forests

*Article*

# Needle Structure in Three *Juniperus* Taxa Indigenous to Slovakia

**Martin Galgóci [1,*], Andrej Kormuťák [1], Dušan Gömöry [2], Miroslav Klobučník [1], Peter Turis [3], Veronika Mistríková [1] and Peter Boleček [4]**

[1] Plant Science and Biodiversity Centre, Institute of Plant Genetics and Biotechnology, Slovak Academy of Sciences, Akademická 2, P.O. Box 39A, 950 07 Nitra, Slovakia; andrej.kormutak@savba.sk (A.K.); miroslav.klobucnik@savba.sk (M.K.); veronika.mistrikova@savba.sk (V.M.)
[2] Faculty of Forestry, Technical University in Zvolen, T. G. Masaryka 24, 960 53 Zvolen, Slovakia; dusan.gomory@tuzvo.sk
[3] Faculty of Natural Sciences, Matej Bel University in Banská Bystrica, Tajovského 40, 974 01 Banská Bystrica, Slovakia; peter.turis@umb.sk
[4] Faculty of Natural Sciences and Informatics, Constantine Philosopher University in Nitra, A. Hlinku 1, 949 74 Nitra, Slovakia; pbolecek@ukf.sk
* Correspondence: martin.galgoci@savba.sk or galgoci7@gmail.com

## Abstract

Needle structure was analyzed in three *Juniperus communis* taxa from different localities in central Slovakia. The main aim was to test the hybrid origin hypothesis of *J. communis* nothovar. *intermedia* (Schur) Nyman, defined as a cross between *J. communis* L. ssp. *communis* and *J. communis* ssp. *nana* (Hook.) Syme. While DNA-based analyses remain the most reliable tool for inferring evolutionary history, comparative needle morphology can provide complementary evidence, including diagnostic traits for taxonomic delimitation. In this study, we evaluated three morphometric and sixteen anatomical needle traits, measured via microscopy in ten shrubs per taxon. The analyses indicated that most traits in nothovar. *intermedia* matched one of the parents, with only two traits proving strongly diagnostic, separating all three taxa: needle length, which showed an intermediate mean phenotype in nothovar. *intermedia* ($R^2 = 0.824$, $p = 0.011$; between parents), and vascular bundle height, which displayed a transgressive pattern ($R^2 = 0.552$, $p = 0.031$; between parents). Although the diagnostic value of individual traits for hybrid detection was generally weak, a phylogenetic network analysis based on six diagnostic traits that separated individuals of the parental taxa provided evidence for reticulate evolution. These results support the hybrid origin of *J. communis* nothovar. *intermedia* and highlight needle traits with potential value for distinguishing ssp. *communis* and ssp. *nana* in natural populations, which may assist in taxonomic delimitation and inform future conservation assessments.

**Keywords:** junipers; hybrid; needle structure; identification; Slovakia

Academic Editor: David Lee

Received: 1 July 2025
Revised: 28 August 2025
Accepted: 1 September 2025
Published: 2 September 2025

**Citation:** Galgóci, M.; Kormuťák, A.; Gömöry, D.; Klobučník, M.; Turis, P.; Mistríková, V.; Boleček, P. Needle Structure in Three *Juniperus* Taxa Indigenous to Slovakia. *Forests* **2025**, *16*, 1406. https://doi.org/10.3390/f16091406

## 1. Introduction

*Juniperus* is a genus of coniferous plants in the family Cupressaceae, commonly known as junipers. These evergreen shrubs and trees are widely distributed across the Northern Hemisphere, from North America and Europe to Asia, occupying diverse habitats ranging from lowlands to alpine zones [1,2]. Junipers play important ecological roles as pioneer species, providing habitat and food resources for wildlife [3], and are economically valued for timber, essential oils, and traditional medicinal uses [1].

Among morphological approaches for classifying the genus, needle morphometry (i.e., intact needle traits) and anatomy (i.e., internal structure) occupy a prominent place.

The taxonomic division into sections *Caryocedrus*, *Juniperus*, and *Sabina* is principally based on relationships between leaf form, female flower features, and cone scales [4]. Building on this framework, several regional studies have investigated variation in needle traits.

For example, needle length and width in *J. oxycedrus* ssp. *macrocarpa* (Smith) Neilreich showed no significant differences among populations in Italy, with most variation occurring within rather than between populations [5]. Similarly, comparative anatomical analyses in Turkish taxa identified species-specific patterns in secretory canal positions [6], while work on Balkan populations revealed that traits of needles and stem anatomy can partially separate subspecies, including *J. communis* ssp. *communis* vs. *J. communis* ssp. *nana* and *J. communis* nothovar. *intermedia* [7]. More recently, phenotypic traits have supported the recognition of *J. oxycedrus* L. and *J. deltoides* R.P.Adams as distinct species [8], and anatomical studies across Iranian conifers highlighted the diagnostic value of needle cross-sectional features [9]. Together, these studies demonstrate that needle traits are taxonomically informative, but their utility for identifying hybrids and closely related subspecies remains uncertain.

Anatomical features identified across other *Juniperus* species illustrate the kinds of traits that may help resolve such taxonomic challenges. For example, palisade parenchyma is present in *J. communis*, *J. foetidissima* Willd., and *J. oblonga* M.Bieb., but absent in *J. excelsa* M.Bieb. and *J. sabina* L. Other needle characteristics—such as cross-sectional shape, presence of fibers, cuticular ornamentation, number of vascular bundles, and sclerenchyma within vascular bundles—have also been reported to be taxonomically informative. Microstructural and histochemical analyses of shoots and female cones in *J. seravschanica* Kom. from the same geographic region further provided insights into chemical compounds involved in resistance to biotic and abiotic stressors [10].

In the present study, needle morphometry and anatomy were compared among *J. communis* L. ssp. *communis*, *J. communis* ssp. *nana* (Hook.) Syme (syn. var. *saxatilis* Pall, ssp. *alpina* (Suter) Čelak., *J. sibirica* Burgsd.; here treated as subspecies following *Flora Europaea*; see also below), and *J. communis* nothovar. *intermedia* (Schur) Nyman, all naturally occurring in Slovakia. The hybrid taxon, defined as ssp. *communis* × ssp. *nana*, is of particular interest. Its growth habits, needle consistency, and branch arrangement have been cited as primary evidence supporting the hybrid hypothesis [11]. However, our previous study using inter-Primer Binding Site (iPBS) amplification revealed a high level of genetic admixture in this taxon, with neutral differentiation between parents corresponding to the subspecies rather than variety level [12]. Here, we revisited the same locality—Kráľová studňa, which harbors a small population of only 22–25 individuals—for detailed needle structure analysis, aiming to provide additional morphological evidence for the hybrid origin of this population and to identify needle-based diagnostic traits useful for distinguishing these taxa.

## 2. Materials and Methods

### 2.1. Sampling and Locations

Needle morphological analyses, including both intact needle morphometry and anatomy, were conducted on three *J. communis* populations representing distinct taxa native to Slovakia. Samples of ssp. *communis* were collected from the locality Šumiac (880 m a.s.l.), those of nothovar. *intermedia* from Kráľová studňa (1284 m a.s.l.), and ssp. *nana* from Mt. Kráľová hoľa (1846 m a.s.l.). All three sites are located within the Nízke Tatry mountain range, central Slovakia. To minimize the risk of sampling clonal individuals, shrubs were selected randomly at intervals of 10–15 m. One-year-old needles were sampled in autumn 2023 to ensure a uniform developmental stage, minimize environmental noise, and improve comparability among individuals. Needle traits were selected for their evergreen nature, ease of measurement, and established taxonomic value in *Juniperus*. Cones and seeds were not included due to their seasonal availability.

### *2.2. Needle Morphometry and Anatomy*

In total, 19 morphological traits were measured. Morphometric analyses focused on (1) intact needle length and stomatal traits. Fresh material was processed in the laboratory the day after field collection. Needle length was measured in ten individuals per taxon, using ten needles per individual. Measurements were taken using millimeter graph paper as a scale. Stomatal traits, including (2) the number of stomatal rows and (3) the number of stomata per 1 mm segment (i.e., stomatal density), were assessed on the flattened adaxial side of the needle's middle section using a binocular magnifier Leica EZ4 D (16×; Leica Microsystems GmbH, Wetzlar, Germany). For this, five needles per individual were examined in ten individuals per taxon.

Anatomical analyses followed the standard protocol for preparing permanent cytological slides [13]. Middle sections of freshly collected needles were excised using a scalpel and fixed in FAA fixative (formalin–acetic acid–alcohol). After rinsing in tap water, the samples were dehydrated through an ethanol series of increasing concentrations. Ethanol–xylene mixture was used as an intermediate step. The dehydrated needle segments were embedded in paraffin wax with a small proportion of beeswax at 60 °C.

Cross-sections (15 µm thick) were prepared using a rotary microtome CUT4055 (microTec Laborgeräte GmbH, Walldorf, Germany). After paraffin removal with xylene, the sections were rehydrated through a descending ethanol series, rinsed in tap water, and stained with safranin (1% $w/v$ in 50% ethanol) and alcian blue (1% $w/v$ in water). The stained sections were mounted in Entellan, dried, and examined under a light microscope Jena NU-2 (Carl Zeiss AG, Oberkochen, Germany).

The following anatomical (cross-sectional) traits were measured using an eyepiece micrometer (Meopta, Přerov, Czech Republic): (4) needle width and (5) height, (6) vascular bundle width and (7) height, (8) resin duct width and (9) height, and (10) epidermal cell width and (11) height. Measurements of needle, vascular bundle, and resin duct dimensions were taken from five individuals per taxon, using five needles per individual. Epidermal cell dimensions were measured in five individuals per taxon, using five needles per individual and ten cells per needle.

Finally, these cross-sectional data were used to calculate: (12) needle height/width ratio, (13) needle area (height × width), (14) vascular bundle height/width ratio, (15) vascular bundle area, (16) resin duct height/width ratio, (17) resin duct area, (18) epidermal cell height/width ratio, and (19) epidermal cell area.

### *2.3. Data Analyses*

Variation in morphometric and anatomical needle traits was assessed using analysis of variance (ANOVA) under a partially nested design. Population pairwise comparisons for individual traits were evaluated using Duncan's multiple range test at $p < 0.05$. Analyses were performed in SAS® software, version 9.4 [14]. To better illustrate which traits possessed sufficient discriminatory power to be considered diagnostic at the population level, Duncan's groupings with descriptive statistics were plotted as boxplots in R 4.4.0 using the package *ggplot2* [15].

The overall variation between individuals was explored by multivariate Principal Component Analysis (PCA) on z-score standardized data (base R *scale* function, *dplyr* package [16], with morphometric and anatomical traits analyzed separately. PCA was performed in *FactoMineR* [17] and visualized (including 95% confidence ellipses) via *factoextra* [18].

To identify traits significantly differentiating ssp. *communis* and ssp. *nana* at the individual level, permutational multivariate analysis of variance (PERMANOVA) was performed separately for each standardized trait using the *adonis2* function in the *vegan* package [19]. These traits were selected for further clustering analyses.

Using these traits, Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering was performed on the standardized data. The UPGMA phenogram was generated with the *ape* package [20], with bootstrap analysis (1000 replicates) by resampling dataset columns with replacement. A majority-rule consensus tree with bootstrap support was constructed using the *consensus* and *prop.clades* functions from *ape*.

Because the study involved a hybrid taxon, individuals were also clustered using a phylogenetic network reconstruction based on the Maximum Pseudo-likelihood (MPL) algorithm implemented in the Julia 1.11.0 package *PhyloNetworks* [21]. For this, Euclidean distance matrices were computed from the selected standardized traits (*dist* function in R). Then, unrooted Neighbor-Joining "gene" trees with 1000 bootstrap replicates each were constructed, and a majority consensus tree was generated using *ape*. These gene trees, their bootstrap replicates, and the consensus tree (used as the starting topology) were input into the *PhyloNetworks* pipeline. The best network was inferred with *snaq!* (hmax = 5, runs = 10), and robustness was assessed using *bootsnaq* (hmax = 5, nrep = 100, runs = 10). The resulting network was visualized in Dendroscope 3.8.10 [22].

Voucher specimens of all three taxa are deposited at the Plant Science and Biodiversity Centre, Slovak Academy of Sciences, Institute of Botany, Dúbravská cesta 9, SK-845 23 Bratislava, Slovakia, under the following accession numbers: SAV0019117 (*J. communis* nothovar. *intermedia*), SAV0019118 (*J. communis* ssp. *nana*), and SAV0019120 (*J. communis* ssp. *communis*).

## 3. Results

### 3.1. Needle Morphometry

Raw data for all needle traits are provided in the Supplementary Materials. Among the traits measured on intact needles, needle length showed the most pronounced differences among taxa. The longest needles occurred in ssp. *communis* (mean 10.53 mm), while the shortest were in ssp. *nana* (mean 5.90 mm). Nothovar. *intermedia* occupied a statistically intermediate position (mean 9.56 mm), closer to ssp. *communis* than to ssp. *nana*. According to Duncan's grouping, all three taxa formed distinct groups (Figure 1).

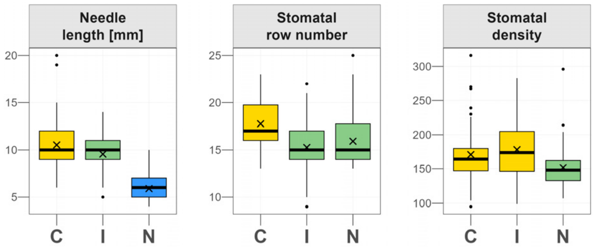

**Figure 1.** Intact needle traits in three *Juniperus communis* populations. C—ssp. *communis*, I—nothovar. *intermedia*, N—ssp. *nana*. Shown are the mean (cross), median (thick bar), interquartile range (box), minimum and maximum (vertical lines), and outliers (dots). Colors represent groupings based on Duncan's multiple range test at $p < 0.05$ (yellow—group A, green—group B, blue—group C).

For stomatal characteristics on the adaxial needle side, ssp. *communis* and ssp. *nana* differed in the number of stomatal rows (17.76 vs. 15.85). The hybrids averaged 15.26 stomatal rows, not significantly different from ssp. *nana*. However, in stomatal density, hybrids resembled ssp. *communis*, reaching 178.28 stomata per 1 mm of needle length (Figure 1).

### 3.2. Needle Shape Diversity

Needle cross-sections revealed substantial variation in shape between taxa, among individuals, and even among needles of the same individual. According to the classification of *Juniperus* needle cross-sectional forms by Lakušić and Lakušić [7], the most common types in ssp. *communis* were those with a concavity on the abaxial side (9 of 25 needles observed from five shrubs; Figure 2d) and the trapezoidal form (6 needles; Figure 2a). Needles with rounded and bent edges, as well as the obtuse triangular form, were also observed (5 needles each).

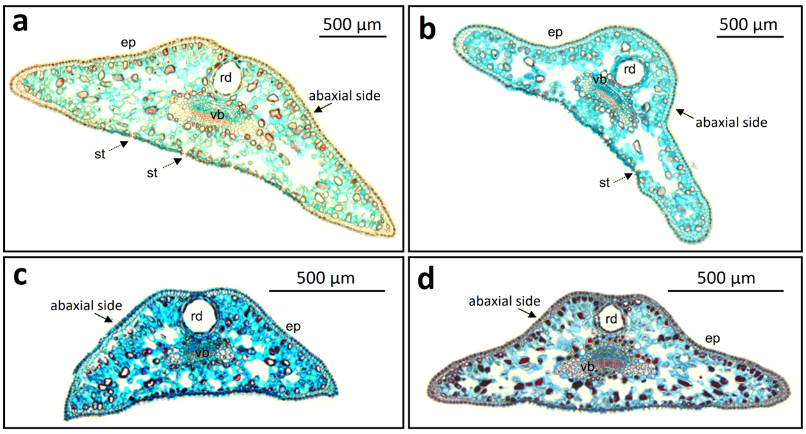

**Figure 2.** Cross-sectional profiles of needles observed in the studied taxa. (**a**)—basic trapezoidal form in *Juniperus communis*, (**b**)—needle with rounded and bent edges (typical of nothovar. *intermedia*), (**c**)—obtuse triangular needle form (typical of ssp. *nana*), (**d**)—needle with a concavity on the abaxial side (typical of ssp. *communis*), ep—epidermis, rd—resin duct, vb—vascular bundle, st—stomata.

In ssp. *nana*, the trapezoidal form was absent, differing from ssp. *communis*. The triangular form dominated (10 needles; Figure 2c), followed by needles with rounded and bent edges (8 needles) and needles with a concave abaxial side (7 needles).

In nothovar. *intermedia*, triangular needles typical of ssp. *nana* were absent, and morphology more closely resembled ssp. *communis*. Interestingly, the trapezoidal form was most common in our population (15 needles). Needles with a concave abaxial side (6 needles) and rounded and bent edges (4 needles; Figure 2b)—the latter considered typical for this variety—were also present.

### 3.3. Needle Anatomy

Quantitative assessment of anatomical traits revealed variable, trait-specific divergence among taxa. For cross-sectional needle parameters, ssp. *communis* and nothovar. *intermedia* clustered together in Duncan's grouping, differing significantly from ssp. *nana*. The only exception was the height/width ratio, where the hybrid grouped with ssp. *nana* (Figure 3).

In vascular bundle traits, the hybrid showed the highest values across all parameters, with significant transgressive segregation (i.e., exceeding the range observed in both parental taxa) for vascular bundle height and area. Notably, no other anatomical traits showed a better diagnostic value than vascular bundle height for distinguishing the populations, as evident from the non-overlapping interquartile ranges (Figure 3).

Resin duct traits were generally less informative (see ANOVA results in Table 1). Significant differences between ssp. *communis* and ssp. *nana* were found only for duct width and area. Although the hybrid showed some indication of intermediacy for duct height, it was not significant, and the hybrid resembled either ssp. *communis* (in width) or ssp. *nana* (in area) (Figure 3).

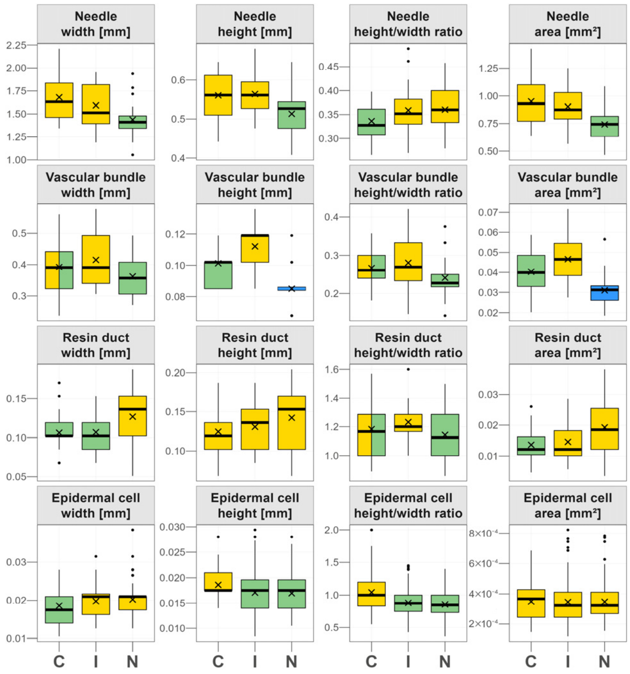

**Figure 3.** Needle anatomical traits in three *Juniperus communis* populations. C—ssp. *communis*, I—nothovar. *intermedia*, N—ssp. *nana*. Shown are the mean (cross), median (thick bar), interquartile range (box), minimum and maximum (vertical lines), and outliers (dots). Colors represent groupings based on Duncan's multiple range test at $p < 0.05$ (yellow—group A, green—group B, blue—group C).

**Table 1.** Summary of ANOVA results for needle anatomical traits in three *Juniperus communis* populations.

| Source of Variation | DF | Width | | Height | | Height/Width Ratio | | Cross-Sectional Area | |
|---|---|---|---|---|---|---|---|---|---|
| | | F-Test | *p* | F-Test | *p* | F-Test | *p* | F-Test | *p* |
| | | | | Needles | | | | | |
| Population | 2 | 12.46 | <0.0001 | 8.48 | <0.0006 | 3.88 | <0.0260 | 14.32 | <0.0001 |
| Shrub (population) | 12 | 4.39 | <0.0001 | 3.72 | <0.0003 | 5.02 | <0.0001 | 4.82 | <0.0001 |
| | | | | Vascular bundles | | | | | |
| Population | 2 | 4.90 | <0.0107 | 30.02 | <0.0001 | 3.58 | <0.0341 | 28.89 | <0.0001 |
| Shrub (population) | 12 | 5.41 | <0.0001 | 2.86 | <0.0035 | 1.72 | <0.0855 | 7.79 | <0.0001 |
| | | | | Resin ducts | | | | | |
| Population | 2 | 4.49 | <0.0152 | 1.95 | <0.1515 | 3.09 | <0.0530 | 4.29 | <0.0181 |
| Shrub (population) | 12 | 1.76 | <0.0768 | 1.11 | <0.3665 | 3.77 | <0.0003 | 1.44 | <0.1736 |

Note: DF—degrees of freedom; F-test and corresponding *p*-values are shown for each trait; statistical significance was evaluated at $p < 0.05$.

In epidermal cell dimensions, a characteristic feature was the strong similarity between ssp. *nana* and the hybrid, which differed from ssp. *communis*. Only the cell area exhibited no significant differences between the three studied populations (Figure 3).

Combining morphometric and anatomical data, 14 traits distinguished the ssp. *communis* population (often with a higher mean phenotype) from that of ssp. *nana*. The hybrid population resembled ssp. *communis* in 5 traits, ssp. *nana* in 6 traits, and showed 3 key diagnostic traits, which differentiated the hybrid population from both parents: intermediate needle length, and transgressive vascular bundle height and area.

### 3.4. Individual-Level Multivariate Analysis and Phenotypic Clustering

PCA of intact needle traits, performed at the individual level, revealed a considerable separation among taxa. The first two components explained 63.1% and 28.3% of the total variation, with ssp. *communis* (yellow circles; showing the greatest within-population variation) being clearly distinct from ssp. *nana* (blue squares). Hybrid individuals (green triangles) occupied an intermediate position, closer to ssp. *communis* (Figure 4). As evident from Figure 1, needle length was the primary driver of separation, while stomatal traits contributed secondarily, reflecting a roughly equal combination of traits resembling each parent.

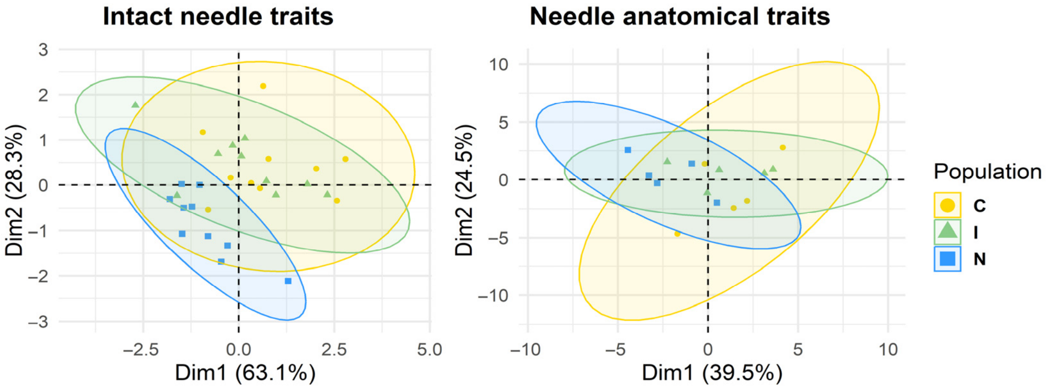

**Figure 4.** Principal component analyses (PCA) of *Juniperus communis* individuals based on needle morphometry and anatomy. The (**left**) plot is based on three intact needle traits (10 individuals/population), while the (**right**) plot is based on sixteen anatomical traits (5 individuals/population). Population codes: C—ssp. *communis*, I—nothovar. *intermedia*, N—ssp. *nana*.

For anatomical traits, the first two components explained 39.5% and 24.5% of the variation. Differentiation was weaker but followed the same overall pattern (Figure 4). Here, the intermediate position of hybrids was mainly due to combining parental traits rather than being defined primarily by intermediate values. Two traits—vascular bundle height and area—were transgressive, obscuring the intermediate position the most (Figure 3).

To identify traits distinguishing individuals of ssp. *communis* and ssp. *nana*, each trait was independently tested using separate PERMANOVAs. Seven potentially diagnostic traits were found: needle length ($R^2 = 0.824$, $p = 0.011$), stomatal row number ($R^2 = 0.565$, $p = 0.027$), vascular bundle height ($R^2 = 0.552$, $p = 0.031$), stomatal density ($R^2 = 0.501$, $p = 0.032$), needle width ($R^2 = 0.416$, $p = 0.047$), needle area ($R^2 = 0.382$, $p = 0.050$), and resin duct area ($R^2 = 0.334$, $p = 0.043$). For subsequent clustering analyses, needle area was excluded due to its marginal significance and strong correlation with needle height and width, rendering it a highly non-independent trait.

Phenetic relationships of five hybrid individuals in relation to ssp. *communis* and ssp. *nana* populations are shown in Figure 5a. The UPGMA phenogram exhibited 100% bootstrap support for the basal split between ssp. *communis* and ssp. *nana*, with hybrids distributed between them. Within these major clades, however, bootstrap support values fell below the conventional threshold (i.e., <75%), reflecting limited discriminatory power of the selected trait set for distinguishing hybrids at the individual level (Figure 5a).

Nevertheless, the MPL network recovered a hybridization event involving *intermedia*_23, with contributions from *intermedia*_22 (0.41) and ssp. *nana* (0.59). As in the UPGMA, internal branch support within clades was weak, consistent with hybridization between ssp. *communis* and ssp. *nana*, rather than within-lineage structuring (Figure 5b).

Overall, multivariate analyses supported the hybrid origin hypothesis of nothovar. *intermedia*, as evidenced by both PCAs. This phenotypic intermediacy, however, was largely driven by a single trait—needle length—while most other traits contributed through an approximately equal combination of parental phenotypes. The hybrid nature of nothovar. *intermedia* was also supported by the clear separation of parental taxa and the ambiguous placement of hybrids—both within UPGMA and across clustering methods—suggesting reticulate evolutionary histories, as further confirmed by the MPL network.

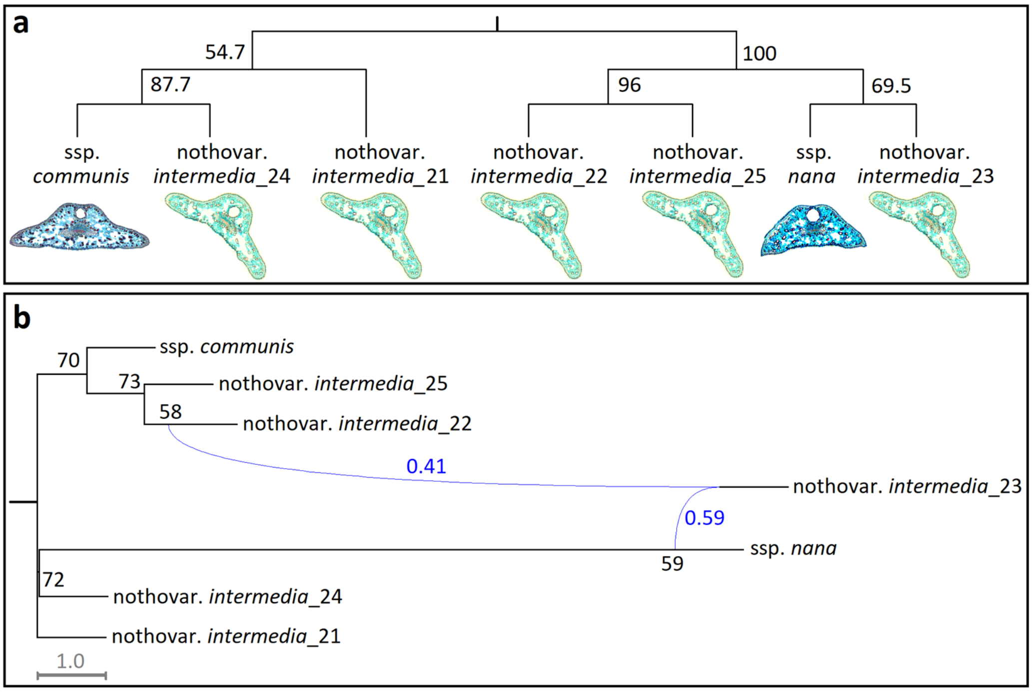

**Figure 5.** UPGMA phenogram (**a**) and Maximum Pseudo-likelihood (MPL) network (**b**) showing similarity and phylogenetic relationships based on diagnostic needle traits of *Juniperus communis*. The analysis included five nothovar. *intermedia* individuals and two populations: ssp. *communis* and ssp. *nana*. Black numbers indicate bootstrap support values for nodes, while blue numbers denote parental contributions to detected hybridization events.

## 4. Discussion

Anatomical traits of *Juniperus* needles have long been recognized for their taxonomic value in distinguishing closely related taxa, even at the infraspecific level. Previous studies identified stomatal number, needle cross-sectional shape, and resin duct dimensions as reliable traits for differentiating *J. communis* ssp. *communis* and *J. deltoides* [7]. Our results extend these findings by demonstrating that stomatal traits—specifically the number of stomatal rows and stomatal density per 1 mm of needle—effectively discriminate between *J. communis* ssp. *communis* and *J. communis* ssp. *nana*. Notably, hybrid individuals (*J. communis* nothovar. *intermedia*) exhibit a distinctive combination of traits: their number of stomatal rows aligns with ssp. *nana*, while stomatal density resembles ssp. *communis*.

High morphological variation within populations, however, limits the utility of some diagnostic traits at the individual level, emphasizing the importance of population-based taxonomy. Of the 19 traits assessed, 14 distinguished populations of ssp. *communis* and spp. *nana*, while only seven traits—needle length, needle width, needle area, stomatal row number, stomatal density, vascular bundle height, and resin duct area—provided meaningful differentiation between their individuals. For hybrids, only three traits—intermediate needle length and transgressive vascular bundle height and area—offered a clear distinction at the population level, and clustering analyses failed to separate individual hybrids as a distinct group based on the most informative traits. In the case of vascular bundle width, variation within taxa was even greater than variation between them. This high within-taxon variability reflects a pattern similar to that reported in genetic studies of *J. communis* populations, where most variation occurs within rather than between populations [23,24].

Intermediate populations between ssp. *communis* and ssp. *nana* are systematically recognized as their natural hybrid, *J. communis* nothovar. *intermedia* (Schur) Nyman [11,25] (see also Plants of the World Online, Royal Botanic Gardens, Kew). Previous iPBS data [12] confirmed its hybrid origin and highlighted this taxon as an evolutionarily significant unit (ESU). Our needle structure analyses provide additional evidence, but hybrids rarely display intermediate or transgressive values in individual traits (e.g., needle length, vascular bundle height). Hybrid status becomes apparent mainly when multiple traits are considered jointly, making field identification challenging. In the field, needle length remains the most reliable diagnostic trait, complemented by needle consistency: ssp. *communis* has stiff, rigid needles, whereas nothovar. *intermedia* needles are softer and more flexible, resembling ssp. *nana*.

By contrast, morphological intermediacy has been the main criterion for identifying spontaneous hybrids in other *Juniperus* taxa, such as between *J. ashei* J.Buchholz and *J. virginiana* L. in Central Texas and the White River region of Arkansas and Missouri [26]. In that case, needle dimensions, density, color, and stomatal distribution were emphasized as key diagnostic traits. Subsequent work then categorized hybrids into three phenotypic groups: resembling one parent, intermediate, or resembling the other parent [27]. Our findings align with this general pattern but suggest that nothovar. *intermedia* is best characterized by a mosaic of parental traits.

These findings, coupled with previous genetic evidence [12], support the conclusion that nothovar. *intermedia* is a natural hybrid of ssp. *communis* and ssp. *nana*. While needle traits provide the main phenotypic data here, other morphological features—such as tree height, trunk form, and reproductive structures—alongside molecular markers remain important for a full understanding of hybrid identity. For instance, investigations of *J. ashei* and *J. virginiana* initially relied on morphological data, but subsequent chemical analyses of terpenoids failed to confirm their hybrid origin [28–30], highlighting the value of integrating multiple lines of evidence.

Molecular studies have also clarified hybridization and introgression in *Juniperus*. For instance, allotriploid hybrids between *J. thurifera* L. and *J. sabina* L. in the French Alps were confirmed via flow cytometry and pollen morphology [31]. Similarly, reassessment of leaf essential oil profiles in populations of *J. maritima* R.P.Adams and *J. scopulorum* Sarg. in British Columbia and Montana demonstrated allopatric introgression of *J. maritima* genes into *J. scopulorum* populations via wind-dispersed pollen [32]. These examples emphasize the importance of combining morphology, chemistry, and genetics to fully resolve hybrid status and evolutionary history in *Juniperus*.

## 5. Conclusions

Variation in needle traits among the studied *Juniperus communis* taxa reflects morphological differentiation consistent with a reticulate evolutionary history. The hybrid nothovar. *intermedia*, however, exhibits only limited intermediate and transgressive traits. Hybridization effects on needle morphology are modest and trait-dependent, with hybrids more often combining parental traits. Although individual traits have limited diagnostic power, several features reliably distinguish the parental subspecies ssp. *communis* and ssp. *nana*, providing useful taxonomic markers.

Overall, our findings suggest that needle morphology alone is often insufficient for definitive hybrid identification but offers valuable complementary evidence alongside genetic data to clarify evolutionary relationships and hybridization within *Juniperus communis*. Future research should investigate the ecological and reproductive dynamics of nothovar. *intermedia* in contact zones between the parental taxa, using multiple lines of evidence for a comprehensive understanding.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f16091406/s1.

**Author Contributions:** M.G. and A.K., Project Administration, Funding Acquisition, Conceptualization, Investigation, Methodology, Writing—Original Draft Preparation; D.G., Methodology, Formal Analysis; M.K., Software, Formal Analysis, Methodology, Data Curation, Visualization, Writing—Review & Editing, Validation; P.T., Investigation; V.M., Methodology, Writing—Review & Editing; P.B., Investigation. All authors have read and agreed to the published version of the manuscript.

**Funding:** We thank the VEGA Grant Agency (project no. 2/0005/23) and the COST Action Committee (project no. CA22136) for financial support. We also acknowledge support from the Operational Program Integrated Infrastructure within the project: Demand-driven Research for Sustainable and Innovative Food (Drive4SIFood 313011V336), cofinanced by the European Regional Development Fund.

**Data Availibility Statement:** All data generated or analyzed during this study are included in this published article. Correspondence and requests for material should be addressed to M. G. Collection of material for research does not require any specific permission and was performed in accordance with the national legislation (Act No. 543/2002 Z. z. on conservation of nature and landscape, §§ 4 and 47, Act No. 220/2004 Z. z. on protection and use of agricultural land).

**Conflicts of Interest:** The authors declare no conflicts of interest.

## References

1. Adams, R.P. *Junipers of the World: The Genus Juniperus*, 2nd ed.; Trafford Publishing: Bloomington, IN, USA, 2014; ISBN 978-1466981986.
2. Farjon, A. *A Handbook of the World's Conifers*; Brill Academic Publishers: Leiden, The Netherlands, 2010; Volume 1, ISBN 978-9004181313.
3. Richardson, D.M. *Ecology and Biogeography of Pinus: An Introduction*; Cambridge University Press: Cambridge, UK, 1998; ISBN 978-0521440917.
4. Köroğlu, A.; Kendir, G.; Şimşek, D.; Miceli, N.; Pinar, N.M. The morphological properties of leaves, cones, seeds of some *Juniperus* species native to Turkey. *Commun. Fac. Sci. Univ. Ank. Series C* **2018**, *27*, 61–84.
5. Klimko, M.; Boratyńska, K.; Boratyński, A.; Marcysiak, K. Morphological variation of *Juniperus oxycedrus* subsp. *macrocarpa* (*Cupressaceae*) in three Italian localities. *Acta Soc. Bot. Poloniae* **2004**, *73*, 113–119. [CrossRef]
6. Gövenç, A.; Hürkul, M.M.; Erdem, A. The leaf anatomy of naturally distributed *Juniperus* L. (*Cupressaceae*) species in Turkey. *Turk. J. Bot.* **2011**, *35*, 251–260. [CrossRef]
7. Lakušić, B.; Lakušić, D. Anatomy of four taxa of the genus *Juniperus* sect. *Juniperus* (*Cupressaceae*) from the Balkan peninsula. *Bot. Serbica* **2011**, *35*, 145–156.
8. Vidaković, A.; Šatović, Z.; Tumpa, K.; Idžojtić, M.; Barišić, A.; Poljak, I. Secondary sexual dimorphism and morphological diversity in two allopatric juniper species: *Juniperus oxycedrus* and *J. deltoides*. *Acta Bot. Croat.* **2024**, *83*, 14–25. [CrossRef]
9. Hamidipour, A.; Radjabian, T.; Charlet, D.; Zarrei, M. Leaf anatomical investigation of *Cupressaceae* and *Taxaceae* in Iran. *Wulfenia* **2011**, *18*, 95–111.
10. Rezanejad, F.; Hakemi, F.G. Microstructural and histochemical analysis of shoots and cones of *Juniperus seravschanica* (*Cupressaceae*). *Microsc. Res. Tech.* **2024**, *87*, 790–799. [CrossRef] [PubMed]
11. Futák, J.; Jasičová, M.; Schidlay, E. *Flora of Slovakia II*; Slovak Academy of Sciences Publisher: Bratislava, Czechoslovakia, 1966; pp. 298–306.
12. Klobučník, M.; Kormuťák, A.; Jurčík, J.; Galgóci, M. Conservation genetic evaluation of *Juniperus communis* sensu lato in Slovakia. *Sci. Rep.* **2025**, *15*, 8398. [CrossRef] [PubMed]
13. Němec, B. *Botanical Microtechnique*; Czechoslovak Academy of Sciences Publisher: Prague, Czechoslovakia, 1962; pp. 356–455.
14. SAS Institute Inc. *SAS® 9.4 Software*; SAS Institute Inc.: Cary, NC, USA, 2021.
15. Wickham, H. *ggplot2: Elegant Graphics for Data Analysis*; Springer: New York, NY, USA, 2016.
16. Wickham, H.; François, R.; Henry, L.; Müller, K.; Vaughan, D. *R Package*, version 1.1.4; *dplyr: A Grammar of Data Manipulation*; The R Project for Statistical Computing: Vienna, Austria, 2025.
17. Lê, S.; Josse, J.; Husson, F. FactoMineR: An R package for multivariate analysis. *J. Stat. Softw.* **2008**, *25*, 1–18. [CrossRef]
18. Kassambara, A.; Mundt, F. *R Package, version 1.0.7*; factoextra: Extract and Visualize the Results of Multivariate Data Analyses; The R Project for Statistical Computing: Vienna, Austria, 2020.

19. Oksanen, J.; Simpson, G.; Blanchet, F.; Kindt, R.; Legendre, P.; Minchin, P.; O'Hara, R.; Solymos, P.; Stevens, M.; Szoecs, E.; et al. *R Package, version 2.8-0*, vegan: Community Ecology Package; The R Project for Statistical Computing: Vienna, Austria, 2025.
20. Paradis, E.; Schliep, K. ape 5.0: An environment for modern phylogenetics and evolutionary analyses in R. *Bioinformatics* **2019**, *35*, 526–528. [CrossRef] [PubMed]
21. Solís-Lemus, C.; Bastide, P.; Ané, C. PhyloNetworks: A package for phylogenetic networks. *Mol. Biol. Evol.* **2017**, *34*, 3292–3298. [CrossRef] [PubMed]
22. Huson, D.H.; Scornavacca, C. Dendroscope 3—An interactive viewer for rooted phylogenetic trees and networks. *Syst. Biol.* **2012**, *61*, 1061–1067. [CrossRef] [PubMed]
23. Van Der Merwe, M.; Winfield, M.O.; Arnold, G.M.; Parker, J.S. Spatial and temporal aspects of the genetic structure of *Juniperus communis* populations. *Mol. Ecol.* **2000**, *9*, 379–386. [CrossRef] [PubMed]
24. Michalczyk, I.M.; Opgenoorth, L.; Luecke, I.; Huck, S.; Ziegenhagen, B. Genetic support for perglacial survival of *Juniperus communis* L. in Central Europe. *Holocene* **2010**, *20*, 887–894. [CrossRef]
25. Kabát, K. *A Key to Flora of Czech Republic*; Academia Publisher: Prague, Czech Republic, 2001; pp. 1–800.
26. Hall, M.T. A hybrid swarm in *Juniperus*. *Evolution* **1952**, *6*, 347–366. [CrossRef]
27. Hall, M.T. Variation and hybridization in *Juniperus*. *Ann. Mo. Bot. Gard.* **1952**, *39*, 1–64. [CrossRef]
28. von Rudloff, E.; Irving, R.; Turner, B.I. Reevaluation of allopatric introgression between *Juniperus ashei* and *J. virginiana* using gas chromatography. *Am. J. Bot.* **1967**, *5*, 600.
29. Flake, R.H.; von Rudloff, E.; Turner, B.I. Quantitative study of clinal variation in *Juniperus virginiana* using terpenoid data. *Proc. Natl. Acad. Sci. USA* **1969**, *62*, 487–494. [CrossRef] [PubMed]
30. Flake, R.H.; von Rudloff, E.; Turner, B.I. Confirmation of clinal pattern of chemical differentiation in *Juniperus virginiana* from terpenoid data obtained in successive years. In *Terpenoids: Structure, Biogenesis and Distribution*; Academic Press: New York, NY, USA, 1973; pp. 215–228.
31. Farhat, P.; Takvorian, N.; Avramidou, M.; Garraud, L.; Adams, R.P.; Siljak-Yakovlev, S.; Kharrat, M.B.D.; Robert, T. First evidence for allotriploid hybrids between *Juniperus thurifera* and *J. sabina* in a sympatric area in the French Alps. *Ann. For. Sci.* **2020**, *77*, 75–93. [CrossRef]
32. Adams, R.P. Allopatric hybridization and introgression between *Juniperus maritima* R.P. Adams and *J. scopulorum* Sarg.: Evidence from nuclear and cpDNA and leaf terpenoids. *Phytologia* **2015**, *97*, 55–65.