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Genetic diversity and wood anatomy of pines in Al-Jabal Al-Akhdar, Libya: A DNA barcoding approach

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Description of the topic. Geographical distribution, historical occurrences, and human activity are some of the variables that affect the genetic diversity of *Pinus* species. Understanding these patterns is critical for conservation efforts and for adapting forest management strategies to climate change.
Objectives. The contrasting levels of genetic diversity among *Pinus* species underscore the importance of species-specific approaches in conservation and management programs. DNA barcoding is an effective method for identifying diverse plant species across taxa and ecosystems.
Method. In this study, *Pinus* species were classified, and their geographical distribution was studied in the Al-Jabel Al-Akhdar region, growing at different altitudes in natural forests and afforested. The anatomical composition of the wood was examined, including tracheid wall thicknesses, latewood thickness, ray parenchyma area, number of resin ducts, ray height, and fiber length. Additionally, DNA barcoding was conducted using *rbcL* primers.
Results. The results of the cross-sectional examination of the wood of *Pinus* species showed significant differences among the species. However, the ray parenchyma area showed no significant differences among the species. Chloroplast *rbcL* regions were used as barcode markers to identify 10 *Pinus* plants. The results highlight the need for an advanced DNA barcode reference library with broad species coverage for accurate species identification.
Conclusions. This study not only provides insights into the diversity and taxonomy of *Pinus* but also contributes to the ongoing conservation of *Pinus* resources and supports sustainable resource management in the region. DNA barcoding technology is critical for taxonomy and biodiversity studies because it allows rapid and accurate species identification in forests.
Keywords. *Pinus* species, highlands, wood properties, biodiversity, Tajima's D, *rbcL*, sequencing.

Diversité génétique et anatomie des pins d'Al-Jabal Al-Akhdar, Libye : une approche par code-barres ADN
Description du sujet. La distribution géographique, les occurrences historiques et l'activité humaine font partie des variables qui influencent la diversité génétique des espèces de *Pinus*. Comprendre ces schémas est essentiel dans les efforts de conservation et pour adapter les stratégies de gestion forestière aux changements climatiques.
Objectifs. Les niveaux contrastés de diversité génétique entre les espèces de *Pinus* soulignent l'importance d'approches spécifiques à chaque espèce dans les programmes de conservation et de gestion. Le code-barres ADN constitue une méthode efficace pour identifier diverses espèces végétales à travers les taxons et les écosystèmes.
Méthode. Dans cette étude, les espèces de *Pinus* ont été classifiées et leur distribution géographique a été étudiée dans la région d'Al-Jabel Al-Akhdar où elles poussent à différentes altitudes dans des forêts naturelles et reboisées. La composition anatomique du bois a été examinée, notamment l'épaisseur des parois des trachéides, l'épaisseur du bois final, la surface du parenchyme des rayons, le nombre de canaux résinifères, la hauteur des rayons et la longueur des fibres. De plus, le code-barres ADN a été réalisé à l'aide des amorces *rbcL*.
Résultats. Les résultats de l'examen en coupe transversale du bois des espèces de *Pinus* ont montré des différences significatives entre les espèces. Cependant, la surface du parenchyme des rayons ne présentait pas de différences significatives entre elles. Les régions chloroplastiques *rbcL* ont été utilisées comme marqueurs de code-barres pour identifier 10 plants de *Pinus*. Les résultats soulignent la nécessité de disposer d'une bibliothèque de référence avancée de codes-barres ADN couvrant un large éventail d'espèces pour une identification précise des espèces.
Conclusions. Cette étude ne fournit pas seulement des éclaircissements sur la diversité et la taxonomie du *Pinus* mais elle contribue également à la conservation continue des ressources en *Pinus* et soutient la gestion durable des

ressources dans la région. La technologie du code-barres ADN est essentielle pour les études de taxonomie et de biodiversité car elle permet une identification rapide et précise des espèces forestières.
Mots-clés. Espèces de *Pinus*, région d'altitude, propriété du bois, biodiversité, Tajima's D, *rbcL*, séquençage.

1. INTRODUCTION

Al-Jabel Al-Akhdar, located in northeastern Libya, is a region characterized by its unique geological, hydrological, and ecological features. The region boasts a diverse flora, with studies identifying 317 vascular plant taxa, including many endemic species. The ecological studies highlight the Mediterranean plant communities, which are crucial for maintaining local biodiversity and ecological balance (Alaib et al., 2017). Taxonomy is concerned with the definition and classification of all plants found on land and in water to date (Vidakovic, 1991). The oldest known classification system is the industrial classification of *Theophrastus*, which divided plants into three groups: trees, shrubs, and grasses. The second period developed in the 16th century, when the Italian botanist Andrea Cesalpino classified plants according to their fruits and seeds. The Swedish botanist Carl von Linné invented the binomial nomenclature system, while Charles Darwin developed basic concepts in his book *On the Origin of Species* (Vidakovic, 1991). Most pine species are found in the northern hemisphere, with fossilized conifers found in Asia, the Soviet Union, and the west coasts of France and the United States. The pine genus has been classified by many scientists, with key factors based on the anatomical and phenotypic characteristics of the needle. In addition, the anatomical properties of pine wood are of great importance in classifying the genus pine through the identification of many traits. The presence of tracheids is the most important one (Ickert-Bond, 2001). The classification of conifers in the Al-Jabal Al-Akhdar region-east of Libya has been studied using phenotype and needle anatomy characters, and an identification key has been developed based on these characters (Tashani & Ali, 2021).

The pine family, known as Pinaceae, comprises a diverse range of species found primarily in the northern hemisphere. This family is characterized by a complex evolutionary history involving significant gene flow and hybridization events among its members. Phylogenetic relationships within Pinaceae have been reconstructed using advanced genomic techniques, revealing intricate patterns of divergence and admixture among different pine species (Jiang et al., 2024). In Libya, about five pine species grow in the Al-Jabal Al-Akhdar region. *Pinus halepensis* L. is a small to medium sized tree with a trunk diameter of 17.73 cm. The bark is orange red in color, of considerable thickness, and with a distinctive fissured texture. The needles are slender yellowish-green, and the cones are narrow and conical. The seeds are 6 mm long, have a 2 cm wing, and are dispersed by wind (Houminer et al., 2022; Harfouch et al., 2003). It currently grows well in the forests of Shehat, Sidi Al-Hamri, Mador Al-Ziton, Marawa-Qandula, Ghout Al-Sultan, Tacness, Slanta, Bilang, and Ras Al-Hilal (Wadi Morcos), where it is planted as trees and shelterbelts, alone or mixed with *Pinus pinea* L., *Pinus brutia* and Italian cypress, *Pinus brutia* Ten. The tree is characterized by its conical shape, dense bark, and rectangular buds. The foliage consists of dark green, thin, serrated needles, while the flowers are produced in inflorescences. The cones are oval and have no stalk, while the seeds are brown to black in color. This species is found in the form of shelterbelts in the Gharika site of the Al-Jabal Al-Akhdar region at an altitude of 780 m (Zunni & Bayoumi, 2006). *Pinus pinea* grows in the Wardama site at an altitude of 625 m. It is also spread in the Al-Rajma forests in Al-Jabal Al-Akhdar region. The tree was characterized by a height of 4.8 m and a diameter of 30 cm, exhibiting a circular crown morphology. Its bark is reddish-brown. The cones are oval or spherical, and the seeds are large, measuring 1.750 cm in length, with a reduced wing that separates quickly (Tashani & Ali, 2021). *Pinus massoniana* var. massoniana is a tree characterized by a narrow conical crown, thick reddish-brown bark, and dark green needles. The dry weight of 100 needles was 10.1 g. The tree produces large, conical cones. The seeds are large, dark gray, and mottled (Eckenwalder, 2009; Farjon, 2010). In the Al-Jabal Al-Akhdar region, it grows in the Fayidia site, which is at an altitude of 767 m. *Pinus heldreichii* H.Christ is a tree that reaches a length of 6.8 m and a diameter of 33.3 cm. It has a broad and spreading crown and a dark gray to black bark. The needles are dark green and grow in two fascicles, measuring 14.5 cm in length. The cones are 8.33 cm long and 3.58 cm wide, have no petiole, and are conical in shape (Vendramin et al., 2008).

To effectively identify and understand the relationships among these diverse *Pinus* species, analysis of robust molecular approaches is necessary. In this context, DNA barcoding emerges as a pivotal technology. The objective of DNA barcoding is to achieve rapid and accurate species identification by sequencing a short DNA sequence or a few DNA regions (Li et al., 2011). However, the amplification

and sequencing of universal nuclear gene primers across different angiosperm taxa is a major challenge. The use of DNA barcodes derived from chloroplast genes is a common practice in plant phylogenetic studies. The *rbcL* (ribulose bisphosphate carboxylase/oxygenase large subunit) locus has been shown to be a valuable tool for comparative analysis at the family and genus level. This marker has been extensively studied in the plastid genome, with broad representation from all major groups and a substantial number of sequences available in GenBank (Newmaster et al., 2006). A substantial body of evidence suggests that *rbcL* should be used as a core barcode marker for the molecular identification of land plants (Hollingsworth et al., 2009; Li et al., 2011). In addition to selecting appropriate DNA barcode fragments, it is also essential to collect a significant number of individuals from diverse populations within a species to establish a comprehensive reference database that can be universally applied (Bolson et al., 2015; Guo et al., 2015). The selection of *rbcL* as a barcode marker for *Pinus* species is based on the complementary and unique characteristics of this marker. *rbcL* is recognized for higher variability and resolving power at the species level (Ismail et al., 2020) and exhibits greater universality and ease of amplification across a wide range of plant taxa (Nurhasanah & Papuangan, 2019).
Nevertheless, the complicated physical characteristics of the genus and the considerable intraspecific variability have made the accurate identification of *Pinus* species challenging. The similarity of morphological traits, including leaf morphology and stem pigmentation between closely related species can lead to misidentification and a subsequent misunderstanding of their ecological functions. Despite the importance of the encyclopedia of Libya's plants and the valuable information that it contains about the plant factions in Libya, it lacks mention of the pine species that are widespread in Libya, especially in the Al-Jabal Al-Akhdar region where only *P. halepensis* and *P. canariensis* were noted. Therefore, the aim of this research is to ensure that conifer species are classified on the basis of wood anatomy and genetic characteristics.

2. MATERIALS AND METHODS

2.1. The study area

The Al-Jabel Al-Akhdar region (JAR) is located between longitude 32° and 33°N and 20° to 23°E. The region spans approximately 360 km in length and 60 km in width from the seashore (**Figure 1**).

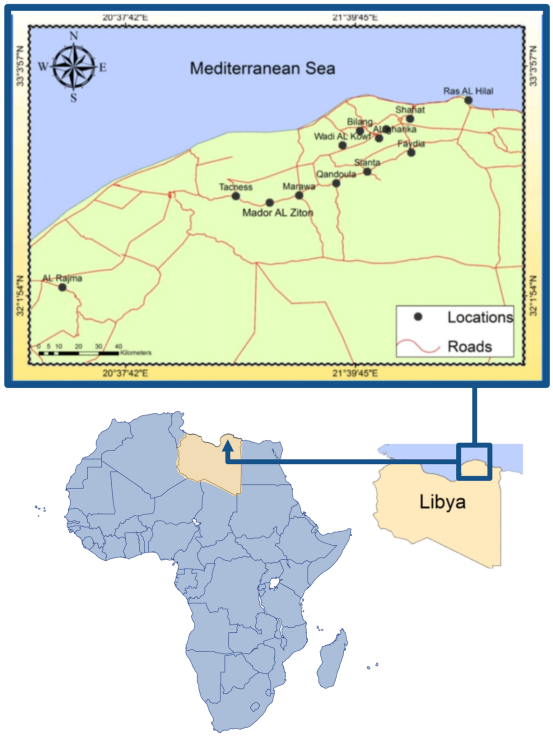

Figure 1. Location map of the study area – *Carte de localisation de la zone d'étude.*

2.2. Plant materials

Needle samples of *P. halepensis*, *P. brutia*, *P. pinea*, *P. massoniana* var.massoniana, and *P. heldreichii* (**Figure 2**) were collected from the Al-Jabel Al-Akhdar region. For each species included in the study, we selected 10 mature and healthy pine trees from various natural growth sites. Within each site, trees were selected in a single row, spaced approximately 20 m apart. This spacing was maintained to minimize potential environmental interference. Needles were randomly collected from well-isolated canopy parts at 1 m ground level from each tree. After collection, needle samples were immediately stored at -80 °C until further use.

Figure 2. Trees of pine species under study– *Espèces de pin étudiées.*

A. *Pinus halepensis*; **B**. *Pinus brutia*; **C**. *Pinus pinea*; **D**. *Pinus massoniana* var.massoniana; **E**. *Pinus heldreichii*.

2.3. DNA isolation, amplification, and sequencing

One gram of needle tissue was frozen in liquid nitrogen and homogenized using the CTAB (cetyl-tetramethyl ammonium bromide) method, according to Doyle (1990). Quantification of total DNA was performed using a Thermo Fisher Scientific Inc. NanoDrop 2000 Spectrophotometer Version 1.4.1. The DNA barcoding gene ribulose 1,5-biphosphate carboxylase (*rbcL*) was performed by Sigma-Aldrich

Company, GATC Company (Germany): *rbcL* -F: 5'-TGT CAC CAC AAA CAG AAC TAA AGC-3' and *rbcL* -R: 5,-GTA AAA TCA AGT CCA CCR CG-3' (Tashani & Aggag, 2020). PCR amplifications were performed in 20 µl: 10 µl PCR master mix (Promega GoTaq® Green), 4 µl H_2O, 2 µl of the forward primer (10 µM), 2 µl of the reverse primer (10 µM), and 2 µl template DNA (50 ng). Reactions were optimized according to the recommended protocol of 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 54 °C for 30 s and extension at 68° C for 40 s, and final extension at 68 °C for 6 min and 16 °C for 2 min. The PCR products were separated by agarose gel electrophoresis (1% agarose) at 80 V for 50 min (Tashani & Aggag, 2020).

2.4. DNA barcode analysis

The resulting PCR product was excised from the gel and purified using a MEGAquick-spin™ (INtRON) total fragment DNA purification kit. The gel-purified DNA bands were sequenced on an automated sequencer using the Sanger method by Macrogene Company (Korea). The generated sequences were deposited at the National Center for Biotechnology Information (NCBI) and the basic local alignment search tool (BLAST) network service (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (S1) was used. The alignment process was conducted manually, with the incorporation of criteria that considered secondary structures and mutational mechanisms. This was done to provide guidance for the strategic placement of gaps, as outlined by Kelchner (2000).

2.5. Genetic diversity analysis

The sequences obtained were also subjected to analysis to determine the average AT% and GC% nucleotide compositions for the *rbcL* marker. A comprehensive investigation into genetic diversity was conducted using the MEGA11 program (Tamura et al., 2021), with a particular focus on segregating sites and nucleotide diversity across the five sequences. Subsequently, the Tajima's D test was applied to assess deviations from neutrality, thereby providing valuable information about the demographic history and evolutionary processes within the studied *Pinus* species.

2.6. Tree-building method

We used MEGA 11 program, a tree-building method (Tamura et al., 2021) to construct a Neighbour-Joining (NJ) tree for the *rbcL* marker. The ratio of successfully identified species from all sampled species was calculated as the proportion of species that were discriminated. The sequence alignments were compared together with other *Pinus* species available in the GenBank database (http://www.ncbi.nlm.nih.gov).

2.7. Wood anatomy and morphometric studies

Wood anatomy properties. Ten trees of each species at different elevations were selected. Two-inch discs (1 × 1 × 3 cm) were prepared in accordance with ISO 13061-14 (2016) and taken from each pine tree. Blocks were cut into 200-micrometer-thick transverse, tangential, and radial sections using a sliding microtome.

Fiber length. The separation of individual wood fibers was performed using Franklin's (1945) method, through which a wood specimen with the dimensions of 15 × 10 × 2 mm was saturated in a mixture (1:1) of acetic acid and oxygenized water in test tubes. Afterwards, the specimens were kept in an oven at 65 ± 3 °C for 48 h. After maceration, the specimens were washed (2-3 times) in distilled water and then immersed in distilled water. Then the shacked and the biometric parameters fiber length were evaluated by light microscopy. From each slice, at least 100 fibers were used for the measurements.

Statistical analysis. This study involved conducting a statistical analysis using SPSS software (version 25). A one-way analysis of variance (ANOVA) was performed to assess significant differences between *Pinus* species for each anatomical parameter. When ANOVA indicated statistically significant differences ($p < 0.05$), post hoc comparisons of means were carried out using Tukey's honestly significant (Tukey HSD) test.

3. RESULTS

Genomic DNA extraction from five *Pinus* samples revealed high molecular weight and comparable concentrations. The efficacy of DNA barcodes is contingent upon the efficiency of polymerase chain reaction (PCR) amplification and the accuracy of the primers employed. In this study, the *rbcL* marker exhibited a 100% success rate in amplification (around 900 bp). **Table 1** presents the species identified by the *rbcL* barcode primer in the BLAST search, along with their respective accession numbers, and compares them with the taxonomist's identification. The results indicate a high degree of similarity among the *Pinus* strains, with most samples producing sequences classified as 99-98%. Moreover, the results illustrate a robust correlation between morphological and molecular identification at the genus level using this marker.

Table 1. Identification of *Pinus* species using *rbcL* barcodes – *Identification des espèces de* Pinus *en utilisant les code-barres* rbcL.

Sample	**Taxonomy identity**	**Species name**	**Gene bank accession number**	**Percentage of homology** (%)	**Query cover** (%)
A	*Pinus halepensis* Mill.	*Pinus halepensis* Mill.	JN854197.1	99.76 98.6	95 F 97 R
B	*Pinus pinea* L.	*Pinus pinea* L.	FN689374.1	99.30 99.05	97 F 96 R
C	*Pinus canariensis*	*Pinus massoniana* var. massoniana	MW537595.1	99.52 99.52	95 F 94 R
D	*Pinus brutia* Ten.	*Pinus brutia* Ten.	AB019820.1	99.52 99.76	96 F 94 R
E	*Pinus nigra* J.F.Arnold	*Pinus heldreichii* H.Christ	MT238042.1	98.63 99.52	98 F 93 R

3.1. Genetic diversity analysis

The study aimed to gain insight into the genetic diversity of pine species by summarizing the genetic diversity observed in the *rbcL* marker across five sequences. Twenty-three segregating sites were identified, as shown in **tables 2** and **3**. Visual inspection of the alignment revealed that these polymorphic sites were clustered in specific regions, particularly at positions 3-9, 425-462, and 879-885. Additionally, specific sites, such as positions 427, 428, 431, 443, and 446, exhibited unique nucleotide states for sequence E, and position 879 was unique for sequence D. These 23 segregating sites yielded a comparatively high nucleotide diversity (π) of 0.0137. The Tajima's D test yielded a value of 0.780, suggesting the potential for positive selection, population expansion, or purifying selection. Haplotype diversity (Hd) was calculated as 1.0. All five sampled *Pinus* sequences represent unique haplotypes, suggesting a high degree of haplotypic variation within this limited sample.

Table 2. Results from Tajima's neutrality test – *Résultats du test de neutralité de Tajima*.

Number of sequences	**Number of segregating sites**	p_s	Θ	π	D
5	23	0.025901	0.012432	0.013739	0.780184

p_s = *S*/total number of sites – *S/nombre total de sites*; $\Theta = p_s/a_1$; π = nucleotide diversity – *diversité nucléotidique*; *D* : Tajima test statistic – *test statistique de Tajima.*

Table 3. Summary of segregating sites in the *rbcL* region - *Résumé des sites ségrégants dans la région* rbcL.

		Nucleotide position (bp)																						
		3	**4**	**5**	**6**	**9**	**425**	**427**	**428**	**431**	**443**	**444**	**445**	**446**	**448**	**449**	**450**	**452**	**453**	**462**	**879**	**882**	**884**	**885**
Samples	**A**	C	T	A	C	T	A	G	A	C	--	G	--	T	T	--	--	--	--	--	G	--	--	A
	B	--	--	A	--	T	A	--	--	A	--	--	--	--	--	G	G	--	--	--	G	--	--	--
	C	C	G	A	A	--	G	--	A	C	--	G	G	T	T	--	G	A	T	--	G	A	C	A
	D	C	G	A	C	--	G	--	A	C	G	G	G	--	T	--	--	--	--	A	--	A	C	A
	E	A	C	T	T	A	C	A	G	--	A	A	A	A	G	T	T	G	A	C	A	C	A	C

A : *Pinus halepensis*; B : *P. pinea*; C : *P. massoniana var.massoniana*; D : *P. brutia*; E : *P. heldreichii*.

3.2. Phylogenetic tree analysis of *rbcL* barcoding gene

The phylogenetic tree (**Figure 3**) was constructed using maximum likelihood methods from sequences retrieved from NCBI. It illustrates the evolutionary relationships among various *Pinus* species based on chloroplast genome regions, primarily the *rbcL* gene. All analyzed sequences demonstrated homology, indicating that they derived from a common ancestral gene or chloroplast region across the diverse *Pinus* species included.

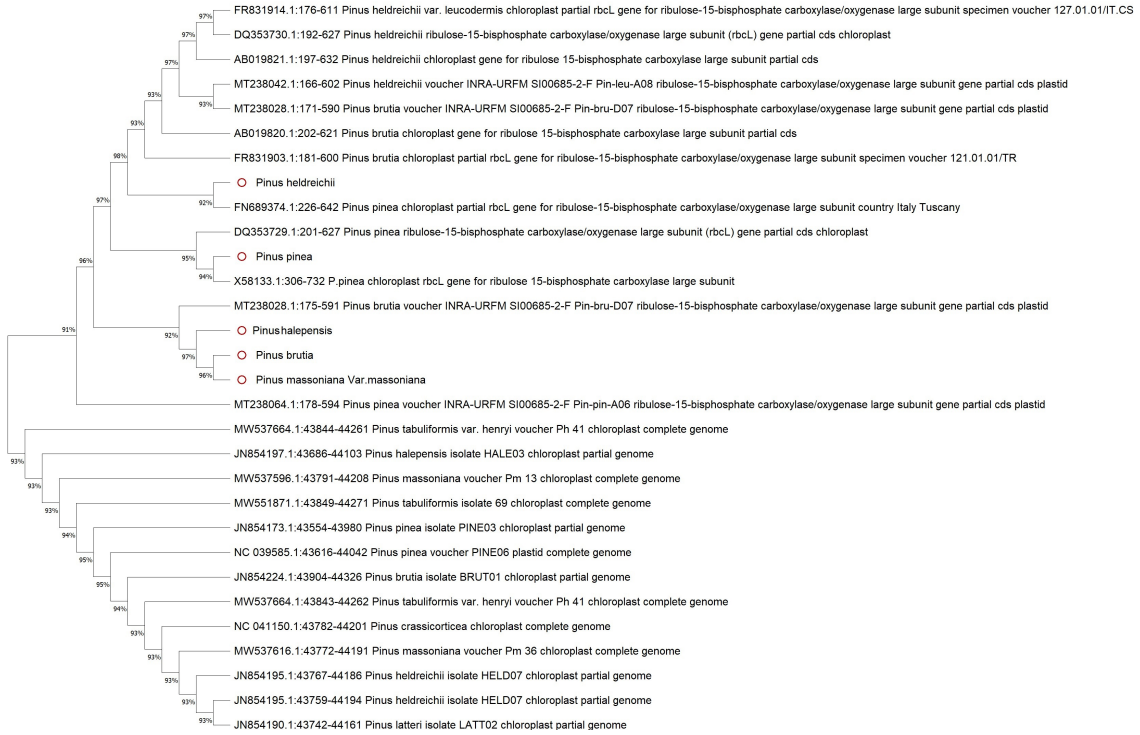

Figure 3. A phylogenetic analysis of *Pinus* species based on *rbcL* gene sequences - *Une analyse phylogénétique des espèces de* Pinus *basée sur les séquences du gène* rbcL.

The *P. heldreichii* cluster is supported by multiple accessions, including FR831914.1, DQ353730.1, AB019821.1, MT238042.1, and JN854195.1, which form cluster closely together, suggesting minimal genetic differences among them. Similar patterns of limited intraspecific variation are observed within the *P. brutia* (*e.g.*, MT238028.1 and AB019820.1) and *P. pinea* (FN689374.1 and DQ353729.1) clades. *Pinus pinea* samples, including accessions FN689374.1, DQ353729.1, X58133.1, MT238064.1, JN854173.1, and NC_039585.1, cluster together, indicating a close evolutionary relationship. *Pinus halepensis* (JN854197.1) forms a distinct lineage. The *P. brutia* samples (MT238028.1, AB019820.1, FR831903.1, and JN854224.1) form another well-defined clade. *Pinus crassicorticea* (NC_041150.1) is identified as a distinct lineage as well. These samples with other *Pinus species*, such as *P. tabuliformis* var. henryi (MW537664.1), *P. massoniana* (MW537596.1, MW537616.1), and *P. latteri* (JN854190.1), show distinct evolutionary relationships. The grouping of *P. heldreichii*, *P. pinea*, *P. halepensis*, *P. brutia*, and *P. crassicorticea* suggests species-specific monophyly. The configuration of the phylogenetic tree is determined by bootstrap values, which indicate the percentage of iterations that support the tree at specific divergence points. As the number of iterations supporting the tree at a given divergence point increases, confidence in the tree's configuration increases.

3.3. Wood anatomy

Table 4 shows the mean values of the anatomical characteristics of wood. Wood is characterized by an anatomical structure consisting of several elements which, in their system of arrangement, are responsible for many of wood natural and mechanical properties. There is also a variation in the shape,

size, and proportion of these elements between wood species, and some of them, making the anatomical arrangement one of the means used to distinguish and define wood species (**Figure 4**).

Table 4. Wood anatomical traits of pine species – *Traits anatomiques du bois des espèces de pin*.

Species	**Tracheids wall thicknesses** (µ)	**Latewood thickness** (%)	**Ray parenchyma area** (%)	**Number of resin ducts**	**Rays heigh** (µ)	**Rays heigh** (cell)	**Fiber Length** (mm)
P. halepensis Mill.	39.2^{a}	32.8^{b}	7.65^{a}	3.00^{a}	223.0^{a}	12^{a}	2.95^{b}
P. massoniana var. massoniana	36.9^{ab}	28.4^{b}	7.97^{a}	3.11^{a}	181.9^{b}	8^{bc}	w
P. brutia Ten.	20.3^{e}	44.0^{a}	7.64^{a}	2.00^{b}	112.4^{d}	7^{c}	2.98^{b}
P. pinea L.	34.0^{b}	45.6^{a}	8.08^{a}	2.78^{ab}	80.6^{e}	5^{c}	2.37^{d}
P. heldreichii H.Christ	27.8^{c}	29.0^{b}	8.19^{a}	2.56^{ab}	81.9^{e}	10^{b}	2.81^{bc}

Similar letters within the same column mean that there is no significant difference at 0.05 level of significance, and different letters mean that there is a significant difference – *Des lettres identiques dans une même colonne signifient qu'il n'y a pas de différence significative au seuil de 0,05 tandis que des lettres différentes indiquent qu'il existe une différence significative.*

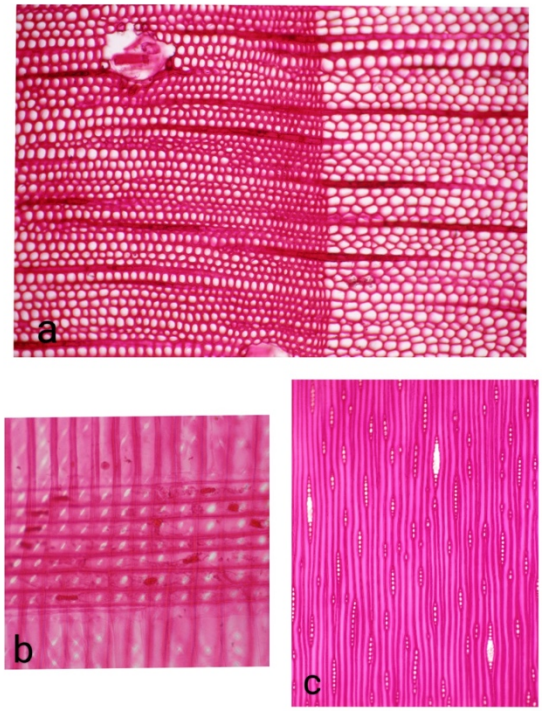

Figure 4. Wood anatomy of *Pinus* species – *Anatomie du bois des espèces de* Pinus.
a. cross section – *section transversale*; **b.** longitudinal radial section – *section longitudinale radiale*; **c.** longitudinal tangential section – *section longitudinale tangentielle.*

3.4. Cross section

The results of the cross-sectional study of coniferous wood species showed significant differences between the species in the following characteristics:
– tracheal wall thickness µ (TWT): the high significant differences in the values of TWT were 39.2, 36.9, and 34.0 µ in *P. halepensis*, *P. massoniana* var. massoniana, and *P. pinea*, respectively. Furthermore, the value of TWT in *P. heldreichii* was 27.8 µ, while the lowest values of TWT were 20.3 µ in P. *brutia*;
– latewood thickness: it was estimated as a percentage of the annual ring width. The results showed a highly significant difference ($p < 0.05$) between species: *P. pinea* and *P. brutia* had the highest percentages at 45.6% and 44.0% respectively, while *P. massoniana* var. massoniana had the lowest at 28.4%;

– ray parenchyma area: the results showed no significant differences in values between the species;
– resin ducts (%): significant differences were observed in the percentage of resin ducts relative to the cross-sectional area among the studied species. *Pinus brutia* had the lowest percentage (0.37%), whereas *P. massoniana* var. massoniana had the greatest (1.18%). At the same time, resin duct diameter varied significantly between species, with *P. massoniana* var. massoniana having the largest diameter (172.3 μ) and *P. pinea* having the smallest (112.4 μ);
– number of resin ducts: the results showed highly significant differences between the species, where the highest number of resin ducts was in *P. massoniana* var. massoniana, while the lowest number was in *P. brutia*;
– longitudinal radial section: the results showed highly significant differences between species for the characteristic of parenchyma ray height and number of cells. The highest value was observed in *P. halepensis* at 223 μ (12 cells), whereas *P. pinea* showed the lowest at 80.6 μ (5 cells), highlighting the significance of this difference;
– longitudinal tangential section: the results of the number of uniseriate rays in 1 cm^2 area showed a highly significant difference between the species values, with the highest number in *P. heldreichii* (86) and the lowest in *P. halepensis* (49). This difference may be due to the relationship between age and the number of rays, where an inverse relationship is observed between the number of rays and age.
– fiber length: the results of the fiber length of the *Pinus* species showed that there is a significant difference in the fiber length between the species, as the *P. massoniana* var. massoniana was characterized by the longest fibers (3.21 mm), while *P. pinea* had the shortest (2.37 mm).

4. DISCUSSION

This study demonstrated the use of DNA barcoding for *Pinus* species identification as a complement to anatomical identification. To find discrepancies between anatomical and DNA identification results, sequences were cross-referenced with morphological identification and compared with GenBank reference sequences. Our results showed that about 60% of the samples were correctly recognized at the species level, although all valid identifications were made at the genus level. Genetic divergence highlights the importance of species identification. DNA barcoding is useful, especially for samples unidentifiable morphologically (Kress, 2017; Dormontt et al., 2018; Antil et al., 2023). Successful barcoding relies on strong morphological identification and comprehensive databases (Bell et al., 2017; Meiklejohn et al., 2019). This technique becomes important for biodiversity assessment and conservation, particularly in diverse regions lacking taxonomic expertise or detailed floristic descriptions (Hebert et al., 2003).
The chloroplast *rbcL* gene has been a foundational DNA barcode marker since early phylogenetic reconstructions (Chase et al., 1993), gaining widespread use for species identification across diverse plant groups (Guo et al., 2015; Kaplan-Levy et al., 2015; Hadi et al., 2016). With over 50,000 sequences in databases (Bell et al., 2017; Omonhinmin et al., 2022), *rbcLs* are key advantages, particularly for *Pinus* species where morphological variation complicates taxonomy (Armenise et al., 2012; Giovannelli, 2017). The Consortium for Barcode of Life (CBOL) recognizes *rbcL* as a universal plant barcode (Antil et al., 2023). Phylogenetic studies reveal most *Pinus* species belong to Ponderosae, Oocarpae, Contortae, Australes, and Sabinianae lineages. The clades like Australes and Ponderosae share common ancestry (Singh et al., 2021). Researchers using *rbcL* and other genes found close relationships among North and Central American *Pinus* species (Gernandt et al., 2005; Hernández-León et al., 2013). Significant genetic diversity in exotic and native *Pinus* was observed in height, cone width, and seed characteristics (Singh & Thapliyal, 2012).
Genetic diversity varies among *Pinus* species and populations. Some, like Scots pine, show high diversity, while others, such as *P. pinea*, exhibit remarkably low variability, hinting at complex evolutionary paths (Tani et al., 1996; Zhang et al., 2005; Vendramin et al., 2007; Kim et al., 2010; Sheller et al., 2023). The high genetic diversity observed in Chinese pine (*Pinus tabuliformis* Carrière) and Henry's pine (*Pinus henryi* Mast.) is due to habitat requirements and historical demographic processes (Li et al., 2008; Liu et al., 2012). Our phylogenetic analysis, based on *rbcL* sequences, supports the monophyly of the genus *Pinus*. This finding is consistent with the results of more comprehensive, multi-locus studies that have also robustly supported the monophyly of the genus using a combination of different barcode regions (Wang et al.,1999). The tree identifies two major clades, which are consistent with previous studies. The close relationship between *P. heldreichii* and *P. pinea* is further supported by

Toromani et al. (2015). However, the long-branch attraction artifact observed for *P. pinea* in clade 2 suggests that additional molecular markers or more advanced phylogenetic methods may be needed to fully resolve the relationships within this clade. Future studies could incorporate a broader sampling of *Pinus* species and explore the potential role of hybridization and introgression in shaping the evolution of this genus. Based on this phylogeny, we can infer that: *P. heldreichii*, *P. pinea*, and *P. brutia* share a recent common ancestor and are more closely related to each other than to other *Pinus* species in the tree. *Pinus halepensis* and *P. brutia* are also closely related, suggesting a recent divergence. The percentage of bases in the genus *Pinus*, especially G+C (43.18%), is significantly lower than that of A+T (56.72%). Studies have shown similar G+C and A+T base percentages (Gernandt et al., 2005; Rinaldi & Sukarjo, 2022).

Despite the use of multiple DNA barcodes, accurately identifying all *Pinus* species remains challenging (Monnet et al., 2021). Thus, a sequential approach, beginning with anatomical and concluding with molecular identification, can precisely determine *Pinus* wood species. The wood anatomy of the pine species exhibits distinct traits that can be used to identify and classify them. The physical contrasts among these species propose changing variations to their surroundings, especially considering dry season and environment changeability. *Pinus halepensis* and *P. brutia* show differentiating reactions to water accessibility, impacting their development and survival. These findings were similar to those reported by Panetsos et al. (1997) and Houminer et al. (2022). However, the dispersal of the axial tissue is important. *Pinus massoniana* var. massoniana has more articulated parenchyma tissue clusters than *P. pinea*.

Understanding the morphological and anatomical characteristics of tree species is fundamental to their classification and conservation. Wood anatomical parameters are valuable for identification, provenance analysis, and ecological studies because they distinguish species and reflect environmental responses (Schweingruber, 2007; Martín et al., 2010). Our study examined traits such as tracheid wall thickness and ray parenchyma area in five *Pinus* species from Al-Jabal Al-Akhdar, and the results reinforce the utility of these traits for species differentiation. Combining these anatomical distinctions with molecular *rbcL* barcoding data provides a comprehensive picture of *Pinus* diversity in Libya. While anatomical traits can overlap, integrating *rbcL* barcoding data provides a more precise method for discriminating between species. Furthermore, recognizing that wood anatomical traits can exhibit site-related variation (Martín et al., 2010), our sampling strategy across different altitudes in the Al-Jabal Al-Akhdar region aimed to capture a broad representation of the species' natural diversity. This acknowledges the potential influence of local environmental conditions on these parameters. Understanding these physical characteristics is essential in the timber trade, as they affect the wood quality and its suitability for different applications. Comprehensive chloroplast genome studies (Ni et al., 2017) have highlighted the complex phylogenetic relationships within *Pinus*. This underscores the need for further research using a multi-locus approach to fully resolve the genus and develop a reliable system for species identification. Therefore, future strategies are expected to provide a more robust genetic framework by using multiple barcode regions in addition to *rbcL*, such as *matK* or *ITS*. This approach enhances the precision of species classification by utilizing additional genetic markers, which can reveal more detailed evolutionary connections among species. As a result, researchers can gain insights into the complexities of biodiversity and evolutionary history.

5. CONCLUSIONS

The results provide further evidence that *rbcL*, a plant nuclear barcode, is capable of successfully identifying a wide variety of plant species. These results showed that the sample from Al-Jabal Al-Akhadar region of eastern Libya is most likely a *Pinus* species, as highlighted by both anatomical and genetic studies. To adapt and survive, *Pinus* species often maintain moderate to high levels of genetic variation. However, the distribution of this diversity varies between species and groups for a variety of reasons, including historical events, human activities, and geographic isolation. To formulate effective management plans for pine species, it is imperative to employ species-specific conservation methods and to leverage complementary techniques such as genetic diversity assessment, ecological niche modeling, and population demographic inference.

Conflict of interest

The authors declare that they have no conflict of interest.

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