rathgeber, C. B. K., Camarero, J. J., Voltas, J., Cherubini, P., & Battipaglia, G. (2019). From xylogenesi to tree rings: wood traits to investigate tree-response to environmental changes. *IAWA Journal* 40(2): in press. DOI: <https://doi.org/10.1163/22941932-40190246>.
- Dünisch, O., Bauch, J., & Gasparotto, L. (2002). Cambial growth dynamics and formation of increment zones in the xylem of *Swietenia macrophylla* King., *Carapa guianensis* Aubl., and *Cedrela odorata* L. (Meliaceae). *IAWA Journal* 23:101–119.
- Dünisch, O., Montóia, V., & Bauch, J. (2003). Dendroecological investigations on *Swietenia macrophylla* King and *Cedrela odorata* L. (Meliaceae) in the central Amazon. *Trees*, 17, 244-250.

- Fioravanti, M., Giulio, G., & Signorini, G. (2016). A non-invasive approach to identifying wood species in historical musical instruments. *Journal of Cultural Heritage*, 27 (supplement), S70-S77.
- Fioravanti, M., Giulio, G., Signorini, G., Rossi Rognoni, G., Sodini, N., Tromba, G., & Zanini, F. (2017). Non-invasive wood identification of historical musical bows. *IAWA Journal*, 38 (3), 285–296.
- García-González, I., & Fonti, P. (2006). Selecting earlywood vessels to maximize their environmental signal. *Tree physiology*, 26(10), 1289-1296.
- Kaennel, M., & Schweingruber, F. H. (1995). Multilingual glossary of dendrochronology: Terms and definitions in English, German, French, Spanish, Italian, Portuguese and Russian. Paul Haupt.
- Haag, V., Kirsch, S., Koch, G., Zemke, V., & Richter, H. G. (2018). Non-destructive investigation of historical Instruments based on 3D-reflected-light microscopy and high resolution μ -X-ray CT. In: Pérez, M. A., Marconi, E. (eds.). *Wooden musical instruments different forms of knowledge: book of end of Wood Musick COST Action FP1302*. Paris: Cité de la musique - Philharmonie de Paris, 143-151.
- Luna-Lugo, A., & Marcano-Berti, L. (2011). Bosques de Venezuela: un Homenaje a Jean Pierre Veillon, *BIOLLANIA* 10, 1-3.
- Lukić, I. (2018). Redefiniranje značaja gubara (*Lymantria dispar*L.) kao šumskoga štetnika u republici Hrvatskoj. Doctoral dissertation, Zagreb, Sveučilište u Zagrebu, Šumarski fakultet, 155 p.
- Novak, K., De Luis, M., Čufar, K., & Raventós, J. (2011). Frequency and variability of missing tree rings along the stems of *Pinus halepensis* and *Pinus pinea* from a semiarid site in SE Spain. *Journal of Arid Environments* 75: 494-498.
- Novak, K., De Luis, M., Gričar, J., Prislán, P., Merela, M., Smith, K. T., & Čufar, K. (2016a). Missing and dark rings associated with drought in *Pinus halepensis*, *IAWA Journal*, 37 (2): 260-274.
- Novak, K., De Luis, M., Saz, M. A., Longares, L. A., Serrano-Notivoli, R., Raventós, J., Čufar, K., Gričar, J., Di Filippo, A., Piovesan, G., Rathgeber, C. B. K., Papadopoulos, A., & Smith, K. T. (2016b). Missing rings in *Pinus halepensis* – the missing link to relate the tree-ring record to extreme climatic events. *Frontiers in Plant Science*, Volume 7, Article 727, DOI: <https://doi.org/10.3389/fpls.2016.00727>.
- Pereyra, J., Vargas, F., Ochoa, G., Oballos, J., & Alvarado, C. (2005). Caracterización de tierras de la unidad experimental, reserva forestal de Ticoporo, Barinas, Venezuela, con fines de ordenamiento. *Interciencia: Revista de ciencia y tecnología de América*, 30 (4), 221-227.
- Prislán, P., Gričar, J., de Luis, M., Smith, K. T., & Čufar, K. (2013a). Phenological variation in xylem and phloem formation in *Fagus sylvatica* from two contrasting sites. *Agricultural and Forest Meteorology*, 180, 142-151.
- Prislán, P., Čufar, K., Koch, G., Schmitt, U., & Gričar, J. (2013b). Review of cellular and subcellular changes in the cambium. *IAWA Journal*, 34 (4), 391-407.
- Prislán, P., Gričar, J., Novak, K., Martínez Del Castillo, E., de Luis, M., Schmitt, U., Koch, G., Štrus, J., Mrak, P., Tušek Žnidarič, M., & Čufar, K. (2016). Cambial rhythm of *Pinus halepensis* and *Pinus sylvestris* from different environments indicate tree adaptation to local conditions. *Frontiers Plant Sciences*, Volume 7, Article 1923, DOI: <https://doi.org/10.3389/fpls.2016.01923>.
- Richter, H. G., & Dallwitz, M. J. (2000 onwards). Commercial timbers: descriptions, illustrations, identification, and information retrieval. In English, French, German, Portuguese and Spanish. Version: May 2000.
- Richter, H. G., Gembruch, K., & Koch, G. (2017). CITES Wood ID –Innovative medium for education, information and identification of CITES protected trade timbers. BN German Federal Agency for Nature Conservation and Thünen Institute, Germany, computer program, version in English 2017.
- Torelli, N. (1990). Les & skorja - wood & bark, slovar strokovnih izrazov. Ljubljana, Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za lesarstvo, 71 p.
- Torelli, N. (1997). Mahagoni (I. del), njegova nomenklatura in zgodovina rabe. *Les*, 49 (11), 345-348.
- Torelli, N. (1999). Cedre (*Cedrus* spp.), cedri (*Cedrela* spp.) in "cedre". *Les* 51 (6), 169-172.
- Torelli, N. (2006). Mahagoni (*Swietenia* spp.) - naravna in kulturna zgodovina. *Gozdarski vestnik*, 64 (5-6), 246-252.
- Vaganov, E. (1990). The tracheidogram method in tree-ring analysis and its application, in *Methods of Dendrochronology: Applications in the Environmental Sciences*, eds E. Cook and L. Kairiukstis (Dordrecht: Kluwer Academic), 63–76.
- Verheyden, A., Kairo, J. G., Beeckman, H., & Koedam, N. (2004). Growth rings, growth ring formation and age determination in the mangrove *Rhizophora mucronata*. *Annals of botany*, 94(1), 59-66.
- Wikipedia, Siguatepeque, URL: <https://en.wikipedia.org/wiki/Siguatepeque> [15. 10. 2019].
- El clima promedio en Siguatepeque Honduras. URL: <https://es.weatherpark.com/y/13702/Clima-promedio-en-Siguatepeque-Honduras-durante-todo-el-a%C3%B1o#Secciones-Sources> [15. 10. 2019].
- Climate Turrialba. URL: <https://en.climate-data.org/north-america/costa-rica/cartago/turrialba-28663/> [15. 10. 2019].